

The bacterial colonization of the large bowel of pre-term low birth weight neonates

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(Received 13 November 1981; accepted 1 December 1981)

SUMMARY

The bacterial colonization of the large bowel of 11 pre-term, low birth weight neonates who were nourished by expressed breast milk was examined by culturing serial faecal samples and compared to that observed in eight breast-fed and seven formula-fed full-term neonates.

Pre-term neonates were colonized by high counts of facultatively anaerobic bacteria from the first days of life while bifidobacteria colonized only six babies during the first week and appeared in only one baby before day 5. *Bacteroides* spp. and clostridia were isolated from seven and six pre-term babies respectively during week 1 and were first observed on day 2.

The intestinal colonization of pre-term infants differed from that in full-term breast-fed infants in the high counts of facultatively anaerobic bacteria and late appearance of bifidobacteria, and from both groups of full-term infants in the early stable colonization by *Bacteroides* spp.

It is postulated that the composition of the normal intestinal microflora of pre-term low birth weight babies contributes to their predisposition to neonatal necrotizing enterocolitis. Results are discussed in relation to the effect of infant feeding regimens on intestinal microbial populations.

INTRODUCTION

In the past 10 years the survival rate of pre-term low birth weight infants has greatly increased due to improved standards of neonatal intensive care. This group of infants represents a new population for which many of the parameters of normal development must be redefined, among them the bacterial colonization of the gastrointestinal tract. There are many factors related to physiological immaturity and methods of neonatal care which are likely to influence the bacterial colonization of pre-term neonates. These infants often spend the first days of life in the relative isolation of a humidicrib screened from many of the exogenous bacteria ingested by the full-term neonate and, being unable to feed directly from the breast, are nourished by expressed breast milk or formula from bottle or tube.

The bacterial populations of the large bowel of pre-term neonates are of

particular interest because of their role in neonatal necrotizing enterocolitis (NEC). This disease occurs primarily in pre-term low birth weight neonates and is generally believed to be due to invasion of a damaged intestinal mucosa by members of the normal gut microflora particularly clostridia and enterobacteria (Bell, Feigin & Ternberg, 1979; Cashore *et al.* 1981; Howard *et al.* 1977). The succession of intestinal bacteria in pre-term infants has not been well documented. It is possible that the composition of the intestinal microflora of pre-term neonates contributes to their predisposition to NEC.

This paper reports results of a prospective study carried out to define the early bacterial colonization of the large bowel of pre-term infants fed expressed breast milk (EBM) and compare it to the colonization of full-term infants who have been breast or formula-fed.

MATERIALS AND METHODS

Subjects

Twenty-six babies born at the Women's Hospital, Crown Street, Sydney were studied. Eleven were pre-term low birth weight babies with gestational ages ranging from 30 to 35 weeks (median 33 weeks) and birth weights ranging from 1440 to 2300 g (median 1920 g). All pre-term babies received EBM which had been pooled from a number of donors, frozen during storage and heated to 100 °C to destroy contaminating bacteria before use. Fifteen were full-term deliveries and of these seven received formula from birth and eight were breast-fed. None of the babies received antibiotic therapy during the course of the survey.

Formula-fed babies received either S26 (Wyeth) or Nan (Nestlé), both of which were prepared by following the manufacturer's instructions.

Specimen collection and transport

Between three and six faecal specimens were collected from each baby during the first week of life. Twelve of the full-term babies were sampled again at 4 weeks of age after they had returned home. Further specimens were collected at weekly intervals from the pre-term babies for as long as they remained in hospital, eight of the babies being sampled beyond one week of age. Faecal specimens (approximately 0.5 g) were collected into bijoux bottles containing 4.5 ml prerduced salts solution (Holdeman & Moore, 1975), 10% glycerol, cysteine HCl (0.5 g/l) and resazurin (1 mg/l) overlaid with a thin layer of paraffin oil. Samples were frozen at -60 °C on the day of collection (Crowther, 1971).

