

Influence of soya-based infant formula consumption on isoflavone and gut microflora metabolite concentrations in urine and on faecal microflora composition and metabolic activity in infants and children

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The urinary excretion of soya isoflavones and gut microflora metabolites was investigated in infants and children who had been fed soya-based infant formulas in early infancy. These infants and children were compared with cows'-milk formula-fed controls, to determine at what age gut microflora metabolism of daidzein to equol and/or *O*-desmethylangolensin (*O*-DMA) was established, and whether exposure to isoflavones in early infancy influences their metabolism at a later stage of development. Sixty infants and children (aged 4 months–7 years) participated in the study; thirty in each of the soya and control groups. There were four age groups. These were: 4–6 months (seven in the soya group and seven in the control group); 7–12 months (seven in the soya group and nine in the control group); 1–3 years (six in the soya group and eight in the control group); 3–7 years (ten in the soya group and six in the control group). Urine samples were collected to measure isoflavonoids by MS, and faecal samples were collected to measure gut-health-related bacterial composition, by fluorescent *in situ* hybridisation with oligonucleotide probes, and metabolic activity. A soya challenge (typically a soya yoghurt alternative product containing 4.8 g soya protein and on average 22 mg total isoflavones) was given to control-group infants (>6 months) and children, and also to soya-group children that were no longer consuming soya, to determine their ability to produce equol and/or *O*-DMA. Urinary genistein, daidzein and glycitein were detected in all infants (4–6 months) fed soya-based infant formula; *O*-DMA was detected in 75 % of infants but equol was detected in only 25 %. In the controls (4–6 months), urinary isoflavonoids were very low or not detected. In the older age groups (7 months–7 years), *O*-DMA was found in the urine samples of 75 % of the soya group and 50 % of the controls, after the soya challenge. Equol excretion was detected in 19 % of the soya-group infants and children, and in only 5 % of the controls. However, in the oldest (3–7 years) children, the proportion excreting *O*-DMA and equol was similar in both groups. Faecal bacterial numbers for *bifidobacteria* ($P<0.001$), *bacteroides* and *clostridia* ($P<0.05$) were significantly lower for the soya group compared with the control group. There appears to be no lasting effect of early-life isoflavone exposure on isoflavone metabolism.

Soya isoflavone metabolism: Equol: Soya-based infant formula: Gut bacterial microflora

There is considerable interest in the contribution that soya isoflavones may make to human health (Wiseman, 2000; Barnes, 2001; Adlercreutz, 2002). The possible health consequences of early soya consumption are also attracting attention (Badger *et al.* 2002; Mendez *et al.* 2002). Although there have been a large number of studies on the metabolism and bioavailability of soya isoflavones in adults (Morton *et al.* 1994; Lampe *et al.* 1998; Watanabe *et al.* 1998; Rowland *et al.* 2000, 2003; Setchell *et al.* 2002*a,b*, 2003*a,b*), there is little information available in infants and children. The gut microflora in early childhood is very different to that in adulthood, therefore it is

important to characterise developmental changes in isoflavone biotransformation in early life.

The intestinal tract of the human fetus is sterile, but after birth it rapidly becomes colonised by bacterial microflora from the mother's faecal, vaginal and skin floras (Heavey & Rowland, 1999). Further bacterial types are then established in the gut from a variety of endogenous and exogenous sources including diet and the environment. The development of the microflora occurs gradually and it can take several years before an adult-type flora is established. This has implications for isoflavone metabolism because the major metabolites, equol and

O-desmethylangolensin (*O*-DMA), are products of gut microflora metabolism. It has been consistently reported in adults consuming soya-protein diets that approximately 35% are able to produce equol (Morton *et al.* 1994; Lampe *et al.* 1998; Rowland *et al.* 2000; Setchell *et al.* 2002a); however, there is far less interindividual variation in the ability to produce *O*-DMA (Rowland *et al.* 2000). The ability to produce equol is of considerable interest because equol is more oestrogenic than the parent isoflavone daidzein (Markiewicz *et al.* 1993) and has the greatest antioxidant activity of all the isoflavones tested in a range of *in vitro* test systems (Hodgson *et al.* 1996; Arora *et al.* 1998; Mitchell *et al.* 1998). A decreased *in vivo* lipid peroxidation (Wiseman *et al.* 2000) and increased resistance of LDL to oxidation *ex vivo* (Tikkanen *et al.* 1998; Jenkins *et al.* 2000; Wiseman *et al.* 2000) have been reported following the consumption of soya-protein diets. Therefore, the superior antioxidant activity of equol compared with other isoflavones and thus the ability to produce equol may provide a greater inhibition of lipid peroxidation and thus a greater reduction in risk for cardiovascular disease. Furthermore, a high urinary excretion of equol has been reported to be associated with a decreased risk of breast cancer (Ingram *et al.* 1997).

Irvine *et al.* (1998a) reported that significant amounts of daidzein and genistein were excreted in the urine of soya-based infant formula-fed infants but not cows'-milk formula-fed infants (up to the age of 4 months). Setchell *et al.* (1997) reported significantly greater amounts of genistein and daidzein in the plasma of 4-month-old soya-based infant formula-fed infants compared with cows'-milk formula-fed infants. Equol was consistently present in the plasma of infants fed cows'-milk formula, and in four out of seven soya-fed infants, but levels were very low. *O*-DMA was not detected in the plasma of any of the infants (Setchell *et al.* 1997).

