Protein-degradation products and bacterial enzyme activities in faeces of breast-fed and formula-fed infants

Patricia M. Heavey[1]*, Shirley-Anne H. Savage[2], Alison Parrett[2],
Cinzia Cecchini[3], Christine A. Edwards[2] and Ian R. Rowland[1]

[1]*Northern Ireland Centre for Diet and Health, Biomedical Sciences, University of Ulster,
Coleraine BT52 1SA, Northern Ireland, UK*
[2]*The Department of Human Nutrition, University of Glasgow, Yorkhill Hospitals, Glasgow G3 8SJ, Scotland, UK*
[3]*Laboratory of Microbiology, Camerino University, 62032 Camerino (MC), Italy*

The aim of the present study was to determine the effects of age and diet (breast milk, formula milk and weaning diet) on metabolic activities in faecal samples from infants aged 1 week to 1 year, and to compare these findings with activities found in samples from adults. Such activities can provide valuable information on functional changes in the microbiota that may have significance for the health of the host. Fresh faecal samples were collected from forty-four breast-fed infants (twenty-four males, twenty females) and thirteen formula-fed infants (three males, ten females) throughout the first year of life. The samples were analysed for protein-breakdown products, including the faecal concentrations of NH_3, phenol and *p*-cresol, and faecal bacterial enzyme activities. There was wide individual variation in all variables measured; however, the values in infants were substantially lower then those found in adults. In pre-weaned infants, faecal NH_3 concentration and β-glucuronidase activity were the only endpoints that were significantly different in breast-fed and formula-fed infants ($P < 0.001$ and $P < 0.05$ respectively). This was not apparent after weaning. There was a significant difference between the breast-fed and formula-fed weaned groups and their pre-weaned counterparts only for NH_3 ($P < 0.05$). β-Glucuronidase activity and phenol concentration were significantly ($P < 0.01$) greater in weaned breast-fed infants compared with pre-weaned breast-fed infants. No differences were observed between pre-weaned and weaned formula-fed infants for any of the variables except for NH_3 concentration. It can be concluded from the present study that there are significant differences in two faecal characteristics between breast- and formula-fed infants and that changes occur as the infants grow older and are weaned onto solid foods.

Infants: Faecal samples: Bacterial protein-degradation products: Enzyme activity

At birth, the gastrointestinal tract is sterile (Escherich, 1885). In an infant's first postnatal week, the gastrointestinal tract is exposed to a wide variety of organisms, reflecting a range of external influences such as the mother's vaginal, faecal and skin floras, and also the extent of bacterial contamination from the perinatal environment (Gareau *et al.* 1959; Bettelheim & Lennox-King, 1976; Sarkany & Gaylarde, 1986). The bacterial contamination of the newborn infant is influenced by mode of delivery and contact with the mother and external environment, which leads to individual patterns of colonisation. Although the infant is exposed to diverse bacteria, not all are able to establish themselves within the neonatal intestine. Bacteria capable of aerobic growth, such as enterobacteria, streptococci and staphylococci, are the first to proliferate in the infant (Mata & Urrutia, 1971; Balmer & Wharton, 1989), as a consequence of a positive oxidation–reduction potential in the gut. As these facultative and aerobic bacteria increase in numbers, they consume O_2, lowering the redox potential and thus allowing anaerobic bacteria to proliferate (Stark & Lee, 1982). Anaerobic bacteria most commonly found in the newborn include bifidobacteria, clostridia and bacteroides (Stark & Lee, 1982).

Once a feeding regimen has commenced, a pattern begins to emerge in the microbiota of the gut and here differences have been reported between breast- and formula-fed infants (Yoshioka *et al.* 1983; Benno *et al.* 1984; Heavey & Rowland, 1999). Further, it would

* **Corresponding author:** Dr Patricia Heavey, fax +44 28703 324 965, email p.heavey@ulster.ac.uk

appear that breast-fed infants have a simple microbiota, consisting of bifidobacteria, enterococci and enterobacteria, with formula-fed infants having more clostridia and bacteroides than their breast-fed counterparts. Infants usually acquire a microbiota similar to that of adults within 2 years (Stark & Lee, 1982).