Culture and identification of organisms

Faecal specimens were diluted and cultured inside an anaerobic chamber containing an atmosphere of 10% H₂ in CO₂. The following range of media was used: supplemented brain heart infusion agar (Holdeman & Moore, 1975) for the non-selective culture of all anaerobes; vancomycin-kanamycin agar (Finegold, Sugihara & Sutter, 1971) for *Bacteroides* spp., *Fusobacterium* spp. and *Veillonella* spp.; tomato juice agar (Finegold, Sugihara & Sutter, 1971) for bifidobacteria;

Table 1. Anaerobic bacteria in the faeces of pre-term and full-term infants during the first week of life

Organism	Gestational status	Feeding	Isolation rate*	
Bifidobacteria	Pre-term	EBM†	6/11	(55 %)
	Full-term	Breast	7/8	(87 %)
	Full-term	Formula	5/7	(71 %)
Bacteroides	Pre-term	EBM	7/11	(64 %)
	Full-term	Breast	5/8	(63 %)
	Full-term	Formula	1/7	(14 %)
Clostridia	Pre-term	EBM	6/11	(55 %)
	Full-term	Breast	5/8	(63 %)
	Full-term	Formula	4/7	(57 %)
Anaerobic Gram-positive cocci	Pre-term	EBM	2/11	(18 %)
	Full-term	Breast	1/8	(12 %)
	Full-term	Formula	0/7	—

* Isolation rate expressed as number of babies from whom organism isolated/number of babies examined.

† EBM = expressed breast milk.

Willis and Hobbs agar (Willis & Hobbs, 1959) for clostridia; MRS (de Man, Rogosa & Sharpe – Oxoid) for lactobacilli; sheep blood agar and MacConkey agar for facultatively anaerobic and aerobic bacteria. Faecal suspensions were heat shocked at 80 °C for 12 min before inoculation of Willis and Hobbs agar and all incubations carried out inside the anaerobic chamber for 48 h except for MRS plates which were incubated in a candle jar and plates inoculated for aerobic growth.

Identification of anaerobic isolates was based on Gram stain morphology, G.L.C. analysis of volatile fatty acids and non-volatile acids produced as end-products of carbohydrate metabolism, spore tests and sugar fermentation reactions. The methods used have been described in detail elsewhere (Stark & Lee, 1982).

RESULTS

Serial faecal samples from 11 pre-term low birth weight babies were cultured to identify the major groups of bacteria colonizing the large bowel during the neonatal period. Patterns of colonization were compared to those observed in 15 full-term neonates, eight of whom were breast-fed and seven formula-fed. All of the pre-term babies were colonized by facultatively anaerobic bacteria during the first week of life and 10 babies were also colonized by anaerobic bacteria, the most common genera being *Bifidobacterium*, *Bacteroides* and *Clostridium*. Isolation rates for these anaerobes varied between pre-term and full-term infants as did the ages when colonization commenced, as shown in Table 1 and Fig. 1.