The aim of the present investigation was to investigate the excretion of isoflavones in the urine of soya-based infant formula-fed and cows'-milk formula-fed infants and children (mostly following a soya challenge), from the ages of 4 months to 7 years. This was to determine at what age gut microflora metabolism of daidzein to equol and/or *O*-DMA was established and to assess whether exposure to soya isoflavones in early infancy influences the children's metabolism at a later stage of development. In addition, the aim of the study was to determine the influence of soya-based infant formula on the developing gut microflora in the infant, both in terms of gut health-related, overall bacterial composition and bacterial metabolic activity. The major effects of the type of infant feeding regimen on the gut microflora are likely to be in the early stages of development, so gut bacterial analysis was performed on faecal samples from a subgroup of infants aged 4–12 months.

Subjects and methods

Study design

The study was approved by the University of Ulster ethics committee and the mothers of the infants and children gave their informed consent.

Sixty infants and children (4 months–6 years) participated in the study; thirty in each of the soya and control groups. There were four age groups: 4–6 months, 7–12 months, 1–3 years and 3–7 years.

In the 4–6 month age group there were seven subjects in the soya group; five males aged 5, 5, 6, 6 and 6.5 months, and two females aged 6 and 6.5 months. There were seven subjects in the control group; three males aged 4.5, 5.5 and 6.5 months and four females aged 4, 4, 4.5 and 6.5 months.

In the 7–12 month age group there were seven subjects in the soya group; three males aged 8.5, 9 and 10 months and four females aged 8.5, 9, 10 and 11 months. There were nine subjects in the control group; five males aged 7, 9, 9.5, 11 and 12 months and four females aged 8.5, 9, 10 and 11 months.

In the 1–3 year age group there were six subjects in the soya group; three males aged 22.5, 25.5 and 26.5 months and three females aged 13, 13, and 15.5 months. There were eight subjects in the control group; five males aged 13, 17.5, 17.5, 30 and 31.5 months and three females aged 13, 29 and 35 months.

In the 3–7 year age group there were ten subjects in the soya group; five males aged 49, 57, 64, 64 and 71.5 months and five females aged 44.5, 52, 54.5, 82 and 83 months. There were six subjects in the control group; three males aged 40.5, 42.5 and 58.5 months and three females aged 43.5, 48 and 68 months.

The study recruited infants and children aged between 4 months and 7 years of age, who had been fed soya-based infant formula in early infancy. In the youngest age group (4–6 months) soya-based infant formulas were consumed from 3 months or earlier. Many of the soya group infants and children had continued to consume soya products after weaning. Subjects, also aged 4 months to 7 years, who had consumed cows'-milk formula as babies and who subsequently were not given soya products, were recruited as controls. On the third day of the study a urine sample was collected. A faecal sample was collected from the 4–12-month subgroup. The control-group infants (>6 months only, due to parental unwillingness in the younger age group) and children, and also the soya-group children that were no longer consuming soya, were given a soya isoflavone challenge to establish whether they were capable of converting daidzein to equol and/or *O*-DMA. Typically the soya challenge was a soya yoghurt alternative product containing 4.8 g soya protein and on average 22 mg total isoflavones (Wiseman *et al.* 2002) and was consumed on days 3 and 4. Urine samples were collected on days 4 and 5 and pooled.

Subjects

Subjects were recruited from general practitioner medical doctors' practices in Northern Ireland. Local paediatric dietitians and health visitors were also involved in recruitment. A medical history was obtained by the general practitioner, and only healthy infants and children were recruited. Subjects were excluded if they were on medication or treatment for any pre-existing disease, had gastrointestinal disease or infection, chronic diarrhoea or antibiotic use 3 months before commencing the study.

Sixty infants and children participated in the study. For the soya-based infant-formula group, sixty-three parents of potential subjects were contacted, thirty-seven agreed to participate and thirty infants and children participated in the study. For the control (cows'-milk formula-fed) group, ninety-nine parents were contacted, forty-five agreed to participate and thirty infants and children participated in the study. The heights and weights were obtained by the subjects' health clinic, where possible, or by the researcher. The participants were allocated to one of the four age groups. The parents of the subjects were asked to keep 3 d weighed food records so that the nutrient intakes of the subjects could be analysed. Nutrient analysis of the food records was carried out with Weighed Intake Analysis Software (version 1.28; Tinuviel Software, London).

Collection and handling of samples

The details of the study and methods for collecting urine and faecal samples were explained during a visit to the parents before commencing the study.

Infant formula milk sample collection. The parent transferred dry powder samples of the soya-based infant formula or cows'-milk formula into a universal container. These were stored at -20°C for subsequent isoflavone analysis.

Urine sample collection. Spot urine samples were collected using an adhesive plastic urine collection bag (Hollister Ltd, Wokingham, Berkshire, UK). The isoflavonoid values measured were expressed per mg urinary creatinine. Ascorbic acid (approximately 0.2 g) was added to the urine samples as a preservative and the samples were stored at -70°C until analysed.

