Effect of various milk feeds on numbers of *Escherichia coli* and *Bifidobacterium* in the stools of new-born infants

By J. H. HEWITT* AND JANET RIGBY†

*From the Division of Hospital Infection, Clinical Research
Centre, Watford Road, Harrow, Middlesex HA1 3 UJ*

SUMMARY

Escherichia coli was found in a similar proportion of stool specimens from infants who were breast-fed and from others fed on three different artificial-milk preparations. When *E. coli* was present its mean colony count in the stools of breast-fed infants was within the range of the mean counts for infants receiving the artificial-milk feeds.

There was no consistent relation between high counts of bifidobacteria (*Lactobacillus bifidus*) and low counts of *E. coli*. This suggests that measures aimed at implanting or stimulating the growth of bifidobacteria in the large intestine of artificially fed infants may not greatly influence the *E. coli* population therein.

The results are discussed in relation to the protection of artificially fed infants from *E. coli* enteritis.

INTRODUCTION

It has long been recognized that breast-feeding protects new-born babies from gastro-enteritis generally and from *Escherichia coli* enteritis in particular (Svirsky-Gross, 1958; Tassovatz & Kotsitch, 1961; Mata & Urrutia, 1971) but the relative importance of the different contributory factors is uncertain.

Ever since the observations of Tissier (1899) on the predominance of bifidobacteria (*Lactobacillus bifidus*) in the stools of breast-fed babies, there has been interest in a possible relation between the 'bifidus flora' and protection against gastro-intestinal infections. The observations were apparently convincing enough for paediatricians and bacteriologists to attempt to establish bifidobacteria or aerobic lactobacilli in infants receiving artificial feeds either by adding to the cows'-milk feeds substances stimulatory for the growth of the bifidobacteria already present (György, Norris & Rose; 1954; Petuely, 1956, 1957; Raynaud, 1959), or – usually in addition to this – the direct feeding of preparations of bifidobacteria (Tissier, 1906; Laboratoire du Lyo-Bifidus, 1969). Most interest in these techniques has been on the continent of Europe, though some studies have been made in Scotland (MacGillivray, Finlay & Binns, 1959; McWilliam, 1960). The preventive

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* Present address: Public Health Laboratory, Dulwich Hospital, East Dulwich Grove, London SE22 8QF.
† Present address: Department of Microbiology, Maelor General Hospital, Croesnewydd Road, Wrexham, Clwyd LL13 7TD.

and therapeutic value of these measures is difficult to assess (Tassovatz, 1964; Schneegans *et al.* 1966) and is sufficiently in doubt that it has not led to their widespread adoption. Nevertheless, the claimed bifid-stimulating properties of some artificial milks have been put forward as a beneficial feature (Mayer, 1966; Wyeth & Brother, 1970).

The inhibition of *E. coli* and other enterobacteria by fermentation products of bifidobacteria has been demonstrated *in vitro* (Delbove, 1932; Ruschmann, 1958), but it is uncertain that this occurs in the conditions prevailing in the human intestinal tract.

Assessment of the numbers of all but the dominant components of the faecal microbial flora is very difficult, whether by examination of Gram-stained smears or by colony counting methods, unless highly selective culture media and incubation conditions are used. We here report the results of colony counts of bifidobacteria and *E. coli* in the stools of healthy new-born infants receiving either breast feeding or one of three different artificial feeds, at as close as possible to 7 days of age, when the faecal bacterial flora has usually achieved some stability (Tissier, 1905; Haenel, 1970; Mata & Urrutia, 1971).

<div align="center">MATERIALS AND METHODS</div>

The babies

In the maternity units of three hospitals, the babies for study were chosen at random, mainly during the period March 1972 to February 1973. The hospitals were:

No. 1 – Northwick Park Hospital, Harrow, Middlesex;

No. 2 – Queen Charlotte's Maternity Hospital, London W14;

No. 3 – Kingsbury Maternity Hospital, London NW9.

Milk feeds

The four feeds studied were:

(i)   Breast-feeding by the mother (hospitals nos. 1 and 3), 24 infants.

(ii)   SMA 'ready to feed' (J. Wyeth Labs.) (hospital no. 1), 20 infants.

