Supplementation of difructose anhydride III enhanced elevation of plasma equol concentrations and lowered plasma total cholesterol in isoflavone-fed rats

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Equol, a derivative of daidzein produced by enterobacteria, has greater activity as a phyto-oestrogen compared with daidzein. Difructose anhydride III (DFAIII) is a newly manufactured non-digestible disaccharide with unique fermentation properties. The present study evaluated the prebiotic effects of DFAIII on equol production and on plasma cholesterol concentrations related to the changes in equol production. We compared plasma equol concentrations at 10.00 and 18.00 hours and faecal isoflavone excretion in three groups of seven rats (male Wistar-ST strain, 6 weeks old) fed a basal diet or a DFAIII or fructooligosaccharide (15 g/kg diet) diet containing 1 g soya isoflavones/kg diet for 20 d. Equol concentrations in the DFAIII group were higher than in the control and fructooligosaccharides groups, especially in the later phase of the light period (18.00 hours) throughout the experiment. Daizein and genistein concentrations did not change between the diet groups. The faecal ratios of equol:daidzein were very high in all groups, but the ratios were higher in the DFAIII group than the control and fructooligosaccharide groups on day 3, and this tendency continued throughout the experiment. On day 20, the plasma total cholesterol concentration was lowest in the DFAIII group. Additionally, the cholesterol concentrations were inversely correlated to plasma equol concentration in all the rats. In conclusion, DFAIII efficiently enhanced plasma equol concentrations, which may be associated with an increase in equol production and a decrease in equol degradation by enterobacteria. Higher plasma equol concentrations may contribute to the hypocholesterolaemic effect of DFAIII feeding.

Difructose anhydride III: Fructooligosaccharides: Equol: Total cholesterol

Many studies have shown that soya isoflavones have beneficial effects on bone (Setchell & Cassidy, 1999; Setchell & Lydeking-Olsen, 2003), on the cardiovascular system (Setchell & Cassidy, 1999) and on the prevention of various kinds of cancer (Horn-Ross *et al.* 2003; Akaza *et al.* 2004). It has recently been shown that equol, an enterobacterial metabolite of daidzein, plays a critical role in the prevention of prostate cancer (Akaza *et al.* 2004) and has more of an oestrogenic and antioxidative potential than is seen for genistein and daidzein (Setchell *et al.* 2002). There have been previous reports on the hypocholesterolaemic effect of isoflavones in rodents (Song *et al.* 2003; Ali *et al.* 2004; Kawakami *et al.* 2004). A study involving human subjects showed that there was a hypocholesterolaemic effect that was induced by the intake of soya-based milk only in equol producers (Meyer *et al.* 2004), which suggests that equol may have an important role in the beneficial effects that are observed after feeding isoflavones. However, the contribution of equol to this hypocholesterolaemic effect after soya isoflavone ingestion has yet to be clarified.

In an attempt to discover the beneficial effects of isoflavones, several studies have been conducted to examine the effects of prebiotics (Ohta *et al.* 2002; Mathey *et al.* 2004; Zafar *et al.* 2004) or probiotics (Bonorden *et al.* 2004; Nettleton *et al.* 2004, 2005; Tamura *et al.* 2004*a*) on equol production. Feeding fructooligosaccharide (FOS) with isoflavones increased plasma equol and bone mineral density in ovariectomised mice (Ohta *et al.* 2002) and rats (Mathey *et al.* 2004). In contrast, inulin lowered serum equol concentrations in rodents (Zafar *et al.* 2004). The probiotics tested had no effect on equol production in human subjects (Bonorden *et al.* 2004; Nettleton *et al.* 2004, 2005), whereas *Lactobacillus gasseri* lowered plasma equol concentrations in mice (Tamura *et al.* 2004*a*). Prebiotic effects via equol production on cholesterol metabolism have not previously been reported.

Difructose anhydride III (DFAIII) is a newly manufactured non-digestible disaccharide, which is fermentable in rats (Suzuki *et al.* 1998; Mitamura *et al.* 2002; Afsana *et al.* 2003). In contrast to almost all other non-digestible oligosaccharides, there was no assimilation of this sugar by *Bifidobacterium* (Kituclin *et al*, unpublished results). In previous studies employing a human breath H_2 test, we observed that DFAIII

was barely fermentable (Tamura *et al.* 2003, 2004*b*). The ingestion of DFAIII by healthy subjects for 4 weeks did not significantly change the faecal denaturing gradient gel electrophoresis profile, although a change in profile was noted in some subjects with chronic constipation (Minamida *et al.* 2004). Only with the *Ruminococcus* sp. M-1, which was isolated from the caecum in rats fed DFAIII, has DFAIII assimilation been observed (Minamida *et al.* 2005). Therefore, DFAIII may have different prebiotic effects compared with other non-digestible saccharides with unique fermentability.

The aims of the present study were to examine the effect of DFAIII on equol production from a soya isoflavone preparation, and to evaluate the potential to reduce plasma cholesterol concentration with regard to equol production in rats fed isoflavone with DFAIII.

Materials and methods

Animals and diets

Experiment 1: Effects of DFAIII ingested with isoflavones on equol production and plasma cholesterol concentration. Male Wistar-ST rats (*n* 21), obtained from Japan SLC, Inc. (Hamamatsu, Japan) at 6 weeks of age, were housed individually in stainless steel cages. The cages were placed in a room with controlled temperature (22–24°C), relative humidity (40–60%) and lighting (lights on from 08.00 to 20.00 hours).