Faecal sample collection. Samples were processed within 3.5 h of collection. The parents were supplied with a pre-weighed container into which they placed the sample immediately after defecation. The date and time of collection were noted. The researcher was then telephoned so that the sample could be collected from the home and transported to the laboratory. The sample was placed on ice or in a refrigerator before collection. In the laboratory the sample was weighed and a 1 g sample was removed and processed for analysis of the gut microflora composition by fluorescent *in situ* hybridisation with group-specific 16S rRNA-targeted oligonucleotide probes. A 200 g/l faecal suspension was prepared with saline (9 ml/l) for subsequent measurement of NH_3 and short-chain fatty acid (SCFA) concentration, pH and activities of the bacterial enzymes, β -glucosidase and β -glucuronidase.

Analytical methods

Measurement of isoflavone concentrations in infant formula samples. Sample extraction was an adaptation of the method of Coward *et al.* (1993). Single replicates of powdered formulas (0.5 g) and $5\ \mu\text{g}$ [$^{13}\text{C}_3$]daidzein (internal standard) were dispersed by sonication and extracted with 5 ml aqueous ethanol (800 ml/l) by stirring for 1 h at 60°C . The mixture was cooled and centrifuged at 8000 g

for 5 min, the solvent extract aspirated off and the residue pellet re-suspended in further aqueous ethanol ($2 \times 2.5\ \text{ml}$). The combined extract was reduced in volume (5 ml) and lipids removed by partitioning into and discarding a hexane wash ($4 \times 20\ \text{ml}$). The extract was dried under an N_2 stream and the residue dissolved in 10 ml aqueous methanol (500 ml/l). A sample (2 ml) of the extract was filtered ($0.45\ \mu\text{m}$) before analysis by liquid chromatography (LC)-MS with selective ion monitoring, according to the method of Wiseman *et al.* (2002). Concentrations were expressed as mg/kg whole food.

Measurement of isoflavonoid concentrations in urine. Urinary isoflavones were measured by isotope dilution LC-MS, by an adaptation of the extraction method of Lu *et al.* (1995) and the chromatographic method of Wang & Murphy (1994). ^{13}C internal standards ($5\ \mu\text{g}$ of each of [$^{13}\text{C}_3$]daidzein and [$^{13}\text{C}_3$]genistein; Dr Nigel Botting, University of St Andrews, UK) were added to urine samples (3 ml), which were deconjugated by incubating overnight at pH 5.0 (acetate buffer) with β -glucuronidase-sulfatase (*Helix pomatia* extract; Sigma-Aldrich Chemical Co., Poole, UK). Samples were neutralised with ammonium carbonate then allowed to adsorb onto Chem-elut CE1010 solid-phase extraction cartridges (Chrompack, London, UK). Analytes were eluted with ethylacetate, followed by diethyl ether. Solutions of extracts in aqueous acetonitrile (150 ml/l) were analysed using LC-MS in +APCI mode by selective ion-monitoring mode. LC-MS separations were performed on a YMC-Pack ODS-AM HPLC column (Crawford Scientific, Strathaven, UK) using a water-acetonitrile gradient containing glacial acetic acid (5 ml/l). The mean values and mean intra-assay imprecision for analytes in a naturally incurred quality-control sample were as follows: daidzein, 3.99 mg/l (CV 1.0%); genistein, 1.60 mg/l (CV 2.0%); glycitein, 0.04 mg/l (CV 11%); O-DMA, 5.11 mg/l (CV 0.3%); equol, 2.76 mg/l (CV 1.2%). A diagnostics kit (Sigma, procedure no. 555; Sigma-Aldrich Co., Poole, UK) was used for the quantitative spectrophotometric determination of creatinine in urine.

Faecal bacterial counts. Bacterial numbers in faecal samples were measured by fluorescent *in situ* hybridisation with group-specific 16S rRNA-targeted oligonucleotide probes (Harmsen *et al.* 1999). The faecal samples were suspended (100 g/l) in PBS and bacterial cells fixed with paraformaldehyde (40 ml/l) overnight at 4°C . After centrifugation, the resulting cell pellet was washed in PBS and then re-suspended in ethanol (480 ml/l) in PBS, and stored at -20°C until analysed. Subsamples of PBS-ethanol stock were added to hybridisation buffer and hybridised for 30 min, at the temperatures stated, with the following group-specific 16S rRNA-targeted oligonucleotide probes labelled with cyanine (cy3) fluorescent marker dyes. These were BIF 164 (probe for genus *bifidobacterium*), 50°C ; BAC 303 (probe for genus *bacteroides*), 45°C ; LAB 158 (probe for *lactobacilli* + *enterococci*), 45°C ; HIS 150 (probe for *clostridium*, subgroups *perfringens* and *histolyticum*), 50°C . Total bacterial counts were estimated by staining the cells with 4,6-diamino-2-phenylindole. After hybridisation, the cell suspensions were filtered through $0.2\ \mu\text{m}$ Millipore filters, the filters were

placed on microscope slides and the cells were counted under a fluorescent microscope (at least fifteen fields, with at least ten bacteria per field).