(iii*a*) Cow and Gate Special Formula A for 3 days followed by Cow and Gate Baby Milk 1; both 'ready to feed' (hospital no. 2), 20 infants.

(iii*b*) Cow and Gate Half-cream Powdered Milk (i.e. Baby Milk 1) for 3–5 days followed by the Full-cream Powdered Milk (Baby Milk 2) (hospital no. 3), 10 infants.

(iv)   Golden Ostermilk 'ready to feed' (hospital no. 1), 4 infants.

Details of the composition of these feeds is given by Barrie, Martin & Ansell (1975).

Collection of specimens

We tried to obtain samples from healthy infants who had been fed exclusively on one type of milk. This proved difficult for the breast-fed infants, because they usually received complementary feeds of bottled milk (SMA or Cow and Gate) during the first 2–4 days of life and for one feed during the night after this. Most specimens from breast-fed infants were from infants fed in this way.

From each infant on, or as near as practicable to, the seventh full day of life (range 6–9 days) a specimen of freshly passed stool was collected by a member of the nursing staff. The specimen, approximately $1 \cdot 0$ g wet weight, was transferred by spoon into 9 ml. of 10 % glycerol-broth in a weighed bottle, and frozen on solid CO_2 in a vacuum flask, then stored at $-30°$ C. The methods for collection of specimens could not prevent contamination from the infant's skin. However, if this did occur, it probably had only a slight effect on the total results. Colony counts were normally made within 2–5 days of collection of the specimens.

Media and cultures

The media and methods were based on those of Drasar & Crowther (1971) but without the use of an anaerobic glove-box.

MacConkey agar (Oxoid CM7) was used for counts of *E. coli* and other enterobacteria. Plates were incubated aerobically for 24 h. at $37°$ C.

Winkler's medium (Wiel-Korstanje & Winkler, 1970) + kanamycin sulphate (50μg./ml.) was used for the enumeration of bifidobacteria. Incubation was anaerobic for 2–3 days at $37°$ C. Baird and Tatlock jars with three sachets of palladium catalyst were used with a commercial gas mixture of 10 % hydrogen, 10 % carbon dioxide and 80 % nitrogen.

Reinforced clostridial agar (Oxoid CM151) + 1 % Liver Digest (Oxoid L27) and $7 \cdot 5$ % (v/v) defibrinated horse-blood (RCMB agar) was used for subculture of suspected anaerobic organisms. Incubation was anaerobic for 24 hr. at $37°$ C.

All plates were dried, open, in a $37°$ C. incubator for 2 hr. Those of Winkler's and RCMB agar were subsequently stored at $4°$ C. in an oxygen-free atmosphere (in anaerobic jars) and returned to room temperature 1–2 hr. before use. At the time of inoculation these plates were exposed to the air for as short a time as possible – up to 20 min. – before incubation.

The surface counting method of Miles, Misra & Irwin (1938) was used. After thawing, and weight determination (by difference), specimens were dispersed by gentle agitation with sterile glass beads. Each was treated as a first tenfold dilution. Seven more tenfold dilutions, down to 10^{-8}, were made in freshly steamed and cooled Brain–Heart Infusion Broth (Oxoid CM225) containing $0 \cdot 05$ % (w/v) cysteine hydrochloride as a reducing agent; single $0 \cdot 02$ ml. drops of each dilution were then deposited on the surface of one plate each of MacConkey agar and Winkler's medium. A culture of *Clostridium sporogenes* (NCTC no. 533) was included in each anaerobic jar to confirm anaerobiosis.

After incubation a representative of each colonial type on each medium was subcultured and Gram-stained for purification and identification; and colony counts were made from the most suitable dilution. With the exception of some repeated counts of enterobacteria on MacConkey agar, counts were made on only one plate of each medium.

Identification of organisms

E. coli *and other enterobacteria.* Representative colonies from MacConkey agar were subcultured to blood agar and/or MacConkey agar as a check for purity.

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Lactose-fermenting Gram-negative rods growing on MacConkey medium, indole-positive by Kovac's test, citrate-utilization-negative (Simmon's medium), and urease-negative were regarded as *E. coli*. All such isolates were screened by slide-agglutination with polyvalent antisera (Wellcome nos. 2 and 3), for ten of the commoner enteropathogenic serotypes, but none was detected during our study. Other aerobic organisms were identified by the methods of Cowan & Steel (1965).