For the first 3 d, a non-cellulose and non-isoflavone containing diet was fed *ad libitum*. After this dietary treatment, the rats were randomly divided into three groups (*n* 7) and assigned one of the following test diets: a control diet based on the AIN-93G formulation, or a diet containing 15 g DFAIII or FOS per kg diet (Table 1). All diets contained 2·5 g commercial soya germ extract per kg diet (1 g isoflavones/kg diet (Fujiflavone P40; Fujicco Co., Ltd., Kobe, Japan), 24·6 g daidzin or its derivatives, and 5·5 g genistin or its derivatives per 100 g soya germ extract). Diets and tap water were given *ad libitum* throughout the experimental period for 20 d. Under pentobarbital anaesthesia on day 20,

Table 1. Composition of the basal diet (g/kg diet)

Ingredients*	g/kg diet
Casein	200
Dextrin	453
Maize oil	70
Mineral mixture†	35
Vitamin mixture†	10
Choline bitartate	2·5
L-Cystine	3
tert-Butylhydroquinone	0·014
Cellulose	80
Fujiflavone P-40	2·5
Sucrose	143·986

In the DFAIII and FOS diets, 15 g cellulose were substituted by each sugar in the basal diet.
* Casein (ALACID; New Zealand Dairy Board, Wellington, New Zealand), dextrin (Pine-Dex #4; Matsutani Chemical Industry, Hyogo, Japan), cellulose (Avicel PH102; Asahi Chemical Industry, Tokyo, Japan). Fujiflavone P-40 is a commercial soya germ extract that contains 400 g isoflavones.
† Prepared according to the AIN-93G formulation.
For details of diets and procedures, see this page.

blood was collected from the abdominal aorta for determination of blood lipid and glucose concentrations, after which the animals were killed. Their caecums were dissected and immediately frozen. All samples were stored at −30°C until subsequent analysis.

Blood for analysis of isoflavones and their metabolites was collected by puncture of the tail vein using a heparinised capillary tube at 10.00 hours (early phase of the light period) and 18.00 hours (later phase of the light period) on days 12 and 19. Faeces were collected over a 2-d period on days 1, 3, 5, 8 and 15.

Experiment 2: Effects of DFAIII on cholesterol without isoflavone. Male Wistar-ST rats (Japan SLC, Inc.) were randomly divided into two groups (*n* 7) and fed either the control or the DFAIII diet described in experiment 1 except that the diets did not contain soya germ extract. Blood collected by puncture of the tail vein on days 0, 14 and 28 was used to measure total cholesterol.

The Hokkaido University Animal Committee approved the study, and the animals were maintained in accordance with Hokkaido University's guidelines for the care and use of laboratory animals.

Analyses

Plasma was obtained by centrifugation. Daidzein, genistein, equol and dihydrodaidzein in the plasma and powdered faeces were analysed by an HPLC method. Briefly, 100 μl plasma were mixed with an equal volume of sodium acetate buffer (100 mmol/l, pH 5·0) containing ascorbic acid (5·7 mmol/l), EDTA (0·27 mmol/l), β-glucuronidase (7800 U; G-0876; Sigma-Aldrich, St Louis, MO, USA) and sulfatase (140 U; S9751; Sigma-Aldrich). The mixtures were incubated at 37°C overnight after adding formononetin (0·8 nmol) as an internal standard. The samples were mixed with ammonium acetate buffer (final concentration 0·2 mmol/l, pH 7·0) and triethylammonium sulfate buffer (final concentration 0·67 mmol/l), heated at 60°C for 10 min, and centrifuged. The supernatant was passed through Sep-Pak C18 cartridges (Waters Co., Ltd., Milford, MA, USA). The methanol eluent of the cartridges was dried, resolved in methanol–1% acetate water solution (80:20 v/v), and analysed using HPLC.

Faeces were freeze-dried and ground to a fine powder. The powdered faeces (0·2 g) were mixed with 5 ml 80% aqueous methanol and 0·4 nmol formononetin, sonicated for 20 min and maintained overnight at room temperature. After the solution had been centrifuged, supernatant was passed through a cellulose acetate filter (0·22 μm) and then analysed using HPLC.

The HPLC analysis employed included some modifications of a previously described method (Tamura *et al.* 2002). Briefly, HPLC was performed using a Capsule-Pack MGII column (4·6 × 150 mm; Shiseido Co. Ltd, Tokyo) at 45°C with an initial gradient consisting of a methanol (containing 1% acetic acid) to water (containing 1% acetic acid) ratio of 30:70 v/v for 10 min. Methanol concentration was increased linearly up to 70% during the following 35 min. A photodiode array detector (SPD-M10A; Shimadzu Co. Ltd., Kyoto, Japan) with UV at 260 nm was used to detect daidzein, genistein and formononetin, and UV at 280 nm was used for equol and dihydrodaidzein.