Measurement of faecal ammonia and short-chain fatty acid concentrations, pH and enzyme activity. For the measurement of faecal NH₃ concentration, the faecal suspensions were diluted and centrifuged at 5000g for 5 min. Then a sample of the supernatant fraction (0.5 ml) was added to 0.5 ml phenol (0.53 mmol/l)-nitroprusside (0.86 mmol/l) solution, 0.5 ml alkaline hypochlorite solution (2 g/l) (Sigma-Aldrich Co., Poole, UK) and 3.5 ml water and left at room temperature for 40 min. NH₃ concentration was measured by spectrophotometry according to the method of Solorzano (1969). For the measurement of faecal SCFA concentration, the faecal suspensions were centrifuged at 5000g for 5 min. A sample (1 ml) of each suspension was spiked with caproic acid (80 nmol) as the internal standard, acidified with the addition of 1 ml H₂SO₄ (0.36 mol/l) and extracted with diethyl ether (0.8 ml). SCFA in the diethyl ether extract were measured by GC with a flame ionisation detector according to the method of Lepage & Roy (1986). The pH of each faecal suspension was measured. For the measurement of β-glucosidase and β-glucuronidase activities, the faecal suspensions were diluted to a final concentration of 200 ml/l in phosphate buffer at a pH value similar to that recorded for each faecal suspension. The suspensions were incubated aerobically at 37°C with *p*-nitrophenyl-β-D-glucopyranoside (3 mmol/l) or *p*-nitrophenyl-β-D-glucuronide (3 mmol/l) (Sigma-Aldrich Co., Poole, UK), respectively.

Release of *p*-nitrophenol was measured spectrophotometrically over time and used as the measure of enzyme activity according to the method of Wise *et al.* (1982).

Statistical analysis

SPSS/PC version 9.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Comparisons between the groups were made using independent *t* tests.

Results

The ages, heights and weights of the infants and children that participated in the present study are shown in Table 1. There were no significant differences between the soya and control groups of infants and children, in terms of age, weight and height, except for the 4–6 month groups, where the heights of the cows'-milk formula-fed infants were significantly lower ($P < 0.05$) than those of the soya-based infant formula-fed infants (Table 1). There were no significant differences between the soya and control groups in terms of dietary macronutrient intake (Table 2).

Table 3 shows the isoflavone aglycone content of the infant formulas that had been fed to the infants participating in the study. In the majority of the cows'-milk formula powders, isoflavones were not detected and, if present, were below the limit of detection (0.5 mg/kg dry powder) (Table 3). Two of the cows'-milk formulas (C and H) contained small amounts of isoflavones (1.2 and 2.1 mg aglycone equivalents/kg respectively) (Table 3). The

Table 1. Age, height and weight of infants and children in the soya and control groups for the different age groups (Mean values and standard deviations)

Age group	Subjects (<i>n</i>)		Age (months)				Height (m)				Weight (kg)			
			Soya		Control		Soya		Control		Soya		Control	
	Soya, all	Control, all	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
4–6 months	7	7	5.9	0.6	5.1	1.1	0.677	0.031	0.636	0.035*	8.0	1.1	7.1	0.7
7–12 months	7	9	9.4	1.0	9.7	1.5	0.730	0.022	0.736	0.034	9.1	0.6	10.2	1.3
1–3 years	6	8	19.3	6.2	23.3	8.9	0.844	0.077	0.893	0.087	11.5	2.6	14.2	3.0
3–7 years	10	6	62.0	13.3	50.2	10.8	1.045	0.114	1.048	0.080	19.0	5.8	17.6	1.9

* Mean value was significantly different to that for the soya group (*t* test) (* $P < 0.05$).

Table 2. Nutrient intakes of the soya- and control-group subjects (Mean values and standard deviations)

Age	Group*	Energy (MJ/d)		Fat (g/d)		CHO (g/d)		Protein (g/d)		NSP (g/d)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
4–6 months	Soya	2.9	0.41	26.5	4.7	94.0	10.6	20.3	4.2	2.8	1.6
	Control	2.7	0.49	27.4	3.5	86.8	18.1	18.6	6.7	2.4	1.7
7–12 months	Soya	3.5	0.78	33.8	11.2	108.2	14.7	28.4	10.4	4.0	1.1
	Control	3.4	0.59	30.6	7.2	107.1	14.1	28.8	10.6	4.5	1.8
1–3 years	Soya	4.1	0.68	37.5	9.5	136.5	21.1	32.3	8.9	5.4	1.2
	Control	3.6	0.94	36.7	11.9	108.4	27.9	31.4	10.4	5.5	2.4
3–7 years	Soya	4.6	1.3	39.7	13.2	158.3	50.6	27.8	8.2	7.6	2.4
	Control	5.1	0.93	46.9	12.7	169.7	30.2	39.5	11.4	6.4	2.9

CHO, carbohydrate.

* There were no significant differences between the soya and control groups.

