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ESTABLISHMENT OF THE INTESTINAL MICROBIOTA IN INFANCY

INGEGERD ADLERBERTH, ERIKA LINDBERG, FOROUGH NOWROUZIAN, and AGNES E. WOLD

Department of Clinical Bacteriology, Göteborg University, Göteborg, Sweden

SUMMARY

The large intestinal microbiota represents the most diverse and complex bacterial ecosystem of the human body, estimated to harbour more than 500 different, mostly anaerobic, bacterial species in an adult individual. Its establishment commences at birth, and proceeds in a sequential manner during the first years of life.

The first bacteria to colonise the neonatal intestine are aerobic or facultative anaerobic bacteria, which initially reach high population counts. These bacteria consume the oxygen lowering the redox-potential in the gut, making way for the anaerobes. Successively, a range of different anaerobic species colonises the gut. With the establishment of an increasingly complex anaerobic microbiota, the growth of the facultatives is suppressed, due to the accumulation of toxic metabolites, oxygen depletion and substrate competition.

A number of factors influence the establishment of the intestinal microbiota, including delivery and feeding modes, degree of social exposure and environmental bacterial content. Thus, differences in colonisation pattern are observed between vaginally and *sectio*-delivered infants, between breast- and bottle-fed infants, and between infants in industrialised and developing countries.

This chapter describes the establishment of the intestinal microbiota in human infants, and reviews factors affecting the colonisation process. We have recently characterised the intestinal colonisation pattern in a population of Swedish infants born in the late 1990s. Our findings indicate that the intestinal microbiota of Swedish infants might have changed in the last decades, probably due to an increasingly hygienic life-style.

THE SEQUENTIAL ESTABLISHMENT OF THE INTESTINAL MICROBIOTA

During or after birth the neonate first encounters the world of microbes, and bacteria commence to colonise the skin, and the respiratory, genital and intestinal tracts. This is the starting-point for the successive development of diversified bacterial ecosystems at these sites, of which the intestinal tract harbours the most complex microbiota.

The implantation of different bacteria into the microbiota does not occur at random. It is regulated through limitations in the intestinal milieu, and through bacterial interactions. Thus, the growth of some bacteria makes way for others, whereas yet other bacteria might instead be suppressed. During the neonatal period, however, it is quite easy for a wide range of bacteria to settle in the gut because of limited competition from a yet not fully developed microbiota.

The mother's vaginal, faecal and perineal microbial communities are common sources of bacterial strains colonising the neonate. Bacteria may also be acquired from any other person in contact with the baby, and from environmental sources (*Bettelheim* et al., 1974a,b,c; *Fryklund* et al., 1992; *Tannock* et al., 1990). The development of the intestinal microbiota may be influenced by any factor influencing the spectrum and amount of bacteria encountered by the infant, such as delivery mode, feeding mode, the number of social contacts and the degree of environmental hygiene (*Adlerberth* et al., 1998; *Bennet*, 1987; *Bennet* et al., 1991; *Stark* and *Lee*, 1982).

A number of studies over the last decades have investigated the intestinal colonisation pattern of young infants. Below, we summarise data from studies performed since the 1970s regarding the time of acquisition and origin of the major bacterial groups inhabiting the intestine.

Aerobic and facultative anaerobic bacteria

Facultative bacteria can perform both aerobic and anaerobic metabolism, although more energy is generated in the oxygen-dependent, aerobic metabolism. The first colonisers of the neonatal intestine belong to this group: *E. coli* and other enterobacteria, enterococci, streptococci and staphylococci (*Mata* and *Urrutia*, 1971; *Rotimi* and *Duerden*, 1981b; *Stark* and *Lee*, 1982). The intestinal milieu is initially characterised by a positive oxidation-reduction potential (*Grutte* et al., 1965), which favours the metabolism and replication of these bacteria. In the absence of competition from anaerobes, the facultative bacteria commonly reach population levels exceeding 10^{10} colony forming units (CFU)/g faeces, which is roughly 100 times higher than their population levels in adults (*Hoogkamp-Korstanje* et al., 1979; *Stark* and *Lee*, 1982).

E. coli strains that colonise the neonate may be acquired from the mother during delivery (*Bettelheim* and *Lennox-King*, 1976), but this usually occurs in less than one third of the infants in Western countries (*Fryklund* et al., 1992; *Gothefors* et al., 1976; *Murono* et al., 1993). This has been ascribed to the use of enemas, antiseptics and other measures to reduce bacterial exposure during delivery. However, even during home-deliveries in Pakistan, less than half of the infants acquired maternal *E. coli* strains (*Adlerberth* et al., 1998). Probably, giving birth lying on the back, and mechanical cleaning to avoid faecal contamination is sufficient to reduce exposure of the baby to maternal faecal bacteria.

In maternity wards, neonates may pick up *E. coli* strains spread between infants by the hospital staff (*Bettelheim* and *Lennox-King*, 1976; *Tullus* et al., 1988b). Such transfer is avoided if "rooming in" is practiced, i.e. when only the parents handle the baby (*Bettelheim* et al., 1983).