Caecal contents and walls were weighed, and a pH meter with a glass electrode was used to directly measure the pH of the caecal contents. Caecal SCFA were measured using a method previously described (Afsana *et al.* 2003). Briefly, caecal contents were diluted with four volumes of deionised water and homogenised using a Teflon homogeniser (Iuchi-Seleido, Osaka, Japan). The homogenate was mixed with NaOH solution (final concentration 20·8 mmol/l) containing a final concentration of 4·17 mmol/l crotonic acid. The mixture was then centrifuged and passed through a filter disk with a pore size of 22 μm. Filtered samples were analysed by HPLC that used a solvent delivery system (SLC-10 AVP; Shimadzu, Co. Ltd.), a double ion-exchange column (Shim-Pack SCR-102 h, 8 × 300 mm; Shimadzu) and an electroconductivity detector (CDD-6A; Shimadzu).

Concentrations of glucose, triacylglycerol, total cholesterol and HDL-cholesterol in the plasma were enzymatically measured by an automatic analyser (Dri-Chem 3500; Fujifilm Medical Co., Ltd., Tokyo, Japan).

Statistical analysis

Grubbs-Smirnov's test for outliers was performed on the caecal succinic acid data. ANOVA was first performed to test for any significant effect of diets, sampling day or time. Subsequently, Fisher's protected least significant difference test was applied to determine whether there were significant differences between all of the groups. Stat View version 5.0 (SAS Institute, Inc., Cary, NC, USA) was used for statistic analysis. Differences were considered significant at $P < 0.05$ for all analyses.

Results

There was no difference between the groups for final body weight and body weight gain (Table 2). Food intake was, however, lower in the DFAIII group and also tended to be lower in the FOS group ($P = 0.071$) compared with the control group. Although the caecal pH was lower in the DFAIII and FOS groups compared with the control group, the weights of the caecal contents and caecal wall were not influenced by these saccharides (Table 3). The caecal acetate pool was greater in the DFAIII group than in the control and FOS groups, and the total SCFA pool was greater in the DFAIII group than in the control group. Additionally, the propionate pool in the FOS group tended to be greater than that seen in the control group ($P = 0.065$). There were no significant differences for any other organic acids among the three groups.

Table 2. Final body weight, body weight gain and total food intake in rats fed a basal diet (control) or a diet containing difructose anhydride III (DFAIII) or fructooligosaccharides (FOS)

(Mean values and standard deviations)

	Control	DFAIII	FOS	Pooled SD
Final body weight (g)	268·0	260·0	267·0	12·90
Body weight gain (g/d)	5·0	4·6	4·9	0·52
Food intake (g/d)	18·0[a]	16·5[b]	16·8[a]	1·28

[a,b] Mean values with unlike superscript letters were significantly different ($P < 0.05$). For details of diets and procedures, see p. 443.

Within the same diet group, the plasma daidzein concentrations at 10.00 hours were higher than the concentrations seen at 18.00 hours on each day. There were, however, no significant differences between the three diet groups for the same time periods. DFAIII group plasma equol concentrations were higher than the concentrations in the control group at all time points, and were higher than those in the FOS group at 18.00 hours on each day. The FOS group equol concentrations were higher than those observed in the control group at 10.00 but not at 18.00 hours on each day. Within the FOS group, equol concentrations on each day were higher at 10.00 than 18.00 hours. There were no differences between all of the diet groups for all time points for the plasma genistein concentrations, although the plasma genistein concentrations in the control group were higher at 10.00 than at 18.00 hours on day 12 (Fig. 1).

The faecal excretion of isoflavones on days 1, 3 and 15–16 is shown in Table 4. The major isoflavone in the faeces was equol. Faecal daidzein and genistein contents in the DFAIII group were lower, or tended to be lower, than those of the control group on days 1, 3 and 15–16. The faecal equol and total isoflavone (sum of daidzein, dihydrodaidzein, equol and genistein) excretion in both non-digestible saccharide groups tended to be lower than that observed in the control group on day 1.

Periodic changes in faecal ratios of dihydrodaidzein:daidzein, and equol: daidzein, are shown in Fig. 2. The dihydrodaidzein:daidzein ratio in the DFAIII group was higher than in the other two groups only on day 1, although the higher ratio concentrations were maintained throughout the remainder of the testing period. The ratios in the control and FOS groups increased after the first day, and were higher on day 8 in the control group and on days 3, 5, 8 and 15–16 in the FOS group when compared with the day 1 values within each group. The equol:daidzein ratio in the DFAIII group was higher than that in the control group on days 3 and 5, and in the FOS group on days 3, 8 and 15–16. Within the same diet group, ratios in the control and DFAIII groups on days 3, 5, 8 and 15–16, and in the FOS group on days 5, 8 and 15–16, were higher compared with day 1.

The ingestion of DFAIII or FOS with isoflavones did not cause any changes in the concentrations of glucose, triacylglycerol and HDL-cholesterol. The plasma total cholesterol concentration was lower in the DFAIII group than the control and FOS groups (Table 5). The plasma total cholesterol concentrations but not the HDL-cholesterol concentrations on day 20 showed an inverse correlation ($R - 0.631$, $P = 0.002$) to the plasma equol concentrations at 18.00 hours on day 19 (Fig. 3). Plasma daidzein concentration did not inversely correlate with plasma total cholesterol concentration (R 0·351, $P = 0.120$; data not shown).