Table 3. Isoflavone content (as aglycone equivalents) of soya-based infant formulas and cows'-milk formulas*

Infant formula	Isoflavone aglycone equivalents						Total (mg/kg)
	Daidzein		Genistein		Glycitein		
	mg/kg	% Total isoflavones	mg/kg	% Total isoflavones	mg/kg	% Total isoflavones	
Cows'-milk formulas							
A	Nd		Nd		Nd		Nd
B	Nd		Nd		Nd		Nd
C	Nd		0.7		0.5		1.2
D	Nd		Nd		Nd		nd
E	Nd		Nd		Nd		nd
F	Nd		Nd		Nd		nd
G	Nd		Nd		Nd		nd
H	Nd		2.1		Nd		2.1
Soya-based formulas							
A	93	26.8	232	67.1	21	6.1	346
B	67	26.5	146	57.9	39	15.5	252
C	98	29	217	64.2	23	6.8	338

Nd, not detected.

*For details of procedures, see p. 609.

three soya-based infant formulas contained large quantities of genistein, daidzein and glycitein, (predominantly in the form of the glycosides, genistin, daidzein and glycitin). The relative proportions of the isoflavones were consistent between the three soya-based infant formulas (Table 3); genistein was the most abundant and formed 63 (SD 5) % of the total, whereas daidzein and glycitein formed 27 (SD 1) and 10 (SD 5) %, respectively, of the total.

The isoflavone intake in soya-based infant formula was estimated for the soya-group infants aged 4–6 months old. The consumption of soya-based infant formula by these infants ranged between 341 and 625 ml/d (526 (SD 110) ml/d). Intakes of isoflavones (as aglycone equivalents) ranged between 17.5 and 33.0 mg/d (28 (SD 6) mg/d). On a body weight (bw) basis, this intake equates to 1.7–4.4 mg/kg bw per d (3.6 (SD 1.2) mg/kg bw per d).

For those infants (aged 4–6 months) fed cows'-milk formula, an estimation of isoflavone content was carried out for those infants fed cows'-milk formula H, which was one of only two cows'-milk formula found to contain isoflavones, although at low concentrations, and contained

the highest amounts by about 2-fold (see earlier; p. 610). The consumption of this formula milk ranged between 497 and 824 ml/d (654 (SD 164) ml/d). For those infants (aged 4–6 months) fed cows'-milk formula, the isoflavone intake ranged between 0.16 and 0.27 mg/d (0.21 (SD 0.1) mg/d), equivalent to 0.02–0.03 mg/kg bw per d (0.028 (SD 0.006) mg/kg bw per d).

Table 4 shows the genistein, glycitein, daidzein, *O*-DMA and equol concentrations in urine from the infants and children in the soya and control groups. Infants in the age group (4–6 months) that consumed soya-based infant formula exhibited significantly higher urinary concentrations of genistein ($P < 0.01$), glycitein, daidzein and *O*-DMA ($P < 0.001$) than the control group (Table 4). In the control-group infants aged 4–6 months old, isoflavonoids in urine were very low or not detected (Table 4).

In contrast to the proportions of the isoflavones in the soya-based infant formulas (Table 3), daidzein and genistein were present in urine in approximately similar concentrations, with glycitein present at considerably lower concentrations (Table 4). In the youngest (4–6 months)

Table 4. Genistein, glycitein, daidzein, *O*-desmethylangolensin (*O*-DMA) and equol concentrations in spot urine samples ($\mu\text{g}/\text{mg}$ creatinine) from infants and children in the soya and control groups† (Mean values and standard deviations)

Age	Group	Soya challenge	Genistein		Glycitein		Daidzein		<i>O</i> -DMA		Equol	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
4–6 months	Soya	No	65*	53	15**	7	60**	32	8**	16	0.05	0.10
	Control	No	Nd		0.02	0.04	Nd		0.02	0.04	Nd	
7–12 months	Soya	No	13	12	4	3	14	10	1	2	0.03	0.06
	Control	Yes	41	53	0.2	0.2	36	38	0.9	0.2	Nd	
1–3 years	Soya	Yes	6	7	1	3	10	11	5	7	0.03	0.08
	Control	Yes	13	12	0.04	0.05	13	17	3	4	0.3	0.6
3–7 years	Soya	Yes	6	6	0.2	0.1	8	6	1	1	0.01	0.04
	Control	Yes	4	4	0.1	0.1	6	8	1	2	Nd	

Nd, not detected.

*Mean value was significantly different to that for the control infants (t test): * $P < 0.01$, ** $P < 0.001$.

†For details of subjects and procedures, see Tables 1 and 2 and p. 609.

age group, all the infants excreted genistein, daidzein and glycitein in their urine, 75 % of soya-based infant formula-fed infants excreted detectable amounts of *O*-DMA and 25 % excreted equol.

In the older age groups (7 months–7 years), *O*-DMA was found in the urine samples of 75 % of the soya group and 50 % of the control infants, after the soya challenge. Equol excretion was detected in 19 % of the soya-group infants and children, and in only 5 % of the control subjects. In the oldest (3–7 years old) children, the proportion excreting *O*-DMA and equol was similar in both the soya and control groups (67 % compared with 71 % and 14 % compared with 16 %).

In the soya-based infant formula-fed subjects, across all the age groups, the majority of subjects (75 %) excreted *O*-DMA, although the amounts varied between 0.2 and 32 µg/mg creatinine. As a percentage of the total urinary isoflavonoids, *O*-DMA contributed between 0.1 and 40 % with a mean of 16.8 (SD 12.8) %. Equol was detected in only 20 % of subjects (0.2 (SD 0.06) µg/mg creatinine) in the soya-based infant formula-fed infants and children.

