BIFIDOBACTERIA IN THE INTESTINAL TRACT OF INFANTS: AN IN-VIVO STUDY

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PLATE XXIII

IT has been recognised for many years that breast-fed babies are relatively resistant to gastro-enteritis (Alexander, 1948; Ross and Dawes, 1954; Hinton and MacGregor, 1958). Various explanations have been put forward to account for this resistance, including passive transfer of antibodies to *Escherichia coli* in colostrum (Sussman, 1961; Michael, Ringenback and Hottenstein, 1971), contamination of artificial feeds during preparation (Neter, 1959), and the nature of the intestinal environment (Ross and Dawes, 1954). The continuing incidence of enteropathogenic *E. coli* infections in young infants, together with the decline in the popularity of breast feeding, has led us to re-examine this problem.

Breast-fed infants differ from bottle-fed infants in the microbiological and physico-chemical properties of the faeces and there can be little doubt that both properties must be greatly influenced by the nature of the feed. From in-vitro studies of breast-fed infants, a number of factors seem likely to influence the production and maintenance of the bifidobacterial flora and low pH value characteristic of the faeces of newborn infants.

There may well be specific factors in human milk that either encourage the growth of bifidobacteria or suppress that of *E. coli*. György (1953) showed that breast milk contained a factor that was essential for the growth of one strain of lactobacillus. An entirely different bifidogenic factor, lactulose, was studied by Petuely and Kristen (1949), and subsequently by MacGillivray, Finlay and Binns (1959). The addition of 1% of lactulose to modified cows' milk preparations induced in infants a predominance of lactobacilli in the faeces, but this was not accompanied by a consistently low pH value.

J. J. Bullen and his colleagues (Bullen, Rogers and Leigh, 1972; Bullen, Rogers and Griffiths, 1974) showed that human milk can have a specific inhibitory effect on *E. coli* due to its high content of iron-binding proteins, predominantly lactoferrin. They found that lactoferrin, in combination with specific antibody to *E. coli*, had a powerful bacteriostatic effect, which was abolished when the lactoferrin was saturated with iron.

The results of our earlier studies (Bullen and Willis, 1971) are in general agreement with the observations and conclusions of Ross and Dawes (1954). Although we do not exclude the possible role of other more specific factors, our findings point to the importance of the ingredients and properties of cows' milk, which seem to provide an intestinal content that is unfavourable both to the growth of bifidobacteria and to the production of an acid environment.

During a feeding trial with an artificial milk designed to mimic breast milk (Willis *et al.*, 1973), it was noted that although the mean pH value of the faeces from the breast-fed group of infants was 5.0, the pH of some individual samples

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was as high as 7.0—yet in all these samples the *E. coli* counts were relatively low. This was a little puzzling because although it is well known that the growth rate of *E. coli* is retarded at pH 5.0, the growth rate of the organism should be unimpeded at pH 7.0. It had been noted also that faeces from breast-fed infants have a “cheesy”, acetic odour (fermentative), in contrast to that of bottle-fed infants whose faeces are always putrefactive. These differences suggested that products of bacterial metabolism may be important determinants of the final bacterial flora.

Early in-vitro observations had shown that acetate buffer (acetic acid and sodium acetate) was a useful agent for the isolation of bifidobacteria, since it inhibited the growth of gram-negative species. Moreover, acetic acid is a common metabolite of saccharolytic bacteria. It was decided, therefore, to seek the presence of an acetate buffer in the faeces of breast-fed infants.

MATERIALS AND METHODS

Test and control feeding groups. A small survey was carried out with normal bottle-fed and breast-fed infants. Weekly faecal samples were examined for their viable bacterial counts, their pH values, and for the volatile products of bacterial metabolism. Eighteen babies were involved, eight breast-fed and ten bottle-fed. In general, samples were examined from birth to the end of the 8th week of life. A 24-h supplementary bottle (dried cows' milk preparation) was fed to all the breast-fed infants for the first 8 days of life. Some of the breast-fed infants began mixed feeding before the age of 8 weeks. Faecal specimens were stored at 4°C for not more than 7 days.

Various factors prevented us from following through all the babies in either group for the full 8-week period. Thus, some infants could not be followed up after discharge from hospital on the 8th day. Some of the breast-fed infants changed to mixed feeding either with cereal or supplementary bottle feeding before the 8-week feeding trial was completed. Consequently, the recorded results show no continuity from week to week as to the number of infants studied in each group. The number of infants providing samples of faeces each week up to the 8th week is given in table I.

Bacterial studies. These included a record of the pH values of 10% faecal suspensions in 0.15M-NaCl solution and viable counts of the aerobic and anaerobic flora with special reference to *Enterobacteriaceae*, staphylococci, streptococci, yeasts, bacteroides, bifidobacteria and clostridia. Viable counts were made by the method of Miles, Misra and Irwin (1938) after incubation at 37°C for 24 h for aerobic organisms. The anaerobic atmosphere contained 10% CO₂. The media and methods were those used by Willis *et al.*, (1973).

