Archives of Disease in Childhood, 1982, **57**, 54–58

Effect of feeding on infants' faecal flora

A SIMHON, J R DOUGLAS, B S DRASAR, AND J F SOOTHILL

Department of Immunology, Institute of Child Health, Department of Microbiology, London School of Hygiene and Tropical Medicine, and Department of Child Health, St Bartholomew's Hospital, London

SUMMARY In newborn English infants, the predominant faecal bacteria were coliforms and bacteroides, as shown by Gram film, culture, and gas-liquid chromatography, whether they were bottle fed or exclusively breast fed. This contrasted with bifidobacterial predominance in faeces from breast-fed Nigerian infants. Presumably environmental factors other than exclusive breast feeding are also important for establishing the flora. No differences were detected between the flora of infants of atopic and non-atopic controls.

There have been many reports,[1 2] including studies from England,[3 4] showing that the faeces of breast-fed infants, unlike those of bottle-fed babies, have bifidobacteria as the predominant organism. In two other recent studies[5 6] bifidobacteria predominated in bottle-fed infants as well as in breast-fed ones. Exclusive breast feeding protects against eczema[7] and probably against other atopic allergy,[8] and the genetic component of atopic disease is, at least partly, one of a number of common minor immunodeficiencies.[9] We have speculated that a Gram-negative flora might have an adjuvant role in atopic sensitisation,[10] and Jarrett *et al.*[11] showed the importance of adjuvants in IgE responses to ingested antigens. We therefore studied the faecal flora of breast- and bottle-fed babies of atopic and non-atopic parents to see whether they differed on either regimen, and were surprised to find Gram-negative rod predominance in all groups.

Materials and methods

Fourteen newborn infants of parents with a history of atopy (that is the mother or father had a history of asthma, hay fever, or eczema), and 15 newborn infants to non-atopic controls were recruited at the maternity wards of St Bartholomew's Hospital, London. Three mothers withdrew from the study, and 2 were not studied for technical reasons. Non-white mothers were excluded because of possible differences in the incidence of atopy,[12] and those without a telephone were excluded. The sample was essentially of middle and lower-middle socio-economic class. No special attempt was made to influence the mothers' decision on the type of feeding. Seventeen infants were exclusively breast fed as reported by nurses and mothers. Two of 12 bottle-fed infants had been breast fed for 5 to 7 days after birth. We also studied the faeces of 12 breast-fed Nigerian infants born under village conditions (age <1 year) which had been frozen in liquid nitrogen as 10% suspensions in beef extract-glycerol broth.[13]

Infants' faeces. Samples at about 2, 4, and 6 weeks of age were collected from the homes by one person (AS) and were processed within 6 hours of evacuation. Eighty-one samples were studied, since only 2 were obtained from 6 of the infants. Broth and saline suspensions were made. Between 0·5 and 1·0 g of faeces were transferred to bottles containing beef extract-glycerol broth to produce 10% suspensions as calculated by weight difference. Two normal saline suspensions were made: one at about 10% and the other at about 25%. Bottles were immediately frozen at -70°C. A smear of fresh faeces was also stained with Gram's stain; 3 morphological groups (Gram-negative rods, Gram-positive cocci, and Gram-positive rods) were numerically graded (few, moderate, very numerous).

pH values. The 10% saline suspensions were tested in a single batch using a Pye Unicam Model 291 pH meter.

Gas-liquid chromatography. These analyses were carried out at the Public Health Laboratory, Luton, using the 25% saline suspension. Volatile fatty acids in each acidified faecal suspension and its non-acidified control were ether-extracted,[4] injected into the column of a Pye Unicam 204 Series Chromatograph, and identified by their elution times in comparison with a standard mixture.