Anaerobes. Representatives of the different colonial forms on Winkler's medium were subcultured in duplicate from well-isolated colonies to RCMB agar and incubated aerobically and anaerobically. This served the dual purpose of purification of cultures – often particularly difficult with anaerobes – and establishing whether organisms were obligate anaerobes or not. Bifidobacteria were identified by the following characters: obligately anaerobic, or occasionally growing weakly on aerobic subculture; characteristic darkening of blood agar around colonies; Gram-positive rods – readily decolorized even in young cultures – but showing characteristic branching, clubbed, swollen, or sinuous forms; not filamentous.

Gram-stained smears. A thin smear was prepared from each specimen after dispersion and stained by Gram's method. The relative abundance of the different cell types was judged by microscopical observation of several fields, and scored on a semi-quantitative basis ($\pm$, $+$, $++$, $+++$), without reference to the results of the colony counts.

pH measurements. The pH of some of the faecal suspensions in transport medium was determined with a meter. The values obtained are probably not directly comparable with other determinations because the transport medium exerted a partial buffering effect on specimens that were of near neutral pH.

Agar-plate tests for Bifidobacterium – E. coli *inhibition.* (Direct-streaking method of Parker & Simmons, 1959.) Pure cultures of bifidobacteria isolated from breast-fed infants were heavily streaked on Reinforced Clostridial Blood Agar plates (glucose content 0·5 %) and incubated anaerobically for 48 or 96 hr. Broth suspensions of *E. coli* and other enterobacteria were then streaked up to the line of bifid growth and anaerobic incubation was contined. Plates were examined for inhibition of *E. coli* after 24 hr.

RESULTS

Occurrence and numbers of various organisms

E. coli *and bifidobacteria.* The incidences of detection and the mean counts for specimens that were positive are shown in Table 1. In some circumstances failure to detect bifidobacteria may have resulted from the use of an insufficiently selective medium. In particular, high counts of viridans streptococci, obtained from stools of infants on Cow and Gate and Ostermilk feeds on Winkler's medium, may have masked or inhibited their growth. *E. coli* – as confirmed by biochemical tests – occurred in a similar proportion of specimens from infants on each of the four feeds. When they did occur the mean count was highest (7×10^9 per g) with SMA feed, and lowest (2–3×10^8 per g) with Cow and Gate Feed. The breast-fed infants had a mean count of 3–8×10^8 per g, and those fed on Ostermilk a mean of 1×10^9 per g. Repeat counts of *E. coli* in all specimens, performed in triplicate on

Table 1. *The occurrence and mean viable counts of* E. coli *and bifidobacteria in stools of week-old infants**

Type of feed	Hospital no.	No. of specimens examined (one per infant)	E. coli		Bifidobacteria	
			Number (%) of specimens yielding *E. coli*	Mean count per g of positive specimens	Number (%) of specimens yielding bifids	Mean count per g of positive specimens
Breast-fed	1	20	13 (65)	8×10^8	17 (85)	2×10^{10}
	3	3	3 (100)	3×10^8	3 (100)	1×10^{10}
SMA	1	20	14 (70)	7×10^9	11 (55)	2×10^{10}
Cow and Gate	2	20	12 (60)	2×10^8	11 (55)	3×10^9
	3	2	2 (100)	3×10^8	1 (50)	5×10^7
Ostermilk	1	10	6 (60)	1×10^9	3 (30)	9×10^9

* Includes only data from specimens in which the identity of *E. coli* isolates was bio-chemically confirmed.

MacConkey medium, confirmed the results obtained in the initial screening. Bifidobacteria were detected, in large numbers, in all but four of 24 specimens from breast-fed infants. Similar numbers occurred in specimens from about half of the infants receiving SMA and Cow and Gate feeds, and in a third of those from infants receiving Ostermilk.