Other enterobacteria than *E. coli*, e.g. *Klebsiella* and *Enterobacter*, colonise many infants in the first weeks of life and often reach high population levels (*Balmer* and *Wharton*, 1989; *Bennet* and *Nord*, 1987a; *Gothefors* et al., 1976; *Lundequist* et al., 1985). These bacteria are less common than *E. coli* in the in-

testinal microbiota of adults (*Finegold* et al., 1983; *Tannock*, 1995), and the neonatal strains are rarely of maternal origin (*Adlerberth* et al., 1998; *Fryklund*, 1994; *Fryklund* et al., 1992; *Shinebaum* et al., 1979). These enterobacteria are frequently spread between neonates via the hospital staff (*Fryklund* et al., 1992; *Shinebaum* et al., 1979), and may also be acquired from feeds and other non-human sources (*Adlerberth* et al., 1998).

Enterococci are isolated from most neonates and commonly reach high population levels in the gut (*Rotimi* and *Duerden*, 1981b; *Stark* and *Lee*, 1982; *Yoshioka* et al., 1983). Their route of transmission to the neonate has not been studied, but most likely both horizontal and vertical transfer occurs. Their natural niche is the intestinal microbiota of humans and animals, but they are also sturdy bacteria that resist various hygienic measures, which make them easily spread in e.g. the hospital milieu (*Kearns* et al., 1995).

Staphylococci, both *S. aureus* and coagulase-negative staphylococci, may colonise the neonatal intestine and reach high population levels in the first days of life (*Balmer* and *Wharton*, 1989; *Lindberg* et al., 2000). Staphylococci are found in the intestine of more than 50% of adults, but in much lower population counts than observed in neonates (*Finegold* et al., 1983). These bacteria are well recognised as members of the skin microbiota, and strains colonising the neonate usually derive from parental skin (*Lindberg* et al., 2004).

Streptococci are found less frequently than enterobacteria, enterococci or staphylococci (*George* et al., 1996; *Rotimi* and *Duerden*, 1981b). Other aerobic bacteria, such as the Gram-negatives *Aeromonas, Pseudomonas* and *Acinetobacter* may transiently colonise the neonate during the first weeks of life (*Adlerberth*, 1996; *Pazzaglia* et al., 1990; *Rotimi* and *Duerden*, 1981b).

Anaerobic bacteria

Obligate anaerobes have an anaerobic metabolism independent of oxygen, and are therefore not dependent on oxygen for growth. In addition, they are often extremely sensitive to the presence of oxygen, because they lack enzymes which detoxify atmospheric oxygen, which is a highly dangerous molecular species (*Gregory* and *Fridovich*, 1973). During the first days of life, the oxygen tension in the infantile gut is quite high, and obligate anaerobes cannot thrive. However, the aerobic and facultative anaerobic bacteria soon consume the oxygen and the redox-potential in the gut changes from positive to negative. This makes way for the anaerobes (*Hoogkamp-Korstanje* et al., 1979; *Mata* and *Urrutia*, 1971; *Stark* and *Lee*, 1982), and bacteria of the genera *Bacteroides*, *Bifidobacterium* and *Clostridium* soon appear in the microbiota (*Balmer* and *Wharton*, 1989; *George* et al., 1996; *Mata* and *Urrutia*, 1971; *Roberts* et al., 1992; *Rotimi* and *Duerden*, 1981b; *Sepp* et al., 2000; *Stark* and *Lee*, 1982). In classical studies, these bacteria are isolated from a majority of infants in the first week of life and may reach population levels of $10^{9\text{-}11}$/g faeces (*Mata* and *Urrutia*, 1971; *Rotimi* and *Duerden*, 1981b; *Stark* and *Lee*, 1982; *Yoshioka* et al., 1983).

Bifidobacteria are aerotolerant anaerobes, and isolates from infants often show some scanty growth under aerobic conditions (*Stark* and *Lee*, 1982). Thus, they may survive quit well outside the intestine, which facilitates their horizontal transfer. Infants' intestinal carriage of bifidobacteria varies between different maternity wards, indicating acquisition from non-maternal sources (*Bezirtzoglou* and *Romond*, 1990a; *Lundequist* et al., 1985; *Mitsuoka* and *Kaneuchi*, 1977). However, transfer of bifidobacteria from mother to infant during delivery also occurs (*Tannock* et al., 1990). As

bifidobacteria may also be part of the oral microbiota of healthy adults, transfer from this source is another possibility.

Bacteroides are strictly anaerobic bacteria that only survive for a short time in the presence of oxygen. Therefore, they require close contact for transfer, and if they are not acquired during delivery, their appearance in the microbiota may be significantly delayed (*Gronlund* et al., 1999; *Neut* et al., 1987). The *Bacteroides fragilis* group (e.g. *B. vulgatus* and *B. thetaiotamicron*) is most common in the intestinal microbiota of newborn infants as well as in adults (*Benno* et al., 1984; *Rotimi* and *Duerden*, 1981a). These bacteria are usually restricted to the intestinal tract and are not part of the normal microbiota at other sites, which may further hamper their spread between individuals.

Clostridia are anaerobes that form spores when the environmental conditions are unfavourable. Clostridia colonising neonates commonly belong to the species *C. perfringens* and *C. difficile*, which are considered quite pathogenic (*Benno* et al., 1984; *Bolton* et al., 1984; *Rotimi* and *Duerden*, 1981b; *Tullus* et al., 1989). *C. perfringens* is common in adults as well, while *C. difficile* is found in less than 4% of healthy adult individuals (*Finegold* et al., 1983; *Rolfe*, 1988). Disturbance of the intestinal microbiota, as in antibiotic treatment, may result in unrestricted expansion of *C. difficile* in the microbiota, leading to antibiotic-associated diarrhoea and, in worst case, pseudomembranous colitis (*Wilson*, 1993). However, for unknown reasons, young infants remain healthy despite high counts of these toxin-producing bacteria in the faeces (*Bolton* et al., 1984; *Tullus* et al., 1989). Colonisation by *C. difficile* usually declines after some months (*Tullus* et al., 1989), reflecting the establishment of a complex microbiota able to suppress their growth.