In experiment 2, no differences were observed for food intake and final body weight between the control and DFAIII groups (data not shown). There were also no differences in the plasma total cholesterol concentration between rats fed the DFAIII diet without isoflavone and those fed the control diet for any periods (Table 6).

Discussion

The promotion of equol production that depends on intestinal fermentation is a new beneficial function of prebiotics. We

Table 3. Weights of caecal wall, contents, caecal pH and organic acid pools in the caecum of rats fed a basal diet (control) or a diet containing difructose anhydride III (DFAIII) or fructooligosaccharides (FOS)

(Mean values and standard deviations)

	Control	DFAIII	FOS	Pooled SD
Caecal wall (g)	0·84	0·98	0·93	0·15
Caecal content (g)	3·68	5·09	3·95	1·34
Caecal content pH	7·70[a]	7·30[b]	7·30[b]	0·17
Succinate (mmol/caecal contents)	2·33	5·76	3·69	5·42
Lactate (mmol/caecal contents)	5·88	9·03	6·14	2·92
Acetate (mmol/caecal contents)	105·0[b]	172·0[a]	110·0[b]	40·5
Propionate (mmol/caecal contents)	30·2	40·7	48·1	17·0
n-Butyrate (mmol/caecal contents)	17·30	18·00	19·30	8·31
Total SCFA (mmol/caecal contents)	152·0[b]	231·0[a]	177·0[ab]	61·1

[a,b]Mean values with unlike superscript letters were significantly different ($P < 0.05$).
For details of diets and procedures, see p. 443.

examined whether DFAIII, a non-digestible saccharide with unique fermentability, promoted equol production from daidzin in a soya extract. Compared with controls, the plasma equol concentrations of rats fed test diets containing soya isoflavones were consistently higher in the DFAIII group. This is in contrast to the FOS group, for which the concentrations were higher only at 10.00 hours. The ratio of equol: daidzein in the faeces was also higher in the early feeding stage in the DFAIII group.

These results strongly suggest that increases in equol production and absorption are associated with the higher plasma concentrations that occur with DFAIII feeding. The enhancement of conversion from the glycoside daidzin to the aglycone daizein, and from daidzein to equol, in the intestine may possibly be involved in the increase in absorption and in plasma equol concentrations after DFAIII ingestion. However, Steer *et al.* (2003) showed that glycoside hydrolysis occurred very rapidly during *in vitro* faecal bacterial fermentation. Equol production from the aglycone is also likely to be associated with the higher concentrations of plasma equol in the non-digestible saccharide groups.

Compared with the control group, the faecal ratio of dihydrodaidzein:daidzein in the DFAIII group was higher on day 1, unlike the equol:daidzein ratio, which was higher on days 3 and 5. These findings suggest that the ingestion of DFAIII first stimulates dihydrodaidzein production, which is subsequently followed by equol production in the intestine. Dihydrodaidzein is an intermediate metabolite in the conversion of daidzein to equol (Rowland *et al.* 2003). However, different bacteria are responsible for these two steps within the reaction (Atkinson *et al.* 2004). DFAIII ingestion may stimulate growth more than single bacteria do. The reduction in caecal pH and the increase in caecal acetate and total SCFA implies there is caecal fermentation of DFAIII. A previous report has shown that the ingestion of DFAIII increased the level of a specific bacterium, which has a high similarity to *Ruminococcus productus*, found in the caecum of rats (Minamida *et al.* 2005). A strain of *R. productus* has been reported to have an equol-producing ability (Ueno *et al.* 2002). Thus, this DFAIII-assimilating bacterium may contribute to equol production in rats fed DFAIII.

As has been previously shown, FOS also increases plasma equol concentration (Ohta *et al.* 2002; Mathey *et al.* 2004).

We demonstrated, however, that the enhancing effects of FOS on plasma equol depend on time. Caecal acetate and total SCFA were higher in the DFAIII group, whereas propionate tended to be higher in the FOS group ($P = 0.065$; Table 3). These results suggest that fermentation properties are different between DFAIII and FOS. Moreover, it has previously been reported that, when measured by breath H_2 in human subjects, DFAIII is slowly fermentable (Tamura *et al.* 2003, 2004*b*), in contrast to FOS, which is rapidly fermented (Stone-Dorshow & Levitt, 1987). Characteristics of fermentation, especially the rapid fermentation of FOS, may be involved in the limited, time-dependent effect of FOS on the plasma equol concentration.

The present study showed that, even on the first day, the major (80–90 %) isoflavone excreted into the faeces was equol, which indicates that ingested daidzin was rapidly and efficiently converted into equol by the intestinal bacteria for all of the diet groups. On day 1, equol excretion in the FOS and DFAIII groups tended to be lower than that seen in the control group. A slower intestinal transit of these non-digestible saccharide groups may possibly be the reason for this observation.

The reason for the lower tendency of equol excretion is, however, not clear, especially in the DFAIII group. On day 3 in this group, the equol:daidzein ratio reached a peak value, and on days 15–16, when the isoflavone metabolism in the intestine had reached a steady state, the plasma equol concentrations were clearly higher in the DFAIII group. The amount of equol in the faeces is determined by the rates of production, absorption and degradation in the intestine. A decrease in equol degradation may also be involved in the consistently higher concentrations of the plasma equol that were observed in the rats fed DFAIII, as the equol:daidzein ratio in the DFAIII group was similar to that observed in the control group on days 15–16. The higher retention of produced equol may cause higher absorption and higher plasma concentrations of equol in the DFAIII group.