Most studies have focused on the bacteriology of the developing infant; however, there are some reports that these bacterial changes are also associated with marked changes in metabolic activity. For example, several investigators have studied microbiota-associated changes, such as mucin and bilirubin degradation and cholesterol metabolism, in faeces of infants up to 2 years old (Norin *et al.* 1985; Midtvedt & Midtvedt, 1992), and there have been comparisons of carbohydrate metabolism in breast- and formula-fed infants (Lifschifz *et al.* 1990; Midtvedt & Midtvedt, 1993; Parrett & Edwards, 1997). Less well studied have been potentially detrimental metabolic processes in the intestine, in particular, the metabolites produced by bacterial fermentation of proteins and amino acids and also bacterial enzymes that may yield toxic products (Rowland, 1992, 1995). In infants, it has long been established that breast milk is the ideal food for the developing infant and of particular interest is the human-milk protein fraction. The protein concentration of human milk is the lowest of all mammalian species and results in almost complete utilisation of the protein component by the infant. The main products of bacterial protein degradation, which include NH_3, phenol and *p*-cresol, can exert toxic effects, both systemically in the body, and locally on the gut mucosa (Bakke, 1969; Visek, 1978; MacFarlane *et al.* 1986).

It is well documented that the products of bacterial enzymes, such as β-glucuronidase and β-glucosidase, can exert damaging effects on the host (Goldin, 1986; Rowland, 1992). β-Glucuronidase acts on ingested toxicants that have been conjugated to glucuronic acid in the liver and secreted into the gut via the biliary tract. Hydrolysis of the conjugates releases potentially toxic aglycones in the colon. Similarly, β-glucosidase can hydrolyse glucoside conjugates found in plants, releasing biologically active phytochemicals (Gangolli & Rowland, 1999).

Analysis of protein-breakdown products and assays of metabolic processes in the faeces can provide valuable information on functional changes in the microbiota that may have significance for the health of the host (Rowland, 1992, 1995). In the present study, we have investigated such functional changes in developing infants fed breast or formula milk.

Materials and methods

Subjects

Fifty-seven full-term newborn infants were recruited from the Glasgow (Scotland) area. Mothers were approached within 2 d of giving birth, and those who were exclusively breast-feeding or exclusively formula-feeding (Aptamil; Milupa Research, Friedrichsdorf, Germany) were invited to participate in the study. None of these infants or their mothers had received any antibiotics at the time of recruitment or at any time before giving samples. Fresh faecal samples were collected from forty-four breast-fed infants (twenty-four males, twenty females) and thirteen formula-fed infants (three males, ten females) at several time points during the first year. These were usually 1 week, 4 weeks, 2 months, early weaning (4 weeks after first non-milk foods), 7 months, 9 months and 1 year. The mothers chose to wean at ages that were not influenced by the authors. At the time of recruitment, there was a breast-feeding initiative underway to encourage breast-feeding. This resulted in fewer formula-fed infants participating in the study.

Ethical approval

This study was approved by the Ethics Committee of Yorkhill NHS Trust, Glasgow. The mothers of all infants gave their informed written consent.

Faecal samples

Faecal samples were collected from the infants at their homes and were processed within 2 h of defecation. When the mother had detected a soiled nappy, she immediately telephoned the department and the nappy was collected by car. To facilitate this process, mothers and infants were recruited from a zone of 30 min travel from the laboratory. Samples were weighed and a faecal suspension (100 g/l) was prepared with distilled water. The samples were kept frozen ($-20°C$) until subsequent analysis. The pH of the samples was measured when the samples were thawed. Due to their small size, not all variables were measured for each sample. For comparison purposes, faecal samples from nine adults (aged 21–28 years) were collected and subjected to similar treatment.

Faecal ammonia concentration

Concentration of faecal NH_3 was determined by spectrophotometry as described by Solorzano (1969). Briefly, the faecal suspensions were diluted and centrifuged at 5000 *g* for 5 min. A sample of the supernatant fraction (0·5 ml) was added to 0·5 ml phenol-nitroprusside solution, 0·5 ml alkaline hypochlorite solution (both from Sigma-Aldrich Co. Ltd, Poole, Dorset, UK) and 3·5 ml water and left at room temperature for 40 min. Absorbance was measured at 570 nm and related to NH_3 concentration by reference to a standard curve.