As clostridia form spores which resist most disinfectants and are ubiquitous in the hospital milieu and other environments (*Wilson*, 1993), clostridiae are easily spread to newborn infants from environmental sources (*El-Mohandes* et al., 1993; *Kato* et al., 1994; *Martirosian* et al., 1995; *Neut* et al., 1987).

The extent to which lactobacilli colonise the intestines of newborn infants is controversial. Most studies from Western countries report quite low *Lactobacillus* colonisation rates in infants (*Balmer* and *Wharton*, 1989; *Ellis-Pegler* et al., 1975; *Gronlund* et al., 1999; *Matsumiya* et al., 2002; *Stark* and *Lee*, 1982) but some find quite high colonisation rates (*Gil* et al., 1986; *Hall* et al., 1990; *Kleessen* et al., 1995). Variations in methodology may account for much of these differences, since lactobacilli are notoriously difficult to identify by traditional biochemical methods. We have recently performed a detailed longitudinal study on the *Lactobacillus* microbiota of more than 100 Swedish neonates, using PCR-based methods for typing of lactobacilli to the species and strain level (*Ahrne* et al., 2005). Lactobacilli were isolated from the stools of at most 45% of the infants at different time-point during the first 18 months of life, and persistent colonisation with a single strain occurred in less than one fifth of the infants. Interestingly, *Lactobacillus* isolation dropped significantly by twelve months, but increased again thereafter. Before one year of age, *L. rhamnosus*, *L. gasseri* and *L. paracasei* dominated, but the first two species disappeared after that age, being replaced by *L. plantarum, L. acidophilus* and *L. delbrueckii* (*Ahrne* et al., 2005).

Lactobacilli are the dominant bacterial group in the vaginal microbiota of healthy women (*Masfar* et al., 1986). Maternal vaginal lactobacilli may tran-

siently colonise the intestine of the baby, but they rarely persist in the microbiota (*Matsumiya* et al., 2002). Other sources of lactobacilli may be the maternal faecal or the parental oral microbiota. In Swedish adults, *L. plantarum, L. rhamnosus* and *L. paracasei* are the most common *Lactobacillus* species in both the oral and intestinal microbiota (*Ahrne* et al., 1998). Most lactobacilli are aerotolerant, and they are probably quite easily transferred between individuals.

Many other anaerobic bacteria form stable populations in the intestinal microbiota of adults, including bacteria of the genera *Veillonella*, *Eubacterium, Fusobacterium, Peptostreptococcus* and *Ruminococcus* (*Finegold* et al., 1983). Only few studies have investigated their establishment in the infantile intestinal microbiota. *Veillonella* are isolated from 10-90% of neonates (*Benno* et al., 1984; *Ellis-Pegler* et al., 1975; *George* et al., 1996; *Kleessen* et al., 1995; *Rotimi* and *Duerden*, 1981b; *Simhon* et al., 1982), and may reach hundredfold higher population levels in young infants than in adults (*Ellis-Pegler* et al., 1975; *Finegold* et al., 1983). Colonisation rates of *Eubacterium* in the microbiota vary between 0 and 40% during the first weeks of life (*Lundequist* et al., 1985; *Rotimi* and *Duerden*, 1981b; *Sepp* et al., 2000; *Stark* and *Lee*, 1982), and <50% are colonised at 9-12 months of age (*Mata* and *Urrutia*, 1971; *Sepp* et al., 1997; *Stark* and *Lee*, 1982). Peptostreptococci generally do not appear until solid foods are introduced (*Benno* et al., 1984; *Stark* and *Lee*, 1982), but are present in a majority of infants at 12 months of age (*Mata* and *Urrutia*, 1971; *Sepp* et al., 1997; *Stark* and *Lee*, 1982). *Ruminococcus* are only occasionally isolated during the first year of life (*Benno* et al., 1984; *Mata* and *Urrutia*, 1971). In one study including the identification of *Fusobacterium* species, less than 20% of infants were colonised by six months of age (*George* et al., 1996). The origin of these strictly anaerobic bacteria successively establishing in the infantile intestinal microbiota has never been studied.

REGULATION OF BACTERIAL POPULATION LEVELS AND COLONISATION RESISTANCE

The successive establishment of various anaerobic species results in a highly diverse microbiota at a few years of age (*Ellis-Pegler* et al., 1975; *Midtvedt*, 1994). During this process, some early anaerobes disappear or decline in numbers, like *C. difficile* and *Veillonella* (*Ellis-Pegler* et al., 1975; *Rolfe*, 1988, 1995). Furthermore, the facultative bacteria are suppressed by the expanding anaerobic populations (*Mata* and *Urrutia*, 1971; *Stark* and *Lee*, 1982).

However, relatively high numbers of both facultative and anaerobic bacteria may be present during the first months (*Hoogkamp-Korstanje* et al., 1979; *Stark* and *Lee*, 1982), or even years of life (*Ellis-Pegler* et al., 1975). The quite simple anaerobic microbiota of infants is not capable of suppressing facultative bacteria as effectively as the complex adult anaerobic microbiota, estimated to harbour several hundred different anaerobic species (*Finegold* et al., 1983; *Moore* and *Holdeman*, 1974). In a classical study by *Ellis-Pegler* and coworkers (1975), the mean ratio of anaerobic over facultative anaerobic and aerobic bacteria was 1.5:1 in infants before four months of age, 10:1 in 4-12 months old infants and 50:1 in children 1-4 years old, as compared to a ratio of 200:1 in adults.