In all groups, plasma daidzein concentrations were higher at 10.00 than at 18.00 hours for each of the diet groups. Plasma equol concentration, however, showed no such tendency except for the control group (Fig. 1). Previous studies reported that plasma daidzein concentrations reached a peak 1–3 h
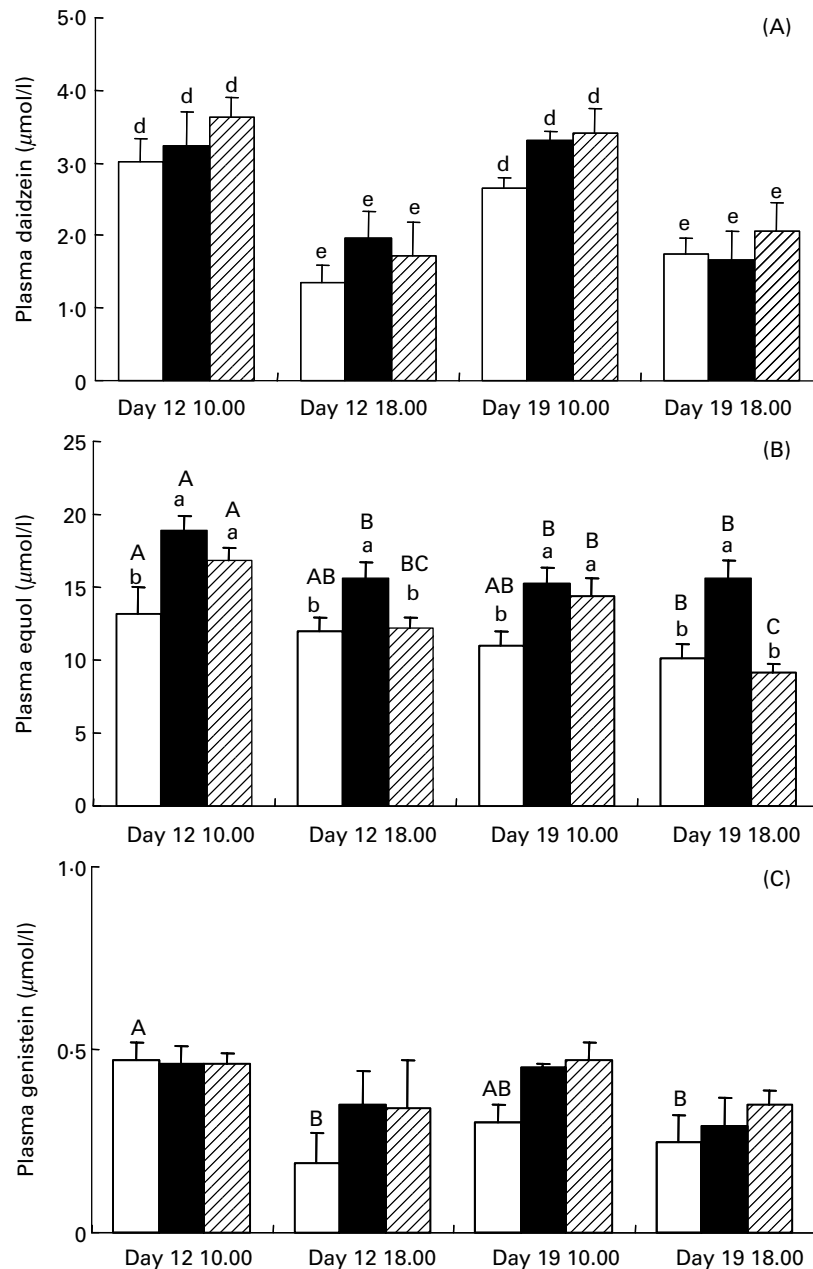
Fig. 1. Plasma concentrations of daidzein (A), equol (B) and genistein (C) at each time point in rats fed the control diet (□) or a diet containing 1·5 % difructose anhydride III (■) or fructooligosaccharides (▨). Means with their standard errors. *P* values for three-way ANOVA: *P* = 0·024 for Diet, *P* = 0·083 for Day, *P* < 0·001 for Time, *P* = 0·052 for Diet × Day, *P* = 0·113 for Diet × Time, *P* = 0·083 for Day × Time, and *P* = 0·039 for Diet × Day × Time for daidzein; *P* = 0·001 for Diet and Time, *P* = 0·033 for Day, *P* = 0·508 for Diet × Day, *P* = 0·008 for Diet × Time, *P* = 0·212 for Day × Time, and *P* = 0·393 for Diet × Day × Time for equol; *P* = 0·770 for Diet, 0·214 for Day, *P* < 0·001 for Time, *P* = 0·074 for Diet × Day, *P* = 0·989 for Diet × Time, *P* = 0·307 for Day × Time, and *P* = 0·090 for Diet × Day × Time for genistein. [a,b,c] Mean values with unlike superscript letters were significantly different between different groups within the same sampling time; *P* < 0·05. [d,e,f] Mean values with unlike superscript letters were significantly different between the different sampling times with the same diet group; *P* < 0·05. For details of diets and procedures, see p. 443.

after a single administration of daidzein (King, 1998; Uehara *et al.* 2001), which suggests a rapid absorption of daidzein. Large amounts of daidzin or daidzein may be retained in the gastrointestinal tract at 10.00 but not at 18.00 hours. Daidzin that reaches the caecum may be rapidly converted to daidzein, as described earlier, and then absorbed from the caecum. The absorption of equol may possibly be slower than that of daidzein, as no difference was noted between the plasma concentrations at 10.00 and 18.00 hours.