Faecal production of ammonia

The rate of production of NH_3 from endogenous N sources in the faeces was determined according to Wise *et al.* (1982). The faecal samples were diluted with 0·1 M-potassium phosphate buffer (at a pH within 0·2 pH units of that of the sample) to a final concentration of 200 g/l. The suspensions were incubated at 37°C for 15 min and samples (0·7 ml) removed at 3 min intervals and kept on ice. The samples were centrifuged at 5000 *g* for 5 min and NH_3 determined as described earlier.

Measurement of phenol and p-cresol

The concentration of phenol and p-cresol in faeces was determined by GC. A sample of faecal supernatant fraction (1 ml) was heated with 0·1 ml internal standard solution (20 mM-p-methoxyphenol), 1·0 ml methanol and 0·2 ml H_2SO_4 (500 ml/l) in a water-bath at 70°C for 50 min. Distilled water (0·2 ml) and chloroform (0·8 ml) were added after the reaction mixture had cooled. The chloroform layer containing derivatised phenolic compounds was extracted, and 5 μl were injected onto a 25 m × 0·25 mm CP-Sil 5 CB column in a Perkin Elmer auto system GC (model 970; Perkin Elmer, Norwalk, CT, USA). The carrier gas (He), H_2 and air were set at flow rates of 25 cm³/s, 45 and 450 ml/min respectively. The injection port temperature was 250°C and the detector temperature was 300°C. Identification of peaks was made by coincidence of retention times with the standards. Quantitative assessment was made by comparison of peak areas of test samples with those of authentic phenol and p-cresol standards.

Determination of β-glucosidase and β-glucuronidase

The pH of the diluted faecal samples was measured and assays for both enzymes were conducted at a buffered pH closest (within 0·2 pH units) to that of the samples. The suspensions were incubated at 37°C with 3 mM-p-nitrophenyl-β-D-glucopyranoside or 3 mM-p-nitrophenyl-β-D-glucuronide (Sigma-Aldrich Co. Ltd) for assessment of β-glucosidase and β-glucuronidase respectively. Release of p-nitrophenol was measured colorimetrically over time and used as the measure of enzyme activity (Wise *et al*. 1982).

Statistical analyses

Data for NH_3 concentration and production and β-glucuronidase and β-glucosidase activities were transformed (log_{10}) and compared using one-way ANOVA, and comparisons between means were compared by the least significant difference criterion. For statistical analyses,

we have included only those infants who provided faecal samples both before and after weaning. In the case of those infants who provided more then one sample within the pre- or post-weaning periods, an average of these samples was taken and treated as a single value.

Results

Age of weaning

All infants were weaned between 12 and 20 weeks inclusive.

Faecal pH

Most infant faecal samples were acidic, with a mean pH of 6·7 (SD 0·9) (range 4·3–8·6). Faecal pH increased gradually as the infants got older (6·5 at < 4 weeks old to 7·2 in infants > 36 weeks old) and there was a significant difference between all infants < 4 weeks old and those > 36 weeks old ($P = 0.002$).

Ammonia concentration

There was wide individual variation in faecal NH_3 concentrations, which ranged from 0·00 to 41·39 μmol/g in infants aged from 1 week to 1 year (Tables 1 and 2). These values were substantially lower than those found in adults, although there was overlap between some older infants and adults (Table 1). In both breast- and formula-fed infants the concentration of NH_3 generally tended to increase with age (Table 2). The results shown in Table 2 demonstrate that many of the variables measured increased over time as a result of either age or diet or a combination of the two. However, statistical analysis was not carried out, since not all infants provided samples for all time points. When the infants were grouped according to weaning status, there was a significant difference between breast- and formula-fed infants prior to weaning ($P < 0.001$, Table 3). However, this was not evident after weaning. There was also a significant difference between pre-weaned and weaned breast-fed infants ($P < 0.001$). NH_3

Table 1. Comparison of faecal bacterial metabolites in infants (1 week–12 months old) and adults*

(Mean values, standard deviations and ranges)