The ability of the established microbiota to suppress the growth of the potentially pathogenic facultative bacteria or *C. difficile* is termed "colonisation resistance". Several mechanisms may be involved, including competition for nutrients and binding sites, and production of toxic metabolites (*Freter*, 1992; *Hentges*, 1983). Not only are many potentially pathogenic members in the microbiota kept at bay, but also the implantation of new bacterial strains into the ecosystem is strongly reduced. With increasing age, and with the establishment of an increasingly complex microbiota, it becomes more difficult for newcomers to colonise the gut (*Cooke* et al., 1971; *Jodal* et al., 1977; *Lari* et al., 1990; *Lodinová* et al., 1973).

BREAST-MILK AND THE INTESTINAL MICROBIOTA

Since the pioneering work by *Tissier* (1900) a number of studies have examined the influence of feeding patterns on the early intestinal microbiota. The results vary greatly between studies. Traditional studies reported a pronounced influence by feeding mode on the microbiota (*Bullen* et al., 1977, 1976; *Mata* and *Urrutia*, 1971; *Stark* and *Lee*, 1982), but in many more recent studies from Western societies, and with the use of modern formulas, there seem to be less difference between the colonisation patterns of breast- and bottle-fed infants.

Mata and *Urrutia* (1971) examined the intestinal microbiota of breastfed indigenous Guatemalan neonates, and found frequent colonisation with and high numbers of *E. coli*, enterococci, clostridia and *Bacteroides* during the first days after birth. However, already by the end of the first week of life the microbiota was completely dominated by bifidobacteria and other bacterial groups were suppressed. *Bullen* and colleagues (1976) while investigating English neonates in the early 1970s, also demonstrated a clear dominance of bifidobacteria in breastfed neonates and low counts of other bacteria. In bottle-fed infants, the bifidobacterial counts were at least one log unit lower than in breastfed infants, and *Bacteroides*, clostridia, *E. coli* and enterococci outnumbered the bifidobacteria. The faecal pH is lower in breastfed than in bottle-fed infants, due to the low buffering capacity of human milk. This was assumed to promote the proliferation of acid tolerant bifidobacteria, whereas *E. coli* and other enterobacteria would be suppressed (*Bullen* and *Tearle*, 1976).

Thus, high bifidobacterial counts were long regarded as the most characteristic feature of the intestinal microbiota of the breastfed infant (*Bullen* et al., 1976, 1977; *Hewitt* and *Rigby*, 1976; *Willis* et al., 1973). However, many of the more recent studies report similar and sometimes very low counts of bifidobacteria in both breast- and bottle-fed infants (*Balmer* et al., 1994; *Balmer* and *Wharton*, 1989; *Kleessen* et al., 1995; *Langhendries* et al., 1995; *Lundequist* et al., 1985; *Rubaltelli* et al., 1998; *Simhon* et al., 1982). There is also no correlation between faecal pH and bifidobacterial counts (*Balmer* and *Wharton*, 1989; *Simhon* et al., 1982; *Willis* et al., 1973).

Some, but far from all, studies show more frequent colonisation and higher counts of *Bacteroides* in bottle-fed than in breastfed infants (*Benno* et al., 1984; *Bullen* et al. 1976, 1977; *Long* and *Swenson*, 1977; *Yoshioka* et al., 1983). More persistent differences include lower counts of clostridia and enterococci in breastfed than bottle-fed infants (*Balmer* et al., 1994; *Balmer* and *Wharton*, 1989; *Benno* et al., 1984;

Bullen et al., 1976; *Simhon* et al. 1982; *Stark* and *Lee*, 1982). More breastfed than bottle-fed infants may harbour a flora dominated by bifidobacteria, mostly due to lower levels of other bacterial groups (*Balmer* et al., 1994; *Balmer* and *Wharton*, 1989; *Benno* et al., 1984; *Rubaltelli* et al., 1998).

Breastfed infants often have higher counts of staphylococci than bottle-fed infants, especially during the first weeks of life (*Balmer* et al., 1994; *Balmer* and *Wharton*, 1989; *Lundequist* et al., 1985; *Simhon* et al., 1982). Probably, staphylococci from the nipple are swallowed during breast-feeding. Staphylococci are commonly isolated from the nipples of lactating mothers, and the same strain is often isolated from the infants' stools (*Lindberg* et al., 2004). However, in a recent study, we found no clear association between breast-feeding and *S. aureus* colonisation (*Lindberg* et al., in manuscript).

There is a tendency in many studies that breastfed infants have lower counts of enterobacteria than bottlefed infants, but, apart from the classical studies mentioned earlier (*Bullen* et al., 1976, 1977; *Mata* and *Urrutia*, 1971; *Stark* and *Lee*, 1982), the difference is usually marginal (*Balmer* et al., 1994; *Balmer* and *Wharton*, 1989; *Benno* et al., 1984). However, the enterobacterial flora differs at the species and strain level between breast- and bottlefed infants. Breastfed infants less frequently harbour enterobacteria other than *E. coli*, such as *Klebsiella* or *Enterobacter* spp. (*Adlerberth* et al., 1991; *Balmer* and *Wharton*, 1989; *Bullen* et al., 1976; *Tullus* et al., 1988a; *Yoshioka* et al., 1983; *Örskov* and *Biering-Sörensen*, 1975). They also carry fewer different *E. coli* strains than bottle-fed infants (*Mevissen-Verhage* et al., 1985b; *Örskov* and *Biering-Sörensen*, 1975).