Chromatographic analysis. This was performed upon 25% aqueous suspensions of faecal material. The procedure for the analysis of acid products was that recommended by Holdeman and Moore (1972), the faecal suspensions being examined before and after acidification, and the volatile fatty acids present recorded.

RESULTS

An acetate buffer in faeces

The faecal material from breast-fed infants produced chromatographic patterns that showed a marked increase in the acetic acid content after acidification (fig. 1). Thus, the acetic acid was present in both the free and combined forms. At no time was an acetate buffer demonstrated in the faeces of bottle-fed infants (fig. 2). All breast-fed infants produced an acetate buffer in their faeces

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FIG. 1.—Chromatograms of the faeces of a breast-fed infant. (a) Acetic acid before acidification of the suspension, and (b) acetic acid after acidification. The difference between the two curves represents the presence of acetate in the original sample.

FIG. 2.—Chromatograms of the faeces of a bottle-fed infant. (a) No acetic acid was present before acidification of the suspension, but (b) acetic acid and other volatile acids were present after acidification.

TABLE I

The distribution of volatile fatty acids in the faeces of breast-fed and bottle-fed infants during the first 8 weeks of life

Type of feeding	Age of infants (weeks)	Number of infants providing a faeces sample	Number of faeces samples in which the stated volatile fatty acid was found						
			Acetic	Propionic	Isobutyric	Butyric	Isovaleric	Valeric	Isocaproic
Breast	1	7	7	3	0	2	0	1	0
	2	5	5	2	0	0	0	0	0
	3	7	7	2	0	1	0	0	0
	4	5	5	1	0	1	0	0	0
	5	4	4	1	0	0	0	0	0
	6	4	4	0	0	0	0	0	0
	7	2	2	0	0	0	0	0	0
	8	1	1	0	0	0	0	0	0
Bottle	1	10	10	10	9	10	9	0	0
	2	4	4	4	3	4	4	2	1
	3	4	4	4	4	4	4	0	0
	4	4	4	4	2	3	3	0	0
	5	3	3	3	2	3	3	1	0
	6	2	2	2	2	2	2	1	1
	7	2	2	2	2	2	2	0	0
	8	2	2	2	2	2	2	1	0

TABLE II

The appearance of volatile fatty acids in the faeces of breast-fed infants following the introduction of supplementary feeding in the 4th week of life

Age of infants (weeks)	Number of infants providing a faeces sample	Number of faeces samples in which the stated volatile fatty acid was found						
		Acetic	Propionic	Isobutyric	Butyric	Isovaleric	Valeric	Isocaproic
4	1	1	1	0	0	0	0	0
5	2	2	2	0	1	0	0	0
6	2	2	2	0	2	1	0	0
7	2	2	2	1	1	1	0	0
8	3	3	2	2	3	2	1	1
9	3	3	3	2	3	2	1	1
10	2	2	2	0	2	1	1	1

at some time during the 8-week period of suckling and for a short time after mixed feeding was introduced.

Volatile fatty acids in faeces

The acidified faecal suspensions from breast-fed babies contained acetic acid at all times throughout the 8 weeks. Propionic and butyric acids appeared occasionally in the early weeks of suckling. The acidified suspensions from bottle-fed babies on the other hand always contained both acetic and propionic acids, and commonly also isobutyric, butyric and isovaleric acids (fig. 2 and table I). Infants that began mixed feeding from the 4th week of suckling showed an increasing variety of volatile fatty acids in the faeces as the supplementary feeding gradually replaced breast feeding (table II).

FIG. 3.—Mean pH values of faecal suspensions from breast-fed infants, ●—●; bottle-fed infants, ⊗—⊗; and breast-fed infants receiving supplementary feeds, ○—○. Observations were made during the first 10 weeks of life.

pH values of faeces

The mean faecal pH values were recorded weekly. Those in breast-fed infants were between pH 5 and 6 throughout the 8 weeks. In the bottle-fed group the values were in the range of pH 8–9 (fig. 3). Breast-fed infants who received supplementary feeding showed values commencing between pH 5 and 6 and rising to between pH 6 and 8 during a 6-week period (fig. 3).

Mean viable bacterial counts

Breast-fed infants. After the 1st week of the breast-fed infants' life, during which supplementary artificial feeds were given, the counts of the faecal bacteria showed a predominance of bifidobacteria over *Escherichia coli* and *Streptococcus faecium* (fig. 4). The clostridia and bacteroides were generally present in small numbers; the counts of bacteroides remained between 10^4 and 10^7 per g, while the clostridia (*Clostridium perfringens* and *Clostridium paraputrificum*) disappeared altogether by the 7th week. No other organism was encountered.

Bottle-fed infants. These differed from breast-fed infants in that the counts of bifidobacteria were well below those of *E. coli* and *S. faecium* (fig. 5). Moreover, after the 1st week of life the number of bacteroides and *C. paraputrificum* remained above 10^7 and counts for *C. perfringens* never fell below 10^5 per g. Other gram-negative organisms (*Proteus* and *Klebsiella* species) were isolated from five infants. Staphylococci and yeasts were not encountered.