	Infants				Adults			
	Mean	SD	Range	n	Mean	SD	Range	n
Ammonia concentration (μmol/g)†	7·68	7·15	0·00–41·39	175	37·86	32·21	8·81–109·79	9
Ammonia production (μmol/g per h)‡	5·86	6·53	0·00–33·99	175	41·35	28·93	12·93–70·33	4
Phenol concentration (mmol/kg)§	0·58	0·84	0·00–6·30	112	2·79	0·38	2·53–3·06	2
p-Cresol concentration (mmol/kg)‖	0·14	0·14	0·00–0·53	112	0·28	0·06	0·26–0·31	2
β-Glucuronidase activity (μmol product formed/h per g faeces)¶	11·87	11·09	0·00–65·22	133	33·32	21·44	17·94–63·74	4
β-Glucosidase activity (μmol product formed/h per g faeces)**	16·55	15·38	0·00–73·47	133	35·61	7·12	28·33–45·64	4

* For details of subjects and procedures, see p. 510.
† Mean age: infants 22 weeks, adults 24·9 years.
‡ Mean age: infants 22 weeks, adults 24·7 years.
§ Mean age: infants 18 weeks, adults 24·5 years.
‖ Mean age: infants 18 weeks, adults 24·5 years.
¶ Mean age: infants 14 weeks, adults 24·7 years.
** Mean age: infants 14 weeks, adults 24·7 years.

Table 2. Ammonia and enzyme activities in faecal samples from infants aged 1 week–1 year*

(Mean values and standard deviations)

Age (weeks)...	≤4			>4–≤12			>12–≤20			>20–≤28			>28–≤36			>36		
	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
Ammonia concentration (μmol/g)																		
All infants	3·72	2·68	37	5·60	4·93	32	6·03	4·14	31	8·00	5·74	22	12·35	9·43	25	12·74	9·57	28
BF	3·11	2·42	26	2·35	1·67	18	5·46	4·20	23	6·70	4·51	17	10·02	6·51	21	12·68	9·98	25
FF	5·18	2·82	11	9·76	4·59	14	8·07	3·83	7	12·43	7·74	5	22·31	16·86	4	13·25	6·36	3
Ammonia production (μmol/h per g)																		
All infants	3·62	3·05	37	6·02	7·52	32	5·12	5·94	31	8·51	7·18	22	5·70	6·40	25	7·52	8·10	28
BF	3·80	3·22	26	3·59	3·03	18	5·39	6·34	23	7·22	5·57	17	5·36	6·75	21	8·19	8·28	25
FF	3·19	2·68	11	9·14	10·20	14	4·77	5·16	7	12·86	10·78	5	5·67	4·66	4	1·91	3·30	3
β-Glucuronidase activity (μmol/h per g)																		
All infants	9·39	9·43	23	9·31	7·47	26	9·90	8·80	27	13·02	10·98	20	18·42	16·99	16	14·23	12·29	21
BF	6·99	5·14	14	6·42	4·55	17	8·49	8·62	16	9·81	6·63	14	11·84	12·06	10	14·46	13·29	18
FF	13·12	13·26	9	13·55	9·34	10	12·87	8·98	10	20·50	15·76	6	29·39	19·32	6	12·85	2·68	3
β-Glucosidase activity (μmol/h per g)																		
All infants	11·77	11·75	23	10·10	6·85	26	16·23	15·73	27	14·38	11·54	20	25·39	15·47	16	25·56	22·13	21
BF	12·03	12·58	14	10·14	7·33	17	17·09	15·65	16	12·65	10·40	14	19·85	10·83	10	25·25	23·05	18
FF	11·38	11·08	9	9·02	6·70	10	16·47	16·59	10	18·44	14·02	6	34·53	18·59	6	27·46	19·32	3

BF, breast-fed; FF, formula-fed.
* For details of subjects and procedures, see p. 510.
All infants were weaned between 12 and 20 weeks inclusive.

concentration was also significantly increased in weaned formula-fed infants compared with their pre-weaned counterparts ($P=0.039$).

Ammonia production

There was wide individual variation in faecal NH_3 production, which ranged from $0.00–33.99$ μmol/g per h in infants aged from 1 week to 1 year (Tables 1 and 2). Overall, these values were substantially less than those found in adults (Table 1). When infants were grouped according to weaning status, there were no significant differences in production rates between any of the groups (Table 3).

Phenol and p-cresol concentration

Phenol and *p*-cresol were not detected in any faecal samples from infants up to the age of 3 weeks, and levels remained low prior to weaning (Table 4). Levels of phenol and *p*-cresol in all infants were lower than those found in adults (Table 1). When the infants were grouped according to weaning status, there was no significant difference between pre-weaned breast- or formula-fed infants for either phenol or *p*-cresol (Table 3). However, phenol concentration was significantly higher in weaned *v.* non-weaned breast-fed infants ($P<0.003$, Table 3).