It is commonly stated that breast-feeding would promote colonisation with lactobacilli. However, most studies do not find any differences regarding *Lactobacillus* colonisation between breast- and bottle-fed infants, and, when present, the differences indicate higher numbers of lactobacilli in bottle-fed infants (*Benno* et al., 1984; *Kleessen* et al., 1995). However, we recently reported that colonisation with *Lactobacillus rhamnosus* was significantly more common in Swedish infants at six months age if they still received breast-milk by that time than in completely weaned infants (*Ahrne* et al., 2005). Thus, certain species of lactobacilli may be favoured by breast-feeding.

A number of factors present in breast-milk have been suggested to influence the intestinal microbiota. These include e.g. secretory IgA, lactoferrin, lysozyme, complex oligosaccharides and nucleotides. However, attempts to add lactoferrin, nucleotides or complex oligosaccharides to infant formulas have not changed the intestinal microflora of the infants studied towards a more "breastfed" pattern (*Balmer* et al., 1989, 1994; *Euler* et al., 2005; *Gil* et al., 1986; *Roberts* et al., 1992). Using formulas of low iron supplement supports the establishment of a microbiota more close, but far from identical, to that of breast-fed infants (*Balmer* and *Wharton*, 1991; *Mevissen-Verhage* et al., 1985a). The complexity of factors in human milk is tremendous, and it is unlikely that artificial feedings will ever be able to completely mimic its composition or effects.

CAESAREAN SECTION, ANTIBIOTICS AND NEONATAL INTENSIVE CARE

The mode of delivery, i.e. vaginal birth or caesarean section profoundly influences the establishment of the intestinal microbiota. The *sectio*-delivered neonate is denied the natural exposure to maternal faecal and vaginal bacteria during delivery, and colonisation occurs exclusively from other sources. In Western countries, this results in a delayed acquisition of several of the common early colonisers, e.g. *E. coli, Bacteroides*, bifidobacteria (*Balmer* et al., 1989; *Bennet* and *Nord*, 1987b; *Gronlund* et al., 1999; *Hall* et al., 1990; *Neut* et al., 1987), and in some studies lactobacilli (*Hall* et al., 1990). It seems as if infants catch up with respect to colonisation with bifidobacteria and lactobacilli quite rapidly (*Bennet* and *Nord*, 1987b; *Hall* et al., 1990; *Neut* et al., 1987), whereas colonisation with *Bacteroides* may be delayed for many months (*Adlerberth* et al., 2005; *Gronlund* et al., 1999). The results regarding *E. coli* varies, but these bacteria may be quite rapidly acquired from environmental sources (*Balmer* et al., 1989; *Bezirtzoglou* and *Romond*, 1990b; *Lennox-King* et al., 1976).

Sectio-delivered infants commonly show an increased colonisation with enterobacteria other than *E. coli* (*Balmer* et al., 1989; *Bennet* et al., 1986; *Long* and *Swenson*, 1977). Many *sectio*-delivered infants are initially cared for at a neonatal ward, where spread of these bacteria may be common (*Fryklund* et al., 1992). In addition, delayed acquisition of anaerobic bacteria in these infants probably makes them more easily colonised with non-*E. coli* enterobacteria, which are less well apt than e.g. *E. coli* to establish in the presence of a complex anaerobic microbiota (*Bennet* et al., 1986).

Clostridia, especially *Clostridium perfringens*, are usually the first anaerobes to colonise infants after *sectio* deliveries (*Bezirtzoglou* et al., 1989; *Neut* et al., 1987). Spores of these bacteria are easily acquired from environmental sources. Furthermore, they do not seem to be dependent on a strictly reduced environment for proliferation, which facilitates their early establishment in the gut (*Bezirtzoglou* and *Romond*, 1991; *Bezirtzoglou* et al., 1989).

Not only *sectio*-delivered neonates, but also most infants cared for in neonatal wards and neonatal intensive care units (NICU's) acquire an intestinal flora which differs from that of healthy, vaginally delivered neonates. The enterobacteria isolated usually belong to the genera *Klebsiella* or *Enterobacter* (*Fryklund*, 1994; *Goldmann* et al., 1978; *Tullus* et al., 1988a), and colonisation with anaerobes is delayed (*Gewolb* et al., 1999; *Hall* et al., 1990; *Hallstrom* et al., 2004; *Sakata* et al., 1985).

Infants cared for in NICU's are often treated with antibiotics, and both oral and parental antibiotics markedly affect the intestinal microbiota of neonates (*Bennet* et al., 1986, 2002; *Bennet* and *Nord*, 1987b). Most anaerobic bacteria are profoundly suppressed, although clostridia may remain detectable in some cases (*Bennet* et al., 1986; *Bennet* and *Nord*, 1987b). *E. coli* decreases, whereas other enterobacteria may increase in numbers (*Bennet* et al., 1986; *Goldmann* et al., 1978). Some recent studies of neonates cared for at NICU's indicate a real paucity of bacterial species in their early intestinal microbiota (*El-Mohandes* et al., 1993; *Gewolb* et al., 1999; *Hallstrom* et al., 2004), most likely a result of heavy antibiotic use and strict hygiene. Coagulase-negative staphylococci are the bacteria most frequently isolated from these infants.