In the DFAIII group, the plasma total cholesterol concentration was lower, and the plasma equol concentration, but not the daidzein or genistein concentration, was higher than that of the control group. This is the first evidence suggesting that the stimulation of equol production by a non-digestible saccharide induces a hypocholesterolaemic effect (Table 5). The present study reveals that the plasma total cholesterol concentration exhibited an inverse correlation to the plasma equol, but not daidzein, concentration (Fig. 3). Additionally,

Table 4. Excretion of isoflavones and their metabolites into the faeces of rats fed a basal diet (control) or a diet containing difructose anhydride III (DFAIII) or fructooligosaccharides (FOS)

(Mean values and standard deviations)

	Control (μmol/d)	DFAIII (μmol/d)	FOS (μmol/d)	Pooled SD
Day 1				
Daidzein	0·630[ABa]	0·348[Bb]	0·462[ABab]	0·219
Dihydrodaidzein	0·061[B]	0·084[B]	0·053[B]	0·040
Equol	2·834[C]	1·720[C]	1·741[C]	0·537
Genistein	0·044[a]	0·021[Bb]	0·033[Bab]	0·016
Day 3				
Daidzein	0·583[ABa]	0·290[Bb]	0·411[Bab]	0·118
Dihydrodaidzein	0·113[B]	0·080[B]	0·117[A]	0·053
Equol	4·338[BC]	4·030[B]	3·774[BC]	0·847
Genistein	0·048[a]	0·024[ABb]	0·045[ABab]	0·017
Days 15–16				
Daidzein	0·443[B]	0·313[B]	0·398[B]	0·101
Dihydrodaidzein	0·096[B]	0·092[B]	0·115[AB]	0·044
Equol	4·335[BC]	3·628[BC]	3·198[C]	0·683
Genistein	0·039	0·028[AB]	0·033[B]	0·012

P values for two-way ANOVA: $P < 0.001$ for Diet and Day, and $P = 0.963$ for Diet × Day for daidzein; $P = 0.770$ for Diet, $P < 0.001$ for Day, and $P = 0.782$ for Diet × Day for dihydrodaidzein; $P = 0.071$ for Diet, $P < 0.001$ for Day, and $P = 0.616$ for Diet × Day for equol; $P < 0.001$ for Diet, $P = 0.007$ for Day, and $P = 0.977$ for Diet × Day for genistein.
[A,B] Mean values with unlike superscript letters were significantly different between the different sampling times within the same diet group; ($P < 0.05$).
[a,b] Mean values with unlike superscript letters were significantly different between different groups within the same sampling time; $P < 0.05$
For details of diets and procedures, see p. 443.

the ingestion of DFAIII without isoflavones had no effect on plasma total cholesterol (Table 6). These findings strongly suggest that the stimulation of equol production by DFAIII is involved in the hypocholesterolaemic effect that is seen in the DFAIII group.

Isoflavones have hypocholesterolaemic effects in rodents (Uesugi *et al.* 2001; Song *et al.* 2003; Ali *et al.* 2004; Kawakami *et al.* 2004; Yousef *et al.* 2004) and human subjects (Setchell & Cassidy, 1999; Wangen *et al.* 2001; Meyer *et al.* 2004), but there is little evidence on what effect equol by itself may have. In a study that examined hypercholesterolaemic and hypertensive human subjects, the intake of soya-based milk and yoghurt induced a hypocholesterolaemic effect only in equol producers (Meyer *et al.* 2004). Daidzin, but not genistin and glysitin, have hypocholesterolaemic effects in ovariectomised rats (Uesugi *et al.* 2001). In both the present and other previous studies, blood concentrations of equol, the major metabolite of daidzein, were higher than daidzein concentrations in rodents (Tamura *et al.* 2002, 2004*a*; Zafar *et al.* 2004). Therefore, the hypocholesterolaemic effect of the soya isoflavones may depend on equol and not daidzein. A potential mechanism for the hypocholesterolaemic effect of isoflavones has been suggested to be an increase in LDL receptor activity, as isoflavones do not reduce plasma cholesterol concentrations in LDL receptor-deficient female mice (Kirk *et al.* 1998). Equol produced from daizein may reduce the plasma cholesterol concentration via an increase in LDL receptor activity, as suggested earlier.

The present study demonstrated that DFAIII reduced plasma cholesterol concentration only in cases in which the diet included soya isoflavones, as mentioned earlier. Soya protein

is well known to reduce plasma cholesterol concentration. In the present study, however, we showed that the concentrations seemed to be lower in rats fed a cholesterol-free, casein-based diet with soya isoflavone compared with a diet without isoflavones. These findings were observed regardless of the non-digestible saccharides and even when the results of two separate experiments were compared. Additionally, when rats were fed soya isoflavones without oligosaccharides in the control groups, plasma equol concentrations were also rather high.