Again, there was no difference between pre-weaned breast- and formula-fed infants for *p*-cresol concentration (Table 3). Similarly, no differences were observed between either breast- or formula-fed infants when comparing pre-weaned and weaned infants (Table 3).

Bacterial enzyme activities

The mean activities of β-glucuronidase and β-glucosidase were less in the infants compared with adults (Table 1), although some individual infants had higher activity for the enzymes than that found in the adults. Bacterial enzyme activities appeared to increase as the infants became older and were introduced to a more varied diet. When the infants were grouped according to weaning status, β-glucuronidase activity was statistically higher in the pre-weaned formula-fed infants compared with the pre-weaned breast-fed infants ($P=0.034$, Table 3). After weaning there was an increase in enzyme activity in both breast- and formula-fed infants compared with their pre-weaned counterparts, although this was not significant.

Table 3 also shows β-glucosidase activity, which was slightly lower in the pre-weaned breast-fed infants compared with the pre-weaned formula-fed infants. There was an increase in β-glucosidase activity in both breast-fed and formula-fed infants post-weaning compared with their pre-weaned counterparts, although again this was not significant.

Discussion

The present study provides observational results with regard to several metabolic activities in breast- and formula-fed infants throughout the first year of life. The results indicate that the pronounced changes reported in microbial composition of the gut flora in early life (Stark

Table 3. Faecal ammonia concentration and production, phenol, *p*-cresol concentrations and enzyme activities in breast-fed and formula-fed infants‡

(Mean values and standard deviations)

Diet...	Breast-fed						Formula-fed					
	Pre-weaned			Weaned			Pre-weaned			Weaned		
	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*
Ammonia concentration (μmol/g)§	3·30	2·31	21	9·67***	6·20	21	7·79***	4·90	11	12·70†	5·85	11
Ammonia production (μmol/h per g)§	4·93	6·47	21	5·30	3·37	21	6·66	7·76	11	7·02	7·15	11
β-Glucuronidase activity (μmol/h per g)‖	7·22	5·62	16	11·97	11·33	16	14·38*	9·18	9	17·69	12·55	9
β-Glucosidase activity (μmol/h per g)‖	12·43	11·30	16	19·04	13·45	16	14·05	8·80	9	20·37	13·27	9
Phenol (mmol/kg)¶	0·20	0·18	13	0·96**	0·93	13	0·48	0·34	8	0·79	0·95	8
p-Cresol (mmol/kg)¶	0·10	0·11	13	0·22	0·19	13	0·20	0·12	8	0·10	0·09	8

Mean values were significantly different from those of pre-weaned breast-fed infants: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Mean value was significantly different from that of pre-weaned formula-fed infants: †$P < 0.05$.

‡ For details of subjects and procedures, see p. 510.

§ Mean age (weeks): breast-fed pre-weaned 6, weaned 26; formula-fed pre-weaned 6, weaned 23.

‖ Mean age (weeks): breast-fed pre-weaned 5, weaned 33; formula-fed pre-weaned 7, weaned 27.

¶ Mean age (weeks): breast-fed pre-weaned 7, weaned 31; formula-fed pre-weaned 6, weaned 27.

& Lee, 1982; Heavey & Rowland, 1999) are reflected in changes in microbial activity in the gut. Overall, the concentrations of phenol, *p*-cresol, NH_3, and NH_3 production, as well as activities of β-glucuronidase and β-glucosidase in infant faeces, were found to be substantially lower in most infants than in adults.

The results from the present study also confirm that there were differences in faecal NH_3 concentrations between infants exclusively fed breast or formula milk, although this effect became less evident as the infants got older and were introduced to a more varied diet. This is comparable with a study conducted by Guérin-Danan *et al.* (1997), who reported an average NH_3 concentration of 6·7 μmol/g faecal sample from infants (*n* 35) aged 10–18 months. However, there was wide variation in faecal NH_3 production and no differences were observed between breast- and formula-fed infants. NH_3 has toxic effects on gut mucosal cells, which include a reduction in the lifespan of these cells. Mammalian cells in culture can only tolerate modest concentrations of NH_3 due to disruptions in intracellular pH (Visek, 1978). However, it is difficult to extrapolate the results from such *in vitro* studies to the concentrations found in the infant and adult faeces.