GLOBAL DIFFERENCES IN COLONISATION PATTERN

The importance of environmental exposure to bacteria as a determinant of infantile intestinal colonisation is shown by the fact that infants in Western societies are colonised later and have a less varied microbiota than infants in developing countries, and infants in the former socialist countries of Eastern Europe (*Adlerberth* et al., 1991, 1998; *Bennet* et al., 1991; *Rotimi* et al., 1985; *Sepp* et al., 1997, 2000). In Western industrialised societies, strict hygienic hospital standards and general household cleanliness is likely to reduce exposure not only to pathogens, but also to a number of commensal bacteria.

In indigenous Guatemalan infants, maternal faeces commonly contaminate the baby during delivery, and a wide range of intestinal bacteria is present in the baby's faeces during the first days of life (*Mata* and *Urrutia*, 1971). These bacteria initially reach high numbers, but already by the end of the first week they are completely outnumbered by bifidobacteria, and this bifidobacterial predominance persists during the period of breastfeeding (*Mata* and *Urrutia*, 1971). The components of this early microbiota are probably acquired during delivery, transferred directly from the mother. The pronounced effect of breastfeeding on the intestinal microbiota in this population, which is not observed in most modern studies from industrialised countries, could relate to the acquisition of a complex microbiota immediately after delivery, possibly containing bacteria facilitating the growth of bifidobacteria but suppressing other bacteria in the intestinal milieu created by breast milk.

In developing countries, not only vaginally delivered infants but also those delivered by caesarean section may acquire many bacteria very early (*Adlerberth* et al., 1991; *Rotimi* et al., 1985), reflecting pronounced exposure from environmental sources. Within three days after birth, all of both vaginally and *sectio*-delivered Nigerian neonates had acquired *E. coli*, and within 6 days most had *Bacteroides*, bifidobacteria and clostridia (*Rotimi* et al., 1985). Regardless of delivery mode, Pakistani infants harboured enterobacteria within three days after birth, often several species simultaneously, whereas less than 60% of Swedish neonates were colonised with enterobacteria by that age, and rarely with more than one species at a time (*Adlerberth* et al., 1991). During the first six months of life, many different *E. coli* strains occur in succession in the intestinal microbiota of Pakistani infants (*Adlerberth* et al., 1998), whereas among Swedish infants a single *E. coli* strain usually dominates for prolonged periods of time (*Kuhn* et al., 1986; *Nowrouzian* et al., 2003).

Colonisation with enterococci and lactobacilli also occurs earlier in Ethiopian than Swedish neonates (*Bennet* et al., 1991). One-year-old Estonian infants carry lactobacilli and *Eubacterium* spp. significantly more often than Swedish one-year-old infants (*Sepp* et al., 1997). Bifidobacteria may also be less common in infants in Western countries (*Simhon* et al., 1982).

In Western infants, intestinal colonisation with "skin bacteria" like staphylococci and ubiquitous environmental bacteria like *C. difficile* are more prominent, most likely due to reduced competition from more "professional" intestinal bacteria. Thus, *S. epidermidis* is more common in Swedish than Ethiopian neonates (*Bennet* et al., 1991), and one-year-old Swedish children are more often colonised with *C. difficile* than Estonian infants (*Sepp* et al., 1997).

A CHANGING COLONISATION PATTERN IN WESTERN SOCIETIES?

We have recently examined the establishment of the intestinal microbiota of Swedish infants born in the late 1990s. One hundred sixteen infants were followed with regular sampling of the intestinal microbiota and major groups of bacteria were quantitatively cultured. To our knowledge, this is the largest birth cohort ever studied with respect to the establishment of the commensal intestinal microbiota. Although differences in methodology preclude direct comparison with earlier studies, there are some indications that the infantile intestinal colonisation pattern might have changes in Sweden in the last decades.

A striking observation was the ubiquitous isolation of staphylococci from faecal samples during the entire first year of life. Coagulase-negative staphylococci were present in high population counts in the intestinal microbiota of all infants within some days after birth. Colonisation persisted throughout the first year of life in 80% of the infants, although the faecal population counts decreased quite significantly as an indication of the failure of these bacteria to withstand the competition from more "professional intestinal bacteria" (*Adlerberth* et al., 2005). *S. aureus* was also quite common in the infants' microbiota, being isolated from the stools of three out of four infants in the first year of life (*Lindberg* et al., 2000). With the exception of some Japanese studies performed in the 1980s, where staphylococci were isolated from almost all neonates but in quite low counts (*Benno* et al., 1984; *Yoshioka* et al., 1983), studies from industrialised countries in the 1970s and 1980s report much colonisation frequencies for staphylococci (*Bullen* et al., 1976; *McAllister* et al., 1974; *Neut* et al., 1987; *Rotimi* and *Duerden*, 1981b; *Stark* and *Lee*, 1982). In studies of Swedish infants in the 1980s, coagulase-negative staphylococci were isolated from 30-70 % in the first week of life (*Bennet* et al., 1986, 1991; *Lundequist* et al., 1985). Thus, staphylococci might have increased in prevalence and numbers in the early intestinal microbiota over the last decades in Sweden. Accordingly, an increase in neonatal staphylococcal colonisation between 1975 and 1995 has been described in France (*Borderon* et al., 1996).