Predominant organisms. The numerically predominant organism(s) in the '7-day' specimens, as shown by the viable counts, are tabulated, according to milk feed, in Table 2. Dominance of bifidobacteria was found in the majority of specimens from breast-fed infants. With Cow and Gate and Ostermilk feeds viridans strepto-cocci (aerobic streptococci failing to grow on or hydrolyse aesculin in an aesculin-bile medium) were the most numerous organisms cultured from most specimens. With SMA feed the results were more variable; bifidobacteria, viridans strepto-cocci, and *E. coli* featured as the most numerous organism in about equal numbers of specimens.

For most specimens there was good agreement between the predominant organ-isms as determined in the viable counts and the estimated abundance of the distinguishable cell types seen in the Gram-stained films prepared direct from the faecal suspensions (Table 3). The main exception was in seven specimens from infants on Cow and Gate feed. In these there were many bifid-like cells in the stained films, but low colony counts of bifidobacteria. Either the organisms were dead – perhaps due to exposure to unfavourable conditions in the stools – or inhibition – perhaps between viridans streptococci and bifidobacteria – could have occurred on the culture medium. Homma, Nishihara & Isoda (1967) have described antagonism between strains of *Streptococcus faecalis* and bifidobacteria on milk-agar plates.

Analysis of the colony counts in conjunction with the results of the microscopic observation of Gram-stained smears of faeces showed that, where enterobacteria were outnumbered, in the colony counts, by more than 100:1, Gram-negative rods of enterobacterial morphology were seen in only 14 % of the smears. They were

Table 2. *Numerically predominant viable organisms*

Type	Hosp. no.	No. of specimens	Specimens (%)* in which predominant organism was							
			Bifido-bacteria	Str. viridans	Str. faecalis	Str. salivarius	E. coli	Klebsiella spp.	Proteus spp.	Bacteroides spp.
Breast	1	20	85	0	5	0	10	5	0	5
SMA	3	4	25	25	0	0	{50	}	0	0
Cow a	1	20	45	30	5	0	30	0	0	5
	2	20	30	80	0	0	0	0	0	0
Osteri	3	4	0	50	0	0	{25	}	25	25
	1	10	20	60	0	10	10	0	0	0

* Some totals exceed 100 % because two organisms gave equal counts.

Table 3. *Numerically predominant cell-types in Gram-stained smears of stools*

		Smears (%)* in which the predominant cell-type was			
Type of feed	No. of specimens	'Bifid type'	Gram-positive cocci	Gram-negative rods	Presumptive *Cl. welchii*
Breast-fed	16	75	6	19	0
SMA	20	50	25	20	5
Cow and Gate	20	65	55	0	0
Ostermilk	10	20	70	40	0

* Some totals exceed 100% because two or three cell types were equally numerous.

seen in 81% of specimens in which the ratio was 10:1 or less. Gram-negative rods were not seen in smears of nine specimens in which the colony counts of *E. coli* were 10^8–10^9 per g; failure to see them in smears therefore cannot be accepted as evidence for absence of *E. coli*.

The relative numbers of bifidobacteria and E. coli

Specimens from infants approximately 7 days old. Fig. 1 shows the relation between the colony counts of *E. coli* and bifidobacteria in 75 specimens, according to the feed received. Although high counts ($> 10^8$ per g) of *E. coli* were obtained from 21 specimens in which bifidobacteria were not detected or from which bifidobacterium counts were $< 10^9$ per g, high *E. coli* counts were also obtained in 17 specimens yielding $> 10^9$ bifidobacteria per g. Failure to detect *E. coli* or counts of $< 10^8$ per g, occurred in 18 specimens in which bifids were not detected or were present at $< 10^9$ per g, and in 19 specimens in which bifid counts exceeded 10^9 per g.

Low counts of *E. coli* occurred with similar frequency in specimens with both high and low counts of bifidobacteria. No direct correlation existed between high bifidobacteria and low *E. coli* counts. These observations are in agreement with the results of Willis *et al.* (1973), who studied infants receiving breast milk, a breast-milk-substitute feed, and an unspecified cow's-milk feed. Their observations extended to infants 14 days of age.