FIG. 4.—Mean viable counts of faecal organisms isolated from breast-fed infants during the first 7 weeks of life: ●—●, bifidobacteria; ○—○, *Escherichia coli*; ▲—▲, *Streptococcus faecium*; □—□, bacteroides; ■—■, clostridia.

Infants during weaning. When mixed feeding was introduced the counts of bifidobacteria fell steadily during the following six weeks (fig. 6). The counts of *E. coli* and *S. faecium* rose to 10^8 per g and above. The clostridia reappeared in the flora and their counts, like that of bacteroides, stabilised at around 10^6 per g. Staphylococci and yeasts were not encountered.

DISCUSSION

The results outlined above endorse the conclusion reached in an earlier feeding trial (Willis *et al.*, 1973) that one important factor that prevents the outgrowth of *E. coli* and other *Enterobacteriaceae* in the gut of the breast-fed infant is the accumulation of acetate buffer. Although it has been customary to attribute the acidic faeces of breast-fed infants to the characteristic preponderance of bifidobacteria, the converse seems more likely.

While we are convinced that the primary factor required to ensure an acidic faeces is a feed of low buffering capacity, production of acid is clearly dependent on the presence of lactose-fermenting organisms. The bacteria most commonly encountered in this investigation, *E. coli*, *S. faecium* and bifidobacteria, all fall

FIG. 5.—Mean viable counts of faecal organisms isolated from bottle-fed infants during the first 8 weeks of life: ●—●, bifidobacteria; ○—○, *E. coli*; ▲—▲, *S. faecium*; □—□, bacteroides; ■—■, clostridia.

into this category. Probably preliminary colonisation of the gut by *E. coli* and *S. faecium* is an essential precursor for the subsequent rapid outgrowth of bifidobacteria. In breast-fed infants these facultative organisms would produce the conditions of a falling pH and Eh which are so favourable to the growth of bifidobacteria. In bottle-fed infants receiving cows' milk, on the other hand, this "starter effect" would be nullified by the high buffering capacity of the feed.

The in-vivo results reported in the present study clearly show that as the pH of the infant's large gut contents falls, significant amounts of acetate buffer accumulate. This not only favours the continued growth of bifidobacteria but produces conditions that are unfavourable to the growth of *E. coli*. This controlling influence that acetate buffer appears to have on the infant's faecal flora is further supported by the results of in-vitro studies (Bullen and Tearle, 1976).

The presence of organisms other than bifidobacteria, *E. coli*, and *S. faecium* in the faeces of infants is characteristic of babies fed on a cow's milk preparation, and has been regarded as the cause of the putrefactive faeces of infants fed on cows' milk as opposed to the fermentative faeces of breast-fed children (Haenel, 1961). Our findings are in accord with these observations; not only were

FIG. 6.—Mean viable counts of faecal organisms isolated from breast-fed infants receiving supplementary feeds from the 4th to the 11th week of life: ●—●, bifidobacteria; ○—○, *E. coli*; ▲—▲, *S. faecium*; □—□, bacteroides; ■—■, clostridia. (↑ = commencement of supplementary feeding.)

organisms such as *Proteus* spp., *Pseudomonas aeruginosa*, and clostridia rarely present in the faeces of breast-fed babies, but such faeces were of the fermentative type. Moreover, in all infants who were followed bacteriologically after changing from breast milk to mixed feeding, these organisms started to appear as the faeces changed from the fermentative to the putrefactive type.

It is interesting that the faeces of breast-fed infants tend to undergo this change at any time when non-breast milk supplements are given. Thus, putrefactive faeces are produced by infants who receive supplementary feeding immediately after birth; once breast feeding is fully established, however, the faeces change to the fermentative type.

It would be unrealistic to suggest that the mechanisms we have studied are the only ones that determine the physiochemical and microbiological nature of the large bowel content of the infant. The studies of Bullen *et al.*, (1972) and Bullen *et al.*, (1974) lay emphasis on suppression of *E. coli* by specific antibody acting in the presence of iron-binding proteins. This, and other possible mechanisms in the defence against infection in the newborn, have been briefly reviewed by Hanson and Winberg (1972).

SUMMARY

Weekly faecal specimens from 18 babies were examined during the first 8 weeks of life. Eight infants were breast fed, ten were bottle-fed. All suckling infants received supplementary feeds for the first 8 days.

A buffer consisting of acetic acid and acetate was demonstrated in the faeces of all the breast-fed infants at some time during the period of examination. This buffer was rarely detected during the 1st week of life when supplementary feeds were given, and buffer already present gradually disappeared with the introduction of mixed feeding. In contrast, at no time was an acetate buffer demonstrated in the faeces of bottle-fed infants. Babies receiving breast milk produced faeces with low pH, high counts of saccharolytic organisms including bifidobacteria and *Streptococcus faecium*, and low counts of *Escherichia coli*, bacteroides and clostridia. Bottle-fed infants on the other hand produced faeces with a high pH and high counts of *E. coli* and putrefactive bacteria, but with low counts of bifidobacteria.

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