In the cows'-milk formula-fed subjects, across all the age groups, 50 % of subjects given a soya challenge excreted *O*-DMA, with a slightly narrower range of urinary concentrations (0.1–11 µg/mg creatinine) than that seen in the soya-based infant formula-fed subjects. When expressed as a percentage of total isoflavonoid excretion, *O*-DMA contributed between 1 and 60 % with a mean value of 16.2 (SD 18.5) %. Equol was detected in only 4 % of the control-group subjects given a soya challenge.

Table 5 shows the bacterial composition of faecal samples from the soya-based infant formula-fed and the cows'-milk formula-fed infants, measured by fluorescent *in situ* hybridisation. The total bacterial count in faecal samples from the soya-based infant formula-fed infants was significantly lower ($P < 0.001$) than that for the cows'-milk formula-fed infants (Table 5). This difference between the soya-based infant formula-fed and the cows'-milk formula-fed infants was reflected in all four major groups of bacteria studied: *bifidobacteria*, *bacteroides*, *clostridia* group, and *lactobacilli* + *enterococci*. The bacterial count in faecal samples from the soya-based

Table 5. Bacterial composition of faecal samples from soya-based formula (SBF)-fed and cows'-milk formula (CMF)-fed infants in the 4–12-month subgroup†

(Mean values and standard deviations)

Bacterial count (bacteria/g faeces)	SBF (n 10)		CMF (n 14)	
	Mean	SD	Mean	SD
Total count	3.9×10^{10}	1.6×10^{10}	7.9×10^{10} **	1.6×10^{10}
<i>Bifidobacteria</i>	3.1×10^9	3.9×10^9	2.5×10^{10} **	2.5×10^{10}
<i>Bacteroides</i>	5.0×10^8	25.2×10^8	6.3×10^9 *	3.2×10^9
<i>Lactobacillus</i> + <i>enterococcus</i>	1.5×10^7	6.3×10^7	6.3×10^7	10×10^7
<i>Clostridia</i>	7.9×10^7	12.5×10^7	7.9×10^8 *	2.5×10^8

Mean value was significantly different to that for the SBF-fed infants (*t* test): * $P < 0.05$, ** $P < 0.001$.

† For details of subjects and procedures, see Tables 1 and 2 and pp. 609–610.

infant formula-fed infants for *bifidobacteria* was significantly lower ($P < 0.001$) than that for the cows'-milk formula-fed infants, and the bacterial count for the *bacteroides* and *clostridia* groups were again significantly lower ($P < 0.05$) for the soya-based formula-fed infants compared with the cows'-milk formula-fed infants. The bacterial count for the *lactobacilli* + *enterococci* group, however, did not differ between the groups (Table 5).

Table 6 shows faecal metabolic activities (NH₃ concentration, β-glucosidase and β-glucuronidase activity) and pH in faecal samples from the soya-based infant formula-fed and the cows'-milk formula-fed infants in the 4–12-month subgroup. There were no significant differences between the groups. Table 7 shows the SCFA concentrations in faecal samples from the soya-based infant formula-fed and the cows'-milk formula-fed infants. There were no significant differences between the groups (Table 7).

Discussion

Three types of soya-based infant formulas were consumed by the infants in the present study, although the majority of these infants consumed soya-based infant formula C. These three formulas had a similar profile of isoflavone content. The predominant isoflavone in all the formulas was genistein, which comprised about 60 % of the total. Of the

Table 6. Faecal bacterial metabolic activities and pH in soya-based formula (SBF)- and cows'-milk formula (CMF)-fed infants in the 4–12-month subgroup*

(Mean values and standard deviations)

Metabolic activity†	SBF (n 13)		CMF (n 15)	
	Mean	SD	Mean	SD
NH ₃ concentration (µmol/g)	23.2	11.5	25.7	12.7
β-Glucuronidase activity (µmol/h per g)	17.0	8.2	11.1	9.2
β-Glucosidase activity (µmol/h per g)	24.2	11.4	22.0	10.6
pH	6.7	0.6	6.7	0.7

* For details of subjects and procedures, see Tables 1 and 2 and p. 610.

† There were no significant differences between the soya and control groups.

Table 7. Short-chain fatty acid (SCFA) concentrations (µmol/g) in faecal samples from soya-based formula (SBF)- and cows'-milk formula (CMF)-fed infants in the 4–12-month subgroup*

(Mean values and standard deviations)

SCFA†	SBF (n 13)		CMF (n 15)	
	Mean	SD	Mean	SD
Acetic	29.7	12.3	32.3	13.5
Propionic	6.4	6.0	7.0	4.2
n-Butyric	7.1	6.5	6.2	4.5
Isobutyric	0.72	0.44	0.66	0.48
Isovaleric	1.2	0.80	1.2	0.86
Valeric	0.40	0.51	0.14	0.17
Total SCFA	45.4	21.1	47.5	18.1

* For details of subjects and procedures, see Tables 1 and 2 and p. 610.