Counts of E. coli *and bifidobacteria in sequential specimens from breast-fed infants.* Fig. 2 shows the counts of *E. coli, Klebsiella,* and bifidobacteria in specimens from eight breast-fed infants at hospitals 1 and 3 from birth up to, when possible, 10 days of age. Of the seven infants in which *E. coli* and bifidobacteria were detected, rising counts of bifidobacteria were accompanied by a definite decline in the numbers of *E. coli* in only one (infant F.R.). In this infant, during the period in which counts were made, our determination of the stool pH showed a drop from 5·7 to 5·1, one of the most acid stools encountered. In three infants (H.A., P.A., W.C.) the *E. coli* counts continued to rise until the 6th–10th day of life despite the presence of very large numbers (10^9–10^{10} per g) of bifidobacteria. In infant F.I. a sharp decline and subsequent recovery of counts of a *Klebsiella* could not be related to a pH effect.
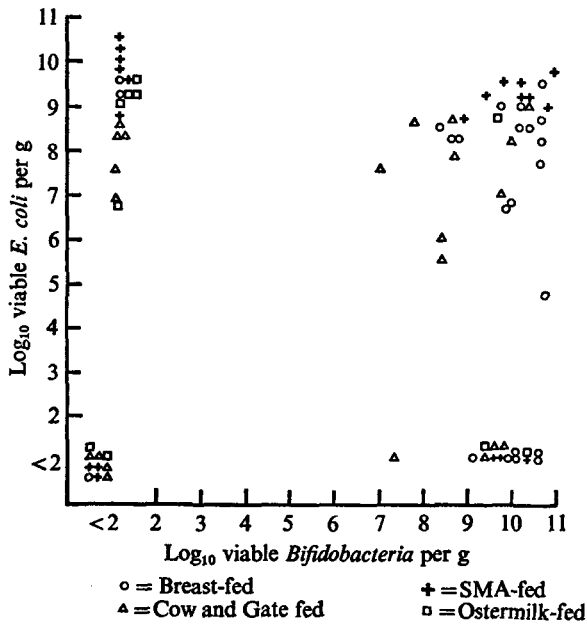
Fig. 1. Relation between viable counts of *E. coli* and of bifidobacteria in faecal specimens from 75 seven-day-old infants on four different milk feeds.
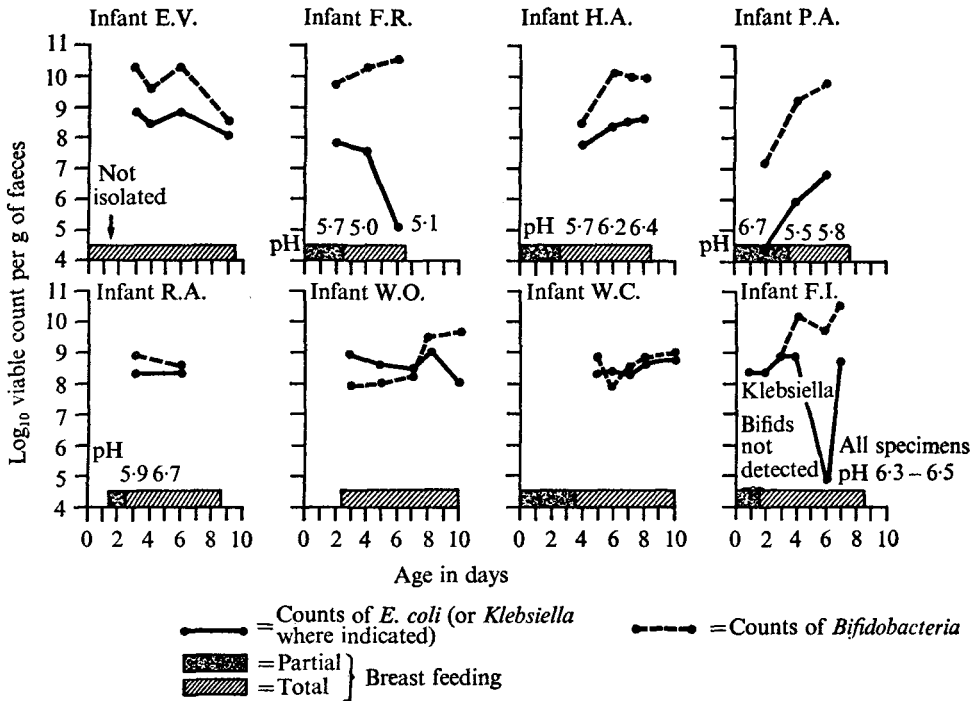

Fig. 2. Sequential counts of *E. coli*, *Klebsiella*, and bifidobacteria in the faeces of eight predominantly breast-fed infants during the first 10 days of life.