In previous reports, a controversy exists regarding the effect of isoflavones on plasma cholesterol in rodents fed a casein-based diet. The ingestion of an isoflavone mixture lowered plasma total cholesterol in male rats (Ali *et al.* 2004; Kawakami *et al.* 2004), and daidzein added to a casein diet also lowered total, HDL- and non-HDL-cholesterol in male Syrian hamsters (Song *et al.* 2003). However, an isoflavone concentrate containing a 1:1 mol ratio of genistein and daidzein had no effect on HDL- and non-HDL-cholesterol in male Golden Syrian F1B hybrid hamsters (Blair *et al.* 2002). Our results suggest that soya isoflavone may have a potential role in the reduction of plasma cholesterol even in a casein-based diet, and, as suggested earlier, these effects are mainly dependent on equol, a fermentation product of daidzein.
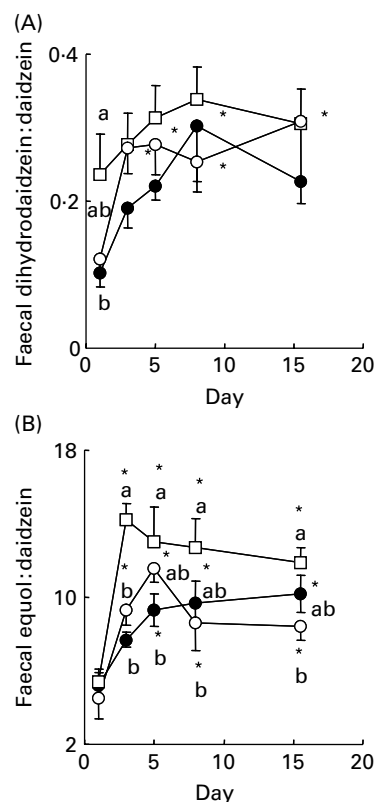

Fig. 2. Changes in the faecal ratio of dihydrodaidzein:daidzein (A) and equol:daidzein (B) after feeding the control diet (●), the diet containing 1·5% difructose anhydride III (□) or the fructooligosaccharide diet (○). Means with their standard errors. P values for two-way ANOVA: $P = 0.012$ for Diet, $P = 0.001$ for Day and $P = 0.792$ for Diet × Day for dihydrodaidzein:daidzein; $P < 0.001$ for Diet and Day and $P = 0.104$ for Diet × Day for equol:daidzein. [a,b] Mean values with unlike superscript letters were significantly different ($P < 0.05$). *Significant difference from the day 1 value within the same diet group. For details of diets and procedures, see p. 443.

Table 5. Plasma glucose, triacylglycerol, total cholesterol and HDL-cholesterol on day 20 in rats fed a basal diet or a diet containing 1·5 % difructose anhydride III (DFAIII) or fructooligosaccharides (FOS)

(Mean values and standard deviations)

	Control (mmol/l)	DFAIII (mmol/l)	FOS (mmol/l)	Pooled SD
Blood glucose	8·80	9·36	8·89	0·74
Triacylglycerol	1·26	1·64	1·54	0·50
Total cholesterol	1·95[a]	1·57[b]	1·99[a]	0·27
HDL-cholesterol	1·08	0·96	1·07	0·23

[a,b] Mean values with unlike superscript letters were significantly different ($P<0.05$). For details of diets and procedures, see p. 443.
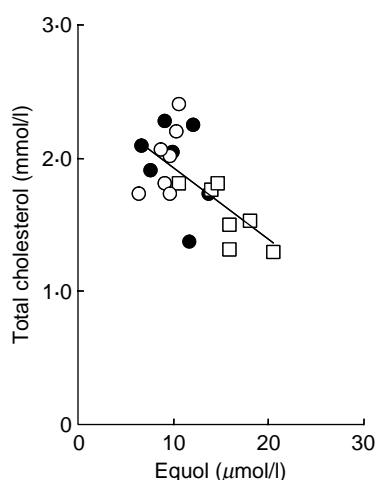

Fig. 3. Correlation between plasma equol at 18.00 hours on day 19 and total cholesterol after feeding the control diet (●), the diet containing 1·5 % difructose anhydride III (□) or the fructooligosaccharide diet (○). Correlation coefficient $= -0.631$ ($P = 0.002$). For details of diets and procedures, see p. 443.

Table 6. Plasma total cholesterol in rats fed a basal diet or a diet containing 3 % difructose anhydride III (DFAIII) or fructo-oligosaccharides (FOS) for 0, 14 and 28 d

(Mean values and standard deviations)

	Control (mmol/l)	DFAIII (mmol/l)	FOS (mmol/l)	Pooled SD
Baseline	2·58	2·62	2·48	0·61
Day 14	2·92	2·95	3·24	0·42
Day 28	3·04	3·00	3·39	0·51

There were no significant differences between the diet groups on any day. For details of diets and procedures, see p. 443.

In conclusion, DFAIII consumed with isoflavones efficiently increases the plasma equol concentration, which may subsequently be associated with a lowering of the plasma total cholesterol.

References

Afsana K, Shiga K, Ishizuka S & Hara H (2003) Ingestion of an indigestible saccharide, difructose anhydride III, partially prevents the tannic acid-induced suppression of iron absorption in rats. *J Nutr* **133**, 3553–3560.