Bacterial culture. Ten per cent faecal suspensions in glycerol broth were stored at −70°C for up to 3 weeks. Serial 10-fold dilutions were made in brain heart infusion containing reducing agents.[14] The media used for counting the various bacteria, with typical dilution ranges, are shown in Table 1. Three or 4 plates containing each medium were inoculated with 50 µl of the dilutions of each sample, distributed with glass spreaders. Aerobic culture, without added CO_2, was kept at 37°C for 24 hours. Anaerobic cultures were incubated at 37°C for 72 hours in Don Whitley anaerobic jars containing 'cold' palladium catalyst under 90% H_2 and 10% CO_2. Single plates for each sample were selected for counting. If two counts of the same organism were made on different media, the higher count was used. The guidelines of Peach *et al.*[14] were followed for the identification of bacteria. All colony types growing anaerobically were Gram-stained, counted, and subcultured aerobically and anaerobically. Gram-negative organisms growing only on anaerobic culture were identified by their antibiotic response.[15]

Table 1 *Culture media for counting bacteria in faeces*

Medium	*Dilutions plated*	*Bacteria counted*
MacConkey's agar without salt (Oxoid CM7b)	10^4–10^7	Coliforms. Enterococci. Staphylococci
Rich medium* + neomycin (20 µg/ml)[2]	10^4–10^7	Anaerobic streptococci. Bifidobacteria. Bacteroides. Clostridia. Enterococci. Staphylococci. Veillonella
Rich medium + neomycin (20 µg/ml) + vancomycin (7·5 µg/ml)	10^4–10^6	Bacteroides. Veillonella
Reinforced clostridial agar (Oxoid CM151) + 1% glucose + 1% liver digest pH 5[3]	10^3–10^6	Bifidobacteria. Enterococci
Lactose-egg yolk agar (Oxoid CM3 + 1% lactose + 5% egg yolk + 0·0025% neutral red) + D-cycloserine (400 µg/ml)[24]	10^2–10^3	Clostridia

*Rich medium: reinforced clostridial agar (Oxoid CM151) plus 1% liver digest, plus 10% defibrinated horse blood.

Immunological tests. Ten of the infants of atopic parents were examined at 12–14 weeks of life, blood was taken, and they were skin-prick tested with *Dermatophagoides* sp., Timothy grass, and cat extracts, and control (Bencard). Serum immunoglobulin G, A, and M were measured by radial immunodiffusion and yeast opsonisation by the method of Levinsky *et al.*[16]

Results

Direct smears of faeces. The predominant category of organisms in Gram-stained smears of faeces was consistent in all samples obtained from each infant, except for 3 which were classified by the category observed twice. Nine of the 12 bottle-fed infants had a predominance of Gram-negative rods. Gram-positive cocci were seen frequently, and were predominant in 2 infants. Gram-positive rods were scanty, and predominated in 1 infant who was bottle fed. In faeces of all 17 breast-fed infants, Gram-negative rods predominated, and Gram-positive rods and cocci were scanty. Very few typical bifidobacteria were seen. By contrast, in faeces of all 12 Nigerian breast-fed infants Gram-positive rods were predominant, and in many cases typical bifidobacteria were seen.

Table 2 *Mean $\log_{10}$ viable bacterial counts/g of faeces (and number positive) of English infants classified by feeding type and history of parental atopy, and of 12 breast-fed Nigerian infants*