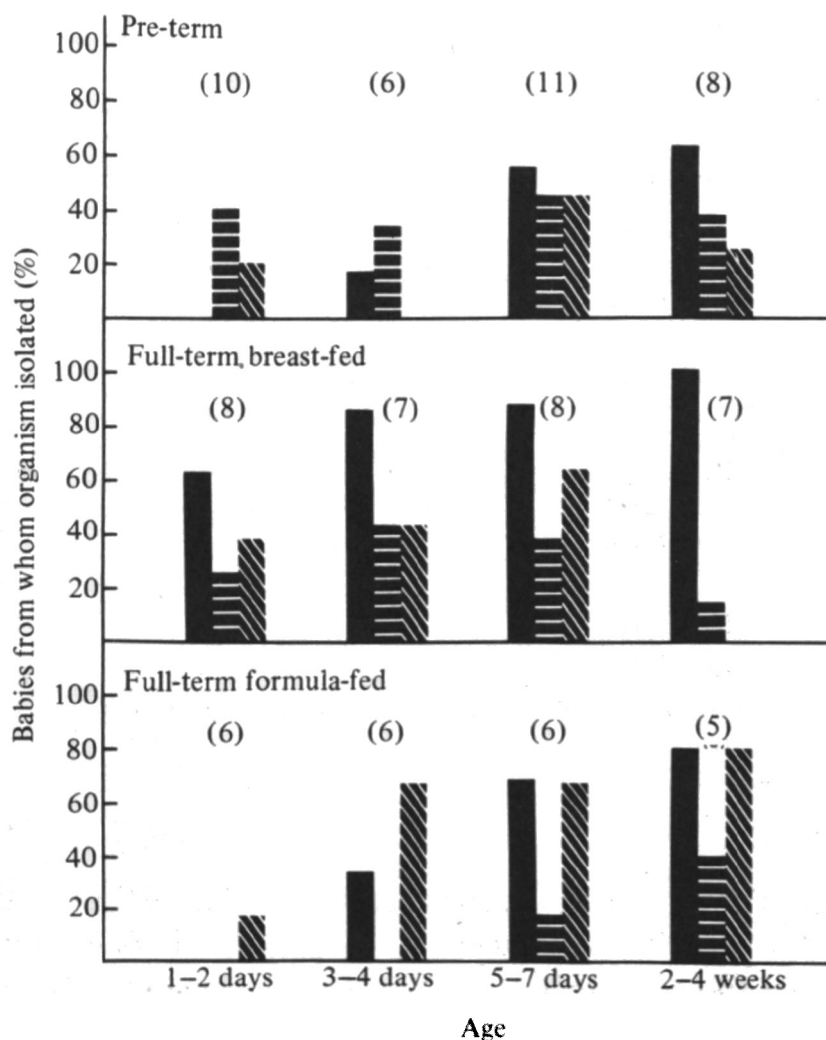


Fig. 1. Colonization of the large bowel of pre-term and full-term neonates by anaerobic bacteria (■, bifidobacteria; ▨, *Bacteriodes* spp; ▩, Clostridia). Number of babies sampled in parenthesis.

(a) *Bifidobacteria*

Bifidobacteria were isolated less frequently from pre-term babies than from full-term babies. Colonization by these organisms was observed in only one pre-term baby before day 5 and in six of the 11 pre-term babies by day 7. Of eight pre-term babies who were followed up beyond 7 days of age, three were still negative on discharge from hospital at between 2 and 4 weeks of age.

By comparison, 12 of the 15 full-term babies were colonized by bifidobacteria during the first week of life. Colonization of the breast-fed babies generally commenced on day 2 whereas in formula-fed infants it was first observed on day 4. Of the 12 full-term infants observed on week 4, only one did not yield bifidobacteria.

Sampling intervals enabled the day of colonization to be determined for eight of the pre-term babies and seven of the full-term breast-fed babies as shown in Table 2. The difference in the time at which colonization commenced in the two groups was found to be significant using a Chi square analysis, $P = 0.05$.

Viable counts of bifidobacteria in full-term babies rose quickly and were within the range 10^9 to 10^{11} organisms/g wet faeces by 4 weeks of age. Levels of the

Table 2. Age of first colonization of pre-term and full-term babies by bifidobacteria

Group of babies	Number of babies first colonized at specified age (days)				Total number of babies
	1-2	3-4	5-7	> 7	
Full-term (breast-fed)	5	2	0	0	7
Pre-term	0	0	3	5	8

organism remained lower in some of the pre-term babies, counts of only 10^5 to 10^7 organisms/g wet faeces being recorded for three babies at last examination between 2 and 4 weeks.

(b) *Bacteroides spp.*

Bacteroides spp. were isolated frequently from pre-term and full-term breast-fed babies but from only one full-term formula-fed baby. Seven of the 11 pre-term babies yielded the organism between 2 and 7 days of age. *Bacteroides spp.* were isolated from three babies who were sampled again at week 3, indicating that colonization had occurred. By comparison, isolations were recorded from five of the eight breast-fed full-term babies during week 1 but from none of this group at week 4, suggesting that *Bacteroides spp.* has only a transient presence in the large bowel of these babies. Only one of the formula-fed babies yielded *Bacteroides spp.* during the first week of life and this baby was still colonized at week 4. Counts of *Bacteroides spp.* fluctuated widely in all babies ranging from 10^4 to 10^{11} organisms/g wet faeces.

(c) *Clostridia*

Clostridia were isolated from six of the 11 pre-term babies and eight of the 15 full-term babies during the first week of life commencing on day 2. Counts in the three groups of babies were similar ranging from 10^3 to 10^7 spores/g wet faeces. Further examinations carried out after the first week yielded clostridia from two pre-term babies and four formula-fed full-term babies but none of the breast-fed full-term babies.

(d) *Facultatively anaerobic bacteria*

Enterobacteria and enterococci colonized all babies from the first days of life. From day 3 onwards counts of enterobacteria in pre-term babies were significantly higher than counts in full-term breast-fed babies but similar to counts in full-term formula-fed babies, as shown in Table 3. Counts of above 10^{10} organisms/g wet faeces were recorded for seven of 11 pre-term babies during the first week compared to four of seven full-term formula-fed infants and only one of eight full-term breast-fed infants.