† There were no significant differences between the soya and control groups.

cows'-milk formulas analysed, only two were found to contain isoflavones and these were present in very small quantities (<2 mg/kg dry powder).

The soya-based infant-formula intake of the infants (aged 4–6 months) was very similar (about 600 ml/d), resulting in a mean exposure to isoflavones of 3.7 mg/kg per d. These values are consistent with previous data on similar numbers of subjects from the UK (mean isoflavonoid intake 4–5 mg/kg per d; Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment 1996, 1998) and New Zealand (2.9–3.8 mg/kg per d; Irvine *et al.* 1998*b*). Values reported for US infants are slightly higher (5–12 mg/kg per d; Murphy *et al.* 1997; Setchell *et al.* 1998). The intake values for infants fed soya-based infant formulas are approximately four to five times that of adult soya consumers (estimated at less than 1 mg/kg per d; Adlercreutz *et al.* 1991; Coward *et al.* 1993).

In contrast, the infants fed cows'-milk formula had negligible isoflavone intake, with a maximum exposure to isoflavones of 0.03 mg/kg per d, over 100-fold lower than that of soya-based infant formula-fed infants.

The urinary excretion data indicate that the isoflavones are readily absorbed from the gut even in infants in the youngest (4–6 month) age group. In this age group, in the absence of a soya challenge, isoflavones could not be detected in the urine of the cows'-milk formula-fed infants. Approximately equal amounts of daidzein and genistein were excreted in urine in the youngest infants, which contrasted with the relative proportions of these two isoflavones in the soya-based infant formulas, where genistein was present at twice the concentration of daidzein. These results are consistent with the limited data available from a previous study (Irvine *et al.* 1998*a*) on relatively small numbers of infants, which reported daidzein concentrations similar to or higher than that of genistein.

The ability of the intestinal microflora of the developing infant to metabolise isoflavones can be assessed from the urinary excretion results. It is clear that the ability to convert daidzein to *O*-DMA is acquired at an early stage in development, because the metabolite was detected in the urine of 75% of the 4–6-month-old soya-based infant formula-fed infants; in one infant it comprised 29% of the total urinary isoflavonoids. In the control group of infants and children, *O*-DMA excretion was detected in a smaller proportion of the older age groups (ages 7 months–7 years) than in the corresponding soya groups (50% compared with 75%).

The other daidzein metabolite, equol, was detected in urine from only 20% of subjects in the soya group, across all ages, and in only 5% of subjects in the control group following the soya challenge. This frequency is much lower than that which has been consistently found in adults consuming soya (approximately 35%; Morton *et al.* 1994; Lampe *et al.* 1998; Rowland *et al.* 2000; Setchell *et al.* 2003*a*), suggesting that the organisms that confer the ability to produce equol are not acquired until later in life. It is difficult to compare data on urinary excretion with the plasma levels measured by Setchell *et al.* (1997), but the latter reported that equol concentrations in the plasma of soya-based infant formula-fed infants were extremely low.

In the oldest (3–7-years-old) children, the proportion excreting *O*-DMA and equol was similar in both the soya and control groups (67% compared with 71% and 14% compared with 16%), indicating that by this age, there is no effect of exposure to isoflavones in early infancy. This suggests that there is no lasting effect on isoflavone metabolism of early-life isoflavone exposure.

Fermentable load, i.e. dietary fibre, is known to influence bacterial populations and metabolism. Using an *in vitro* model of human colonic fermentation it has been shown that the metabolism of daidzein to equol by cultured human microflora can be influenced by the conditions used (Setchell & Cassidy, 1999; Setchell *et al.* 2002*a*). A high NSP content, which stimulates bacterial fermentation, resulted in a rapid conversion of daidzein to equol, whereas under conditions that mimicked low carbohydrate intake, equol was not formed. A study of twenty-four healthy adults (Rowland *et al.* 2000) found that good equol producers consumed less fat as a percentage of energy compared with poor equol producers 26 (SD 2.3) v. 35 (SD 1.6)% and more carbohydrates 55 (SD 2.9) v. 47 (SD 1.7)%. Lampe *et al.* (1998) similarly showed that women but not men who were good equol producers consumed a significantly higher percentage of energy as carbohydrate compared with poor equol producers, and they also consumed more plant protein and dietary fibre. It was suggested that, when consumed by women, dietary fibre or associated components of a high-fibre diet promote the growth and/or the activity of the bacterial populations responsible for equol production in the colon (Lampe *et al.* 1998). A later study (Lampe *et al.* 2001), however, showed no effect on urinary equol excretion, when dietary fibre intake was doubled by the consumption of 16 g wheat bran/d, although this increase in dietary fibre may be insufficient to significantly modulate intestinal bacterial populations. The observed increase in the ability to produce equol and *O*-DMA with increasing age, in the soya-group and control-group (following soya challenge) infants and children in the present study, may thus be partly related to the increase, with increasing age, in the dietary fibre content of the diets of both the soya-group and control-group infants and children. The NSP content of the diets increased from 2.8 (SD 1.6) and 2.4 (SD 1.7) g/d for the soya-group and control-group infants, respectively, in the youngest age group (4–6 months) to 7.6 (SD 2.4) and 6.4 (SD 2.9) g/d for the soya-group and control-group children, respectively, in the oldest (3–7 years) age group. The daily NSP intake in this oldest age group is only around one half the value we found for healthy adults, approximately 15 g/d (Rowland *et al.* 2000), which may also contribute to the lower frequency of equol producers observed in the present study compared with in adults (approximately 15% compared with 35%).