Infants	*Parental atopy*	*Coliforms*	*Bacteroides*	*Veillonella*	*Clostridia*	*Enterococci*	*Bifidobacteria*	*Staphylococci*
English								
Age 2 weeks								
Bottle	Yes (4)	9·5 (4)	10·1 (2)	7·8 (2)	6·5 (3)	9·2 (2)	ND	ND
	No (7)	9·4 (7)	8·5 (4)	6·2 (1)	7·2 (5)	8·9 (5)	7·6 (2)	7·2 (5)**
Breast	Yes (8)	9·4 (7)	9·4 (5)	6·7 (2)	5·5 (7)	7·3 (2)	ND	7·7 (6)
	No (7)	9·4 (6)	8·2 (4)	8·2 (2)	5·9 (6)	7·7 (3)	ND	8·1 (6)
Age 4 weeks								
Bottle	Yes (5)	9·4 (5)	10·3 (2)	5·1 (3)	7·2 (4)	9·5 (5)	ND	ND
	No (7)	9·7 (7)	9·9 (4)	7·5 (3)	7·0 (6)	8·7 (6)	9·6 (1)	ND
Breast	Yes (9)	9·3 (9)	9·5 (6)	7·5 (1)	6·4 (8)	7·0 (5)	4·3 (1)	7·8 (3)
	No (7)	9·5 (7)	9·5 (5)	ND	6·2 (6)	7·0 (4)	ND	7·6 (3)
Age 6 weeks								
Bottle	Yes (5)	9·3 (5)	8·0 (3)	7·4 (4)	6·7 (4)	8·8 (5)	7·2 (1)	ND
	No (7)	9·7 (7)	9·3 (5)	6·4 (3)	6·0 (7)	9·2 (7)	6·4 (4)	8·9 (1)
Breast	Yes (7)	9·6 (7)	8·9 (5)	7·3 (3)	5·8 (7)	8·2 (5)	ND	8·6 (2)
	No (8)	9·6 (8)	9·7 (4)	7·2 (2)	6·9 (6)	7·4 (6)	4·6 (2)	7·1 (2)
Nigerian								
Breast	(12)	6·4 (12)	8·6 (4)	ND	4·8 (2)	8·5 (7)	9·6 (12)	7·7 (2)

ND = not detected; **P=0·045 (Fisher's exact test).

Table 3 *Bacteria in significant excess (in prevalence and counts) in 81 specimens of faeces of 19 bottle-fed babies compared with 12 breast fed-babies*

	Aged 2 weeks		*Aged 4 weeks*		*Aged 6 weeks*	
	Organism	*P*	*Organism*	*P*	*Organism*	*P*
Prevalence†			Enterococci	0·04		
			Veillonella	0·03		
			Staphylococci*	0·02		
Counts‡	Clostridia	< 0·025	Clostridia	<0·05	Enterococci	< 0·05
			Enterococci	<0·01		

*More prevalent in breast-fed babies.
†Fisher's exact test;
‡Mann Whitney U test;

Bacterial culture. The only significant difference in faecal flora between infants of atopic and non-atopic parents was a lack of staphylococci in the faeces of bottle-fed infants of atopic parents at 2 weeks, $P<0.05$, Table 2. Because of this we combined the atopic and non-atopic groups to analyse the effect of breast and bottle feeding; in both feeding groups coliforms and bacteroides predominated, and bifidobacteria were scarce, with a few exceptions. The few significant ($P<0.05$) differences of prevalence (number positive) or count of each bacterial species are shown in Table 3 for each time of study; there were excesses of enterococci, veillonella, and clostridia in bottle-fed babies, and of staphylococci in breast-fed babies, at 4 weeks, only occasionally confirmed at 2 weeks and 6 weeks.

Table 4 shows ratios of different categories of bacteria grown from infants' faeces. The only significant difference was at week 4, when the Gram-negative/Gram-positive ratio was higher in breast than in bottle-fed babies, mainly because of an excess of enterococci in the faeces of the latter. In the faeces of 12 breast-fed Nigerian infants bifidobacteria were consistently the predominant organisms ($\log_{10}$ mean 9.6 ± 0.17); coliforms were consistently fewer ($\log_{10}$ mean 6.4 ± 0.49), Table 3. The ratios of the different categories of organisms are quite different from the English infants (Table 4).