Counts of enterococci were more variable during the first week of life but in the two to four week age group counts in the pre-term babies were again significantly

Table 3. *Levels of facultatively anaerobic bacteria in the faeces of pre-term and full-term infants*

Organism	Group of babies	Viable count* of organisms at specified age			
		1-2 days	3-4 days	5-7 days	2-4 weeks
Enterobacteria	Pre-term	8.7 ± 2.5 (9)†	9.3 ± 1.9 (6)	9.4 ± 0.98 (18)	9.5 ± 0.85 (16)
	Full-term (breast-fed)	8.1 ± 1.2 (6)	7.8 ± 2.2‡	7.2 ± 2.2‡	6.1 ± 1.9‡ (6)
	Full-term (formula-fed)	8.9 ± 0.56 (4)	9.4 ± 1.4 (9)	9.6 ± 0.65 (7)	9.5 ± 0.71 (5)
Enterococci	Pre-term	6.3 ± 1.5 (7)	8.4 ± 1.3 (5)	8.2 ± 0.91 (17)	8.4 ± 1.4 (15)
	Full-term (breast-fed)	8.5 ± 1.8 (6)	9.5 ± 1.1 (7)	7.8 ± 2.1‡ (11)	6.2 ± 1.2 (5)
	Full-term (formula-fed)	9.3 ± 0.28 (3)	8.3 ± 0.99 (5)	9.5 ± 2.1 (7)	9.7 ± 0.47 (5)

* Viable count expressed as a mean log₁₀ number of organisms/g wet faeces ± I.S.D.

† () = number of specimens

‡ Counts which are significantly different from corresponding values in pre-term babies.

higher than in full-term breast-fed babies though lower than full-term formula-fed babies. (All tests of significance made using Student's *t* test, $P < 0.05$.)

(e) *Other genera*

Anaerobic Gram-positive cocci were isolated from two pre-term babies and one full-term breast-fed baby, lactobacilli from one full-term breast-fed baby and *Veillonella* spp. from one full-term formula-fed baby during the first week of life.

DISCUSSION

The aim of this investigation was to compare the bacterial colonization of the large bowel of pre-term and full-term neonates. None of the babies studied received antibiotic therapy during the course of the study. As is usual in investigations of intestinal bacteria in humans, the faecal bacterial populations are assumed to be representative of the bacteria of the lumen of the large bowel. Other authors have verified this assumption (Moore, Cato & Holdeman, 1978). Although the number of babies studied was relatively small, results indicate that the succession of bacterial populations in the large bowel of pre-term neonates fed EBM differs from that in both breast and formula-fed full-term neonates. Differences were observed in the ages at which bifidobacteria and *Bacteroides* spp. became established and in the levels of facultatively anaerobic bacteria.

The succession of bacteria in the faeces of breast-fed neonates has already been described and results found in this study are in broad agreement with earlier reports (Mata & Urrutia, 1971; Rotimi & Duerden, 1981). Facultatively anaerobic

bacteria colonize from the first days of life followed closely by bifidobacteria. Levels of facultatively anaerobic bacteria fall by the third day and Bullen and co-workers (1976*a, b*) have attributed their suppression to the establishment of an acetate and acetic acid buffer of low pH in the intestinal lumen. The bifidobacteria quickly reach high levels to become the predominant organisms although other anaerobes such as *Bacteroides* spp., clostridia and anaerobic streptococci are also found. A long term study previously carried out in this laboratory showed that anaerobes other than bifidobacteria tend not to persist in breast-fed infants during the period of exclusive breast feeding (Stark & Lee, 1982). The succession of organisms in the faeces of formula-fed neonates is marked by higher levels of facultatively anaerobic bacteria while colonization by bifidobacteria generally commences several days later. Hewitt & Rigby (1976) have also found that the incidence of bifidobacteria in seven day old formula-fed infants is lower than that reported for breast-fed infants of the same age. Anaerobic bacteria other than bifidobacteria are also found in the faeces of formula-fed infants during the first week of life and these persist beyond the neonatal period (Stark & Lee, 1982). *Bacteroides* spp. were isolated more frequently from breast-fed neonates than from formula-fed neonates. This finding differs from reports by other authors (Long & Swenson, 1977) and was therefore confirmed by examination of a further 20 babies aged between 4 and 5 days.