Akaza H, Miyanaga N, Takashima N, *et al.* (2004) Comparisons of percent equol producers between prostate cancer patients and controls: case-controlled studies of isoflavones in Japanese, Korean and American residents. *Jpn J Clin Oncol* **34**, 86–89.

Ali AA, Velasquez MT, Hansen CT, Mohamed AI & Bhathena SJ (2004) Effects of soybean isoflavones, probiotics, and their interactions on lipid metabolism and endocrine system in an animal model of obesity and diabetes. *J Nutr Biochem* **15**, 583–590.

Atkinson C, Berman S, Humbert O & Lampe JW (2004) In vitro incubation of human feces with daidzein and antibiotics suggests interindividual differences in the bacteria responsible for equol production. *J Nutr* **134**, 596–599.

Blair RM, Appt SE, Bennetau-Pelissero C, Clarkson TB, Anthony MS, Lamothe V & Potter SM (2002) Dietary soy and soy isoflavones have gender-specific effects on plasma lipids and isoflavones in golden Syrian f(1)b hybrid hamsters. *J Nutr* **132**, 3585–3591.

Bonorden MJ, Greany KA, Wangen KE, Phipps WR, Feirtag J, Adlercreutz H & Kurzer MS (2004) Consumption of *Lactobacillus acidophilus* and *Bifidobacterium longum* do not alter urinary equol excretion and plasma reproductive hormones in premenopausal women. *Eur J Clin Nutr* **58**, 1635–1642.

Horn-Ross PL, John EM, Canchola AJ, Stewart SL & Lee MM (2003) Phytoestrogen intake and endometrial cancer risk. *J Natl Cancer Inst* **95**, 1158–1164.

Kawakami Y, Tsurugasaki W, Yoshida Y, Igarashi Y, Nakamura S & Osada K (2004) Regulative actions of dietary soy isoflavone on biological antioxidative system and lipid metabolism in rats. *J Agric Food Chem* **52**, 1764–1768.

King RA (1998) Daidzein conjugates are more bioavailable than genistein conjugates in rats. *Am J Clin Nutr* **68**, 1496S–1499S.

Kirk EA, Sutherland P, Wang SA, Chait A & LeBoeuf RC (1998) Dietary isoflavones reduce plasma cholesterol and atherosclerosis in C57BL/6 mice but not LDL receptor-deficient mice. *J Nutr* **128**, 954–959.

Mathey J, Puel C, Kati-Coulibaly S, Bennetau-Pelissero C, Davicco MJ, Lebecque P, Horcajada MN & Coxam V (2004) Fructooligosaccharides maximize bone-sparing effects of soy isoflavone-enriched diet in the ovariectomized rat. *Calcif Tissue Int* **75**, 169–179.

Meyer BJ, Larkin TA, Owen AJ, Astheimer LB, Tapsell LC & Howe PR (2004) Limited lipid-lowering effects of regular consumption of whole soybean foods. *Ann Nutr Metab* **48**, 67–78.

Minamida K, Shiga K, Sujaya IN, Sone T, Yokota A, Hara H, Asano K & Tominta F (2005) Effects of difructose anhydride III (DFA III) administration on rat intestinal microbiota. *J Biosci Bioeng* **99**, 230–236.

Minamida K, Sujaya N, Tamura A, Shigematsu N, Sone T, Yokota A, Asano K, Benno Y & Tomita F (2004) The effects of Di-D-fructo-furanose-1,2′:2,3′-dianhydride (DFA III) administration on human intestinal microbiota. *J Biosci Bioeng* **98**, 244–250.

Mitamura R, Hara H, Aoyama Y & Chiji H (2002) Supplemental feeding of difructose anhydride III restores calcium absorption impaired by ovariectomy in rats. *J Nutr* **132**, 3387–3393.

Nettleton JA, Greany KA, Thomas W, Wangen KE, Adlercreutz H & Kurzer MS (2004) Plasma phytoestrogens are not altered by probiotic consumption in postmenopausal women with and without a history of breast cancer. *J Nutr* **134**, 1998–2003.

Nettleton JA, Greany KA, Thomas W, Wangen KE, Adlercreutz H & Kurzer MS (2005) The effect of soy consumption on the urinary 2:16-hydroxyestrone ratio in postmenopausal women depends on equol production status but is not influenced by probiotic consumption. *J Nutr* **135**, 603–608.

Ohta A, Uehara M, Sakai K, Takasaki M, Adlercreutz H, Morohashi T & Ishimi Y (2002) A combination of dietary fructooligosaccharides and isoflavone conjugates increases femoral bone mineral

density and equol production in ovariectomized mice. *J Nutr* **132**, 2048–2054.

Rowland I, Faughnan M, Hoey L, Wahala K, Williamson G & Cassidy A (2003) Bioavailability of phyto-oestrogens. *Br J Nutr* **89**, S45–S58.

Setchell KD, Brown NM & Lydeking-Olsen E (2002) The clinical importance of the metabolite equol – a clue to the effectiveness of soy and its isoflavones. *J Nutr* **132**, 3577–3584.

Setchell KD & Cassidy A (1999) Dietary isoflavones: biological effects and relevance