In-vitro antagonism between bifidobacteria and E. coli. A zone of inhibition of growth of *E. coli* NCTC no. 10418, three laboratory strains of *E. coli* – including a strain of the enteropathogenic serotype O 111 – and a *Klebsiella*, was obtained close to the growth of six strains of *Bifidobacterium*. Five of the latter were recent isolations from breast-fed infants, and one was *Lactobacillus bifidus* NCTC no. 10471. Inhibition was seen only on plates that had been incubated for 4 days before the enterobacteria were streaked on; it was not evident when preincubation had been only 48 hr. This could indicate an insufficient accumulation of inhibitory products during the shorter incubation period. Growth of *E. coli* on these plates was less prolific under anaerobic than under aerobic conditions.

DISCUSSION

Because of interest in the possible application of the bifid–coliform antagonism that had been demonstrated *in vitro*, many earlier studies of the faecal bacterial content of infants fed on different milk preparations have concentrated on the bifid content of the stool. Our observations gave no indication of a suppression of growth of *E. coli* in the stools of the breast-fed infant compared with those receiving other feeds. At the end of the first week of life and in the sequential counts, *E. coli* coexisted in faecal material with large numbers of bifidobacteria. Whether this coexistence could continue over a longer period was not determined. The data of Willis *et al.* (1973) suggest that it can continue until the 14th day of life, and those of Mata *et al.* (1971, 1972) throughout the first 2 years.

Similar conclusions on the co-existence of bifidobacteria and *E. coli* were reached by Schneegans *et al.* (1966), who reported attempts to implant a bifid flora by oral feeding of bifids in carriers of enteropathogenic *E. coli*, and also by MacGillivray *et al.* (1959), who used lactulose to stimulate growth of bifidobacteria.

It seems unlikely therefore that the bifid:*E. coli* antagonism demonstrated in laboratory studies is very active in the colon of the breast-fed infant. This being the case, any protective effect against *E. coli* enteritis possessed by unheated human milk (Tassovatz & Kotsitch, 1961) cannot be attributed to a greatly reduced reservoir of *E. coli* in the colon of the breast-fed infant. It seems more likely that protective action is exerted in the small intestine, which has been shown to be the site of epithelial attachment of *E. coli* in enteritis of pigs (Arbuckle, 1970). There is little information on the bacterial content of the lumen of the small intestine of healthy or diseased infants receiving different feeds. Observations made *post mortem* are likely to be greatly affected by rapid overgrowth of the bacteria present in late stages of the illness. Intubation studies provide the only relevant data (Barbero *et al.* 1952; Giunta & Rosaschino, 1970).

We confirmed previous observations (Barbero *et al.* 1952; Crosse, Wallis, Low & Henly, 1960) that SMA-fed infants develop a faecal bifid flora quantitatively intermediate between that of breast-fed and cow's-milk-fed infants. The acidity of the stools of infants fed on SMA can approach that of breast-fed infants (Barbero *et al.* 1952; Barness *et al.* 1957; Crosse *et al.* 1960). Although it had previously been thought that stool acidity was a direct result of the fermentative activity of

bifidobacteria (Barbero *et al.* 1952), the observations of Willis *et al.* (1973) in breast-fed infants showed that the presence of bifidobacteria was not essential for development of a low stool pH. They suggested that the low buffering capacity of breast-milk was responsible. SMA also has a lower buffering capacity than cows milk (Wyeth & Brother, 1970) but despite this we found that the incidence and mean counts of *E. coli* were higher in the stools of infants fed on SMA than in those of infants fed cow's milk. These observations indicate that the enhancement of bifido bacteria in the colon may not necessarily lead to reduced colonization by *E. coli*, and that not all milks of buffering capacity lower than cow's milk may render the colon contents sufficiently acid to reduce the growth of *E. coli* or other intestinal pathogens.

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