The pre-term babies were also colonized by facultatively anaerobic bacteria from the first days of life and these remained at high levels resembling the full-term formula-fed babies. Thus the control mechanisms limiting the multiplication of these organisms do not operate in the pre-term or formula-fed neonates. Bifidobacteria did not appear during the first few days of life and by 7 days only half the babies were colonized. This delay in colonization was most marked in comparison with breast-fed full-term neonates but the organisms seemed to appear even later than in the full-term formula-fed babies. *Bacteroides* spp. were also found to colonize about half the pre-term babies from the first days of life and unlike their transient appearance in full-term breast-fed infants, they persisted beyond the first week. By 7 days of age two pre-term babies yielded *Bacteroides* spp. as the sole anaerobes, one *Bacteroides* spp. and clostridia, one only clostridia, and one had no anaerobes at all.

There have been few previous reports of the intestinal bacterial colonization of pre-term infants. Gothefors & Blenkarn (1978) have investigated the faeces of four pre-term neonates aged between 21 and 36 days and only the oldest of these yielded bifidobacteria. Bell *et al.* (1978) have identified bacterial groups cultured from rectal swabs from 41 infants, 30 of whom were pre-term, and reported the isolation of Gram positive anaerobes from only 2% of babies. All of these babies had, however, received formula and 75% were receiving antibiotics at the time of sampling.

The intestinal bacterial populations of pre-term infants are of particular interest due to their role in NEC, a disease occurring primarily in this group of neonates. It is widely accepted that NEC results from bacterial invasion of the gut wall following ischaemic damage. Reported isolations from the blood and peritoneal fluid of patients almost invariably include members of the normal gut microflora

(Kliegman, 1979), although some cases have been attributed to definite pathogens such as *Clostridium botulinum* (Edmund, 1979) and salmonella (Stein *et al.* 1972). The composition of the intestinal microflora of pre-term neonates could contribute to the predisposition of these infants to NEC in the following ways. The high counts of faecal enterobacteria indicate that pre-term infants harbour a large pool of potentially opportunistic pathogens. *Klebsiella pneumoniae* and *E. coli* are frequently isolated from the peritoneal fluid of NEC patients (Kliegman, 1979) and Bell *et al.* (1979) have suggested that these organisms are related to the pathogenic process. In addition, the delayed colonization of pre-term babies by bifidobacteria may favour the proliferation of pathogenic organisms should they gain entrance to the gut.

Clostridia have also been implicated as putative pathogens in NEC (Howard *et al.* 1977; Cashore *et al.* 1981). This group of bacteria was isolated with equal frequency and in equal numbers from both pre-term and full-term neonates. Thus the predisposition of pre-term babies to NEC is probably not due to a greater incidence of colonization by these organisms. The presence of clostridia in the intestine of pre-term neonates may, however, be significant at times of damage to the gut wall. Furthermore, the gut ecosystem of the pre-term infant has been shown to be different from that of the full-term infant. Differences in environmental conditions in the gut ecosystem of the pre-term compared to the full-term neonate may potentiate the pathogenic properties of clostridia such as toxin production and invasiveness.

It is difficult to predict the exact reasons for observed differences in colonization patterns. Differences between full-term breast and formula-fed infants are presumably determined by diet, however, the pre-term babies differ from the full-term group not only in diet but also in a variety of physiological parameters associated with their gestational age and in their neonatal care. Pre-term babies received EBM which had been pooled from a number of donors, frozen during storage and heated to 100 °C before use to kill contaminating bacteria. This treatment has been found to destroy macrophage and lysozyme activity, IgA and iron binding capacity by the protein lactoferrin in human milk (Ford *et al.* 1977).

It has been suggested that feeding pre-term neonates on EBM offers protection against NEC. Animal studies have shown that mononuclear phagocytes contained in breast milk protect infant rats from a disease resembling NEC in humans (Pitt, Barlow & Heird, 1977). The present studies suggest that feeding high risk neonates EBM which has been frozen and heated may not be of significant value in this respect, not only because the cellular component of the milk is destroyed but also because of the type of intestinal microflora induced.

These preliminary findings have important implications for the care of pre-term low birth weight infants and emphasize the need for further study of the intestinal bacterial colonization of this group of neonates particularly in relation to the effect of diet. It has been shown that feeding pre-term infants EBM which has been frozen and heated does not induce the intestinal microflora which is typical of full-term breast-fed infants and it is suggested that the altered balance of intestinal

organisms contributes to the predisposition of these individuals to NEC. The possibility that modifying the feeding regimen of pre-term neonates may offer a greater degree of protection against this disease warrants investigation.

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