Table 4 *Ratios* of categories of the faecal flora of 29 English infants and 12 Nigerian infants*

	English infants						*Nigerian infants*
	2 weeks		*4 weeks*		*6 weeks*		*<1 year*
	Bottle	*Breast*	*Bottle*	*Breast*	*Bottle*	*Breast*	*Breast*
Gram-negative / Gram-positive	1	27·8	2·8**	92**	2·2	79·7	0·092
Aerobes / Anaerobes	4	3·3	0·4	0·8	5·9	1·6	0·087
Coliforms / All others	1·5	2·7	0·3	0·8	1·2	1·5	0·0006

* Calculated from mean counts of bacteria in each category. **P <0·05 (Mann Whitney U test of replicate ratios).

pH values. The faeces of breast-fed babies had significantly ($P<0.001$, Mann-Whitney U test) lower pH values than those of bottle-fed babies at each age studied (week 2, mean pH 5·9 and 6·8: week 4, pH 5 and 7·2; week 6, pH 6 and 7·1).

Gas-liquid chromatography. The volatile fatty acid mixtures detected by gas-liquid chromatography in the faeces of 26 infants could each be placed in one of 3 well-known categories in which one or two fatty acids predominated (Table 5). None had the acetic acid predominance which has been described in association with a predominant bifidobacterial flora.[17] Three samples were available for study from each of 10 infants, and 2 from another 10. The different samples from each of these 20 gave consistent results in 17. The fatty acids observed are consistent with the expected metabolic products of the organisms cultured.

Characterisation of atopy. Only 3 of the 10 infants of atopic parents examined at 12–14 weeks had symptoms possibly referable to atopy. Two sneezed frequently and 2 had dry skin, one with cough, wheeze, and colic; only the latter was thought to be of atopic origin and he was bottle fed, but was skin-test negative. Only one child gave one positive skin test response (to *Dermatophagoides* sp.) and he was breast fed. These 2 had the lowest serum IgA (both 4 IU/ml), lower than the department normal range. Yeast opsonisation was normal in all.

Table 5 *Predominant faecal fatty acids in breast- and bottle-fed infants*

Predominant fatty acid	*Bottle (n = 12)*	*Breast (n = 14)*
Acetic	Nil	Nil
Propionic	7	Nil
Butyric	4	11
Acetic + butyric (1:1)	Nil	1
Inconsistent	1	2

In 5 infants with 3 samples, in which the finding in 1 sample differed from the other 2, infants were classified by the latter finding. Where the results differed they were classified as inconsistent.

Discussion

The most striking observation in this study is virtual absence of bifidobacteria in exclusively breast-fed infants born in a London hospital. They, like the bottle-fed group, showed a predominant Gram-negative flora with coliforms the dominating bacteria. The results from the Nigerian infants provided a positive control for our techniques, and confirm previous reports of high numbers of bifidobacteria in faeces of breast-fed infants. In Guatemalan Indians, bifidobacteria became the predominant bacteria by the end of the first week of life reaching counts greater than 10^{10}/g of wet faeces.[2] In the UK Willis *et al.*[3] reported counts between 10^6 and 10^{10}/g in 15 of 22 breast-fed infants studied daily during the first 2 weeks of life, and Hewitt and Rigby[18] and Dolby *et al.*[19] found bifidobacteria in breast-fed infants' faeces in counts of 10^{10} and 10^7–10^{11}/g respectively, though neither of these studies detected a consistent negative relationship between 'bifid' counts and *Escherichia coli* counts. However, Mitsuoka and Kaneuchi[6] in Japan isolated bifidobacteria in all infants, whether breast or bottle fed, at counts greater than 10^{10}/g, although he found streptococci, enterobacteriaceae, and anaerobes other than bifidobacteria were fewer in breast-fed infants than in bottle-fed infants. In a prospective study of the faecal flora of breast-fed and bottle-fed infants during the first 6–8 weeks of life, Bullen *et al.*[4] reported that in breast-fed infants, bifidobacteria greatly outnumbered streptococci, coliforms, bacteroides, and clostridia. Other workers suggested that the bacteriostatic effect of antibody and lactoferrin in human milk[20] may suppress the *E. coli* in the upper gastrointestinal tract. These conditions and the low buffering capacity of human milk could favour the rapid outgrowth of bifidobacteria, and the production of large amounts of acetate; this would lower the pH values even more, and so inhibit coliforms, bacteroides, and clostridia. We observed lower pH values in the faeces of breast-fed infants despite the lack of a bifidobacterial flora, although our values are not as low as those of Bullen *et al.*,[4] and none of our specimens had acetic acid as the sole end product of bacterial metabolism. The mixtures of acetic, propionic, butyric, and other higher fatty acids reflect the dominant presence of coliforms and bacteroides, and clostridia may have contributed to it too. Besides the positive bifidobacteria cultures in the Nigerian samples, the consistency of results of cultures, microscopical examinations, and gas-liquid chromatography provide strong support for the validity of our cultural techniques.