We suggest that an important cause for the expansion of these traditional skin bacteria in the infantile gut microbiota is a lack of competition from "professional" gut bacteria. We found that less than 60% of vaginally delivered infants were colonised with *E. coli* by one month of age (*Adlerberth* et al., 2005; *Nowrouzian* et al., 2003), which is a reduction compared with results from Sweden in the 1970s and 1980s (*Bennet* et al., 1986; *Gothefors* et al., 1976; *Kuhn* et al., 1986; *Lundequist* et al., 1985; *Tullus*, 1988). Short hospital stays, rooming-in and strict hygiene may have reduced exposure of infants to *E. coli* in the hospital. Furthermore, spread of *E. coli* must also be very limited in families and homes, as *sectio*-delivered infants in our study showed delayed acquisition by *E. coli* up to six months of age (*Adlerberth* et al., 2005). Our results suggest that hygienic measures both in hospitals and general life have reduced the circulation of some typical faecal bacteria like *E. coli* in Sweden in the last decades.

In contrast to *E. coli,* the isolation rate of enterococci was as high in the present study as previously reported (*Lundequist* et al., 1985), and *sectio*-de-

livered infants were colonised as early as infants delivered vaginally (*Adlerberth* et al., 2005). As mentioned earlier, enterococci are sturdy bacteria which resist various hygienic measures, and during the last decades they have emerged as important nosocomial pathogens. This indicates that bacteria differ widely in their ability to resist an increasingly hygienic life-style.

Bacteroides colonisation was infrequent compared to Swedish studies performed in the 1980s (*Bennet* and *Nord*, 1987b; *Lundequist* et al., 1985). Thus, only around one third of the vaginally delivered infants harboured *Bacteroides* at one week of age, and this frequency did not increase during the first two months of life (*Adlerberth* et al., 2005). Furthermore, at two months of age, only one of seventeen *sectio*-delivered infants had acquired *Bacteroides*, and not even at one year of age had they caught up with vaginally delivered infants with respect to colonisation by *Bacteroides*. This further indicates a limited circulation of faecal bacteria in the modern Swedish society.

Another finding points towards a poorly developed intestinal microbiota still at one year of age in today's Swedish infants. We observed a persistently increasing *C. difficile* isolation rate throughout the first year of life, and more than 50% of the vaginally delivered infants harboured *C. difficile* by twelve months of age (*Adlerberth* et al., 2005). The incidence was even higher in *sectio*-delivered infants. In a Swedish study performed in the 1980s, colonisation by *C. difficile* peaked at a frequency of 30% around six months of age, and less than 10% of the infants were colonised at twelve months (*Tullus* et al., 1989). As described earlier, *C. difficile* is suppressed by the complex intestinal microbiota that establish with age. The persistent increase of this species in the microflora over the first year of life suggests that there is limited competition from the microbiota.

CONSEQUENCES OF A CHANGED INTESTINAL COLONISATION PATTERN IN EARLY LIFE?

The changes in the early intestinal microbiota indicated by our recent study points towards an increased ratio of Gram-positive to Gram-negative bacteria in the microbiota of young Swedish infants. Colonisation with the Gram-negative enterobacteria (*E. coli, Klebsiella* etc.) and *Bacteroides* seem to have decreased in the last decades, while colonisation frequencies for Gram-positives such as enterococci, bifidobacteria and clostridia remain high and staphylococcal colonisation seem to have increased. As the intestinal microflora is a common source of bacteria causing extra-intestinal infections, it is interesting to note that staphylococci are the most common cause of neonatal septicaemia today (*Kallman* et al., 1997). Furthermore, the intestinal microflora is the major drive for the gut immune system (*Cebra*, 1999). Since Gram-positive and Gram-negative bacteria induce different patterns of immuno-regulatory and inflammatory mediators when they interact with the innate immune system (*Hessle* et al., 2000, 2003, 2005), there is reason to believe that changes in the intestinal colonisation pattern connected to an increasingly hygienic life-style may have global effects on the function of the developing infantile immune system.

NON-CULTURE DEPENDENT METHODS FOR STUDIES OF THE INFANTILE INTESTINAL MICROBIOTA

The data reviewed in this paper on the ecology and diversity of the infantile gut microbiota is based exclusively on studies using traditional culture techniques. Clearly, culture techniques have limitations, since many intestinal bacteria are uncultivable, and they are time-consuming and expensive. However, several alternative approaches for studies of the intestinal microbiota are available today, which may be regarded as useful complements to traditional culturing, and most likely with time will replace culture dependent methods.

The development of the intestinal ecosystem can be followed by assessing biochemical reactions performed by the intestinal bacterial population. Anaerobic bacteria in the human colon produce short chain fatty acids (SCFAs), such as acetate, propionate and butyrate, and the variety of SCFAs increases as a more complex microflora is established (*Midtvedt*, 1994; *Midtvedt* et al., 1988). Other functional activities of intestinal bacteria include, for example, the conversion of cholesterol to coprostanol, transformation of bilirubin to urobilins, degradation of mucins, and inactivation of pancreatic trypsin. By assessing various biochemical parameters in faeces information is achieved on whether bacterial groups responsible for certain key metabolic reactions have established or not. In Swedish children, some biochemical functions characterising a highly complex microbiota are not established yet at five years of age (*Midtvedt*, 1994; *Norin* et al., 1985).