To evaluate the influence of soya-based infant-formula feeding on gut health-related, bacterial composition of the faecal flora and on certain aspects of bacterial metabolism, we focused on a subgroup of the infants aged 4–12 months old, which is a period when there are known to be major changes in the gut microflora (Heavey & Rowland, 1999). In this subgroup, the soya-based infant formula-fed infants had significantly lower

($P < 0.001$) total numbers of bacteria than the cows'-milk-fed infants and this difference was consistent across all the four main groups of bacteria studied although the values for *lactobacilli* + *enterococci* were not significant. There was a significant difference ($P < 0.001$) in *bifidobacteria* numbers, approximately 10-fold lower in the soya-based infant formula-fed infants compared with the cows'-milk formula-fed infants (4–12 months old). *Bifidobacteria* are considered to be of particular importance for gut health (Heavey & Rowland, 1999).

In addition to measuring the bacterial composition of the faecal microflora, a number of bacterial metabolic activities and endpoints (for example, SCFA concentrations) were measured. Such metabolic activities and endpoints can provide more sensitive indicators of changes in bacterial flora in the gut than bacterial counts (Rowland *et al.* 1985). SCFA are considered to be important factors in gut health, by keeping the gut contents acidified, preventing colonisation by pathogenic bacteria (Gibson & Roberfroid, 1995). However, there was no significant difference in faecal SCFA concentrations or pH between the soya-based infant formula-fed and cows'-milk formula-fed infants (4–12 months old). NH_3 , a protein degradation product, which is generated by gut bacterial action on protein, amino acids and urea in the gut, has a number of adverse effects on the intestinal mucosa, including a reduction in epithelial life span, altered DNA synthesis and disruption of intermediary metabolism (Visek, 1978). There were, however, no significant differences in faecal NH_3 concentration or in faecal bacterial β -glucosidase and β -glucuronidase activity between the soya-based infant formula-fed and the cows'-milk formula-fed infants (4–12 months old). These enzymes are inducible by their substrates and it might have been expected that an increase in β -glucosidase activity would be observed in the soya-based infant formula-fed infants compared with the controls, in response to the considerable soya isoflavone glucoside consumption by the soya group (Rowland *et al.* 2003). Following the consumption of isoflavones and their subsequent conjugation and excretion in bile, although higher levels of glucuronide conjugates of isoflavones are likely to have been present in the colon of the soya-based infant formula-fed infants compared with the controls, increased β -glucuronidase activity might not be expected in the soya-based infant formula-fed infants. This is because a number of other endogenous glucuronidated metabolites, such as sterols, are also likely to be present. However, as the total numbers of bacteria in the faecal flora were lower in the soya-based infant formula-fed infants compared with the controls, the activities of faecal bacterial β -glucosidase and β -glucuronidase do appear to be higher in the soya-based infant formula-fed infants compared with the controls, thus suggesting that these enzymes were induced in some bacteria. As β -glucuronidase can additionally hydrolyse glucuronide conjugates of ingested toxicants, releasing potentially toxic aglycones in the colon, and similarly β -glucosidase can hydrolyse glucoside conjugates found in plants releasing biologically active phytochemicals that may be beneficial or harmful, depending on the phytochemical ingested, greater β -glucuronidase activity could exert damaging effects on the host and thus

have adverse health consequences. However, greater β -glucosidase activity could result in either beneficial or harmful health consequences (Rowland, 1992; Gangolli & Rowland, 1999).

In conclusion, therefore, the present data have shown that infants aged 4–6 months fed soya-based infant formulas excreted considerable quantities of genistein, daidzein and glycitein in urine, indicating that the compounds are well absorbed. As the hydrolysis of isoflavone glucosides to aglycones by glucosidases is required for absorption (Setchell *et al.* 2002b; Rowland *et al.* 2003), the results suggest that hydrolytic ability develops by or before 4–6 months of age. In the cows'-milk formula-fed infants (not given a soya challenge), in this age group the isoflavones were undetectable reflecting the low to undetectable levels of the compounds in the formulas. The majority of the soya-based infant formula-fed infants (including those under 6 months) and about one half of the cows'-milk formula-fed group (after a soya challenge) were capable of converting daidzein to *O*-DMA. In contrast, conversion of daidzein to equol was seen in very few children even in the oldest (3–7 years) age group. The results indicate that there appears to be no lasting effect of early-life isoflavone exposure on isoflavone metabolism. These findings have important developmental implications for isoflavone bioavailability.

The results also suggest that the type of infant formula milk consumed influences the microbial composition of the infant gut microflora, with soya-based infant-formula-fed infants (4–12 months) having significantly lower total bacteria counts and *bifidobacteria* counts ($P < 0.001$) and *bacteroides* and *clostridia* counts ($P < 0.05$) than cows'-milk formula-fed infants in the same age group (4–12 months old).

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