Although the work of Bullen *et al.*[17] showed that infant feeding can radically modify the faecal flora, our study and others strongly suggest that other factors are relevant too. One possibility is the dose of bacteria eaten by the child at birth. Intestinal colonisation starts immediately after rupture of the membranes. In many societies, such as the Guatemalan Mayan Indians studied by Mata and Urrutia,[2] and possibly the mothers of the Nigerian infants we studied, women give birth in the kneeling or squatting position and defecation during childbirth is usual. Certainly there was less asepsis, use of enemas, etc. Under these conditions the baby ingests a considerable amount of the mother's faeces which are rich in bifidobacteria.[21] Possibly this also contributes to the acquisition of a bifidobacterial flora, and though such conditions do not apply in Luton, where the studies of Bullen *et al.*[17] were done, differences in obstetric techniques may play a part. Considerable variation of mother-to-newborn interchange of *E. coli* has been described in hospital deliveries. Bettleheim *et al.*[22] showed that 22 of 28 mothers and infants at St Bartholomew's Hospital had the same *E. coli* serotype in their faeces, but a more recent study by Gothefors *et al.*[23] found that only 5 of 20 Swedish mothers and their infants had the same dominant serotype. Changes in obstetric practice may have contributed to this. The one difference noted—namely the excess of staphylococci in breast-fed babies—presumably arose from a larger inoculum from the mothers' skin. We did not identify the source of the *E. coli* in the breast-fed infants; they received only water to drink apart from human milk, and this and nipple cleansing seem unlikely sources.

Our primary interest, a possible adjuvant role of the flora in the development of atopy and other immunopathology, remains to be established. If this is indeed important in preventing such disease, our findings may provide one explanation why exclusive breast feeding does not always prevent eczema[7] and show why atopy is more prevalent in industrialised countries than in developing ones,[12] but there are several other possible mechanisms too. They also suggest that it will only be possible to study the effect of the minor immunodeficiencies which underline atopy on the intestinal flora when more of the environmental factors which produce these big variations are understood and controlled.

We thank Professor C B S Wood, Department of Child Health, and the nursing staff of St Bartholomew's Hospital; the staff of the Microbiology Departments at Queen Elizabeth Hospital for Children, and Dr Willis and staff, Public Health Laboratory, Luton, for help and advice.

The Crohn's in Childhood Research Association and the Wellcome Trust provided financial support.

A S was supported by a British Council Fellowship.