Another approach to study the intestinal microbiota is to perform gas-liquid chromatography of bacterial cellular fatty acids in faecal samples (*Eerola* and *Lehtonen*, 1988; *Vaahtovuo* et al., 2001). Each bacterial species has a typical cellular fatty acid composition, and the cellular fatty-acid profile of a faecal sample thus consists of cellular fatty acids of all bacteria present in that sample. This method may be used to assess changes in the microbiota over time, and differences between individuals or groups of individuals, but cannot directly show which bacteria account for the differences observed. As a technical procedure, GLC is inexpensive and rapid. The method was recently used to indicate differences in intestinal microbiota between vaginally and *sectio*-delivered neonates, and changes in the microbiota occurring with the development of symptoms of necrotising enterocolitis in premature neonates (*Hallstrom* et al., 2004).

With the development of molecular genetic techniques, a variety of methods have recently been established for studies of the intestinal microbiota, most of which are based on the detection of bacterial 16S rRNA genes. So far, however, only a limited number of studies on the infantile intestinal microbiota have been performed with molecular techniques.

Fluorescent in situ hybridisation (FISH) probes targeting 16S rDNA of specific bacterial groups has been used to study the intestinal microbiota of infants. Whole bacterial cells are permeabilised to allow the probes to reach their target. If the 16S rRNA contains a sequence complementary to the fluorescently labelled probe, a hybrid is formed, causing the whole cell to fluoresce, and the fluorescing bacteria can be visualised in the microscope. Although only bacterial groups recognised by the probes currently available can be detected, the number of probes is rapidly increasing (*Blaut* et al., 2002). Using this method, *Harmsen* and co-authors (2000) demonstrated that bifidobacteria dominated the microbiota in breastfed but also in most bottle-fed infants, and

that *E. coli* and *Bacteroides* seemed to constitute a relatively larger proportion of the microbiota in bottle-fed infants. Thus, this study largely confirmed previous results obtained with conventional culture techniques. Another study by the same group demonstrated that formula-fed neonates had higher numbers of bacteria belonging to the *Coriobacterium* group, which includes e.g. *Eggerthella lenta* (formerly *Eubacterium lentum*) and *Collinsella aerofaciens* (formerly *Eubacterium aerofaciens*). This agrees with a previous study by *Benno* et al. (1984) showing higher counts of *Eubacterium* in bottle-fed than in breastfed neonates.

PCR and denaturing gradient gel electrophoresis (DGGE) combined with sequencing of the major ribotypes was used in one study to analyse the development of the intestinal microbiota of two healthy babies. This method involves the extraction of bacterial DNA followed by PCR amplification of fragments corresponding to region V6 to V8 of the 16S rRNA gene, using universal bacterial primers. Subsequent separation of the PCR products in DGGE results in a fingerprint of the microbiota where each band represents a specific bacterium. The identities of the bands are determined by cloning and sequencing of the amplified PCR products. This method permits the identification of hitherto unrecognised bacteria, but only dominant groups of intestinal bacteria are detected. The authors confirmed the early appearance of bifidobacteria in the infantile intestinal microbiota, but *Ruminococcus* was also identified as an early coloniser in these infants (*Favier* et al., 2002). Many of the cloned rDNA sequences exhibited less than 97% identity with sequences of known bacteria, indicating the presence of bacteria not previously identified in the infantile intestinal microbiota.

In a study by *Wang* and co-workers (2004), the faecal microflora of two Swedish infants was monitored over time by terminal restriction fragment length polymorphism analysis (T-RFLP) of amplified 16S rRNA genes from faecal samples. Bacterial DNA from faecal samples was isolated and 16S rRNA genes were amplified in PCR using fluorescently labelled primers. The PCR products were digested with restriction enzymes, and the fluorescently labelled terminal restriction fragments were separated and detected. 16S rDNA clone libraries were constructed from the same faecal samples, and the T-RFLP patterns of the clones were compared with those of the corresponding faecal samples. In this way, dominant bacterial groups present in the samples were identified. The bacterial groups detected most frequently in the early samples were *Enterobacteriaceae, Veillonella, Enterococcus, Staphylococcus* and *Bacteroides*. Bacteria of the genera *Bifidobacterium, Clostridium, Ruminococcus* and *Eubacterium* were identified in both infants, the three latter genera mainly after the first months of life. *Enterobacteriaceae* and *Bacteroides* predominated in both infants during breast-feeding (*Wang* et al., 2004).

As the two infants analyzed above participated in our longitudinal study of the establishment of the intestinal microflora in Swedish infants, we could compare the results using T-RFLP with the results of traditional culture (*Lindberg*, 2004). We found that culture, by use of selective media, was superior to T-RFLP for detection of sub-dominant groups, including facultative bacteria such as *E. coli*, *S. aureus*, as well as anaerobes of low population numbers, mainly lactobacilli. Bacteria that instead were commonly missed by culture were mainly strictly anaerobic bacteria, e.g. those belonging to the genera *Eubacterium*, *Veillonella*, *Ruminococcus* and *Fusobacterium*, for which no selective media

were used. In addition, a wider spectrum of *Bacteroides* species was detected with T-RFLP than with culture.

So far, the results obtained when using molecular methods for analyses of the infantile intestinal microbiota have mostly confirmed what has been found using conventional culture techniques. Although the relative proportion of some bacterial groups in the early microbiota seem to be underestimated by culture (*Harmsen* et al., 2000), a high proportion of the early intestinal colonisers seem to be detectable with traditional culture methods. However, with age the complexity of the microbiota increases, and in samples from older infants it is likely that culturing only detects a fraction of all different bacterial species present. Further development and application of molecular methods in studies of the establishment of the intestinal microbiota will greatly increase our knowledge on intestinal ecology in early life.

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