Although the level of phenol in infants' faeces was substantially lower than those found in those of adults (Smith & MacFarlane, 1996; Table 1), this was not observed for *p*-cresol. There were also no apparent differences between breast- and formula-fed infants for either phenol or *p*-cresol. A likely explanation for this is that the formula feeds currently available, including the products used in the present study, contain similar amounts of tyrosine (the substrate from which gut bacteria generate phenol and *p*-cresol) to levels found in breast milk. Phenols are usually detoxified by either sulfate or glucuronide conjugation in the colonic mucosa and then excreted in the urine or remain unabsorbed and excreted in the faeces (Ramakrishna *et al.* 1989).

Faecal bacterial enzyme activity remained low in most infants compared with adults (Table 1), except for some babies with extremely high levels of both enzymes. This may reflect the specific bacterial flora in the infants,

since different bacterial types express different levels of β-glucuronidase and β-glucosidase activities (Saito *et al.* 1992). Guérin-Danan *et al.* (1997) reported levels less than those found in infants aged 10–18 months compared with levels in adults. Again, there were some individual infants with very high levels of both enzymes.

With regards to β-glucuronidase, our present results show that exclusively formula-fed infants had significantly higher enzyme activities than exclusively breast-fed babies. After weaning, the formula-fed infants exhibited significantly greater β-glucuronidase activity compared with their breast-fed counterparts, although this was not significant. Our present results are consistent with those of Grönlund *et al.* (1999), who reported higher enzyme activity in formula-fed infants (before and after weaning), but not with the study of Mykkänen *et al.* (1997) who found no differences between the groups. The inconsistency between studies may be linked to the different types of formula feed used (Heavey & Rowland, 1999).

β-Glucosidase activity was slightly higher (although not significantly so) in completely breast-fed compared with completely formula-fed infants, possibly due to the presence of bifidobacteria and lactobacilli, which are frequently present at greater concentrations in breast-fed babies. Both bifidobacteria and lactobacilli possess high levels of β-glucosidase (Saito *et al.* 1992; Rowland & Tanaka, 1993). This difference was less evident after weaning and activity of β-glucosidase increased in both groups after weaning.

It has long been recognised that breast milk is the ideal food for the developing infant. The results from the present study demonstrate significant differences in faecal NH_3 and β-glucuronidase concentrations between pre-weaned breast- and formula-fed infants. However, it would appear that changes occur in most faecal biochemical characteristics for all infants as they get older. This may be a response for the introduction of a weaning diet combined with the maturation of the gut and gut enzymes. To what extent early feeding patterns influence adult microbiota activities remains to be established.

Table 4. Phenol and p-cresol in infants (aged 1 week–1 year) faecal samples*
(Mean values and standard deviations)

Age (weeks)…	≤3			>3–≤4			>4–≤12			>12–≤20			>20–≤28			>28–≤36			>36		
	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
Phenol (mmol/kg)																					
All infants	0.00	0.00	15	0.16	0.15	18	0.51	0.58	18	0.49	0.26	20	0.70	0.39	15	0.83	0.45	11	1.91	1.81	12
BF	0.00	0.00	12	0.15	0.14	11	0.31	0.16	12	0.49	0.28	12	0.58	0.29	11	0.88	0.56	7	1.84	1.88	11
FF	0.00	0.00	3	0.11	0.14	7	0.72	0.77	9	0.49	0.24	8	1.03	0.49	4	0.75	0.17	4	2.70	–	1
p-Cresol (mmol/kg)																					
All infants	0.00	0.00	15	0.01	0.10	18	0.15	0.11	18	0.20	0.17	20	0.20	0.14	15	0.22	0.16	11	0.09	0.16	12
BF	0.00	0.00	12	0.01	0.11	11	0.14	0.01	12	0.22	0.18	12	0.17	0.13	11	0.22	0.17	7	0.01	0.17	11
FF	0.00	0.00	3	0.01	0.01	3	0.16	0.13	9	0.16	0.15	8	0.30	0.14	4	0.26	0.15	4	0.15	–	1

BF, breast-fed; FF, formula-fed.
* For details of subjects and procedures, see p. 510.

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