References

[1] Haenel H. Some rules in the intestinal microflora of man. *J Appl Bacteriol* 1961; **24:** 242–51.
[2] Mata L J, Urrutia J J. Intestinal colonization of breast fed children in a rural area of low socioeconomic level. *Ann N Y Acad Sci* 1971; **176:** 93–109.
[3] Willis A T, Bullen C L, Williams K, Fagg C G, Bourne A, Vignon M. Breast milk substitute: a bacteriological study. *Br Med J* 1973; **iv:** 67–72.
[4] Bullen C L, Tearle P V, Willis A T. Bifidobacteria in the intestinal tract of infants: an *in vivo* study. *J Med Microbiol* 1976; **9:** 325–33.
[5] Haenel H, Müller-Beuthow W, Grütte F-K. Zur fäkalen Mikroökologie des Säuglings in Abhängigkeit von der Ernährung: Zusamnensetzung der Mikroflora sowie Vorkommen der *Lactobacillus bifidus*-Typen. *Zentralbl Backteriol I Abt [Orig]* 1970; **215:** 333–47.
[6] Mitsuoka T, Kaneuchi C. Ecology of the bifidobacteria. *Am J Clin Nutr* 1977; **30:** 1799–810.
[7] Matthew D J, Taylor B, Norman A P, Turner M W, Soothill J F. Prevention of eczema. *Lancet* 1977; **i:** 321–4.
[8] Saarinen U M, Kajosaari M, Backman A, Siimes M A. Prolonged breast feeding as prophylaxis for atopic disease. *Lancet* 1979; **ii:** 163–6.
[9] Soothill J F. Some intrinsic and extrinsic factors predisposing to allergy. *Proc Roy Soc Med* 1976; **69:** 439–42.
[10] Soothill J F, Stokes C R, Turner M W, Norman A P, Taylor B. Predisposing factors and the development of reaginic allergy in infancy. *Clin All.rgy* 1976; **6:** 305–19.
[11] Jarrett E E E, Haig D M, McDougall W, McNulty E. Rat IgE production. II. Primary and booster reaginic antibody responses following intradermal or oral immunisation. *Immunology* 1976; **30:** 671–7.
[12] Morrison-Smith J. Skin tests and atopic allergy in children. *Clin Allergy* 1973; **3:** 269–75.
[13] Crowther J S. Transport and storage of faeces for bacteriological examination. *J Appl Bacteriol* 1971; **34:** 477–83.
[14] Peach S, Fernandez F, Johnson K, Drasar B S. The non-sporing anaerobic bacteria in human faeces. *J Med Microbiol* 1974; **7:** 213–21.
[15] Sutter V L, Finegold S M. Antibiotic disc susceptibility tests for rapid presumptive identification of Gram-negative anaerobic bacilli. *Appl Microbiol* 1971; **21:** 13–20.
[16] Levinsky R J, Harvey B A M, Paleja S. A rapid objective method for measuring the yeast opsonisation activity of serum. *J Immunol Methods* 1978; **24:** 251–6.
[17] Bullen C L, Tearle P V, Stewart M G. The effect of 'humanised' milks and supplemented breast feeding on the faecal flora of infants. *J Med Microbiol* 1977; **10:** 403–13.
[18] Hewitt J H, Rigby J. Effects of various milk feeds on numbers of *Escherichia coli* and *Bifidobacterium* in the stools of newborn infants. *J Hyg (Camb)* 1976; **77:** 129–39.
[19] Dolby J M, Honour P, Valman H B. Bacteriostasis of *Escherichia coli* by milk. I. Colonisation of breast fed infants by milk-resistant organisms. *J Hyg (Camb)* 1977; **78:** 85–93.
[20] Bullen J J, Rogers H J, Leigh L. Iron binding proteins in milk and resistance to *Escherichia coli* infection in infants. *Br Med J* 1972; **i:** 69–75.
[21] Gossling J, Slack J M. Predominant gram-positive bacteria in human feces: numbers, variety, and persistence. *Infect Immun* 1974; **9:** 719–29.
[22] Bettleheim K A, Breadon A, Faiers M C, O'Farrell S M, Shooter R A. The origin of O serotypes of *Escherichia coli* in babies after normal delivery. *J Hyg (Camb)* 1974; **72:** 67–70.
[23] Gothefors L, Carlsson B, Ahlstedt S, Hanson L A, Winberg J. Influence of maternal gut flora and colostral and cord serum antibodies on presence of *Escherichia coli* in faeces of the newborn infant. *Acta Paediatr Scand* 1976; **65:** 225–32.
[24] Willis A T. *Anaerobic bacteriology in clinical medicine*, second edition. London: Butterworth, 1964: 35.

Correspondence to Professor J F Soothill, Department of Immunology, Institute of Child Health, 30 Guilford Street, London WC1N 1EH.

Received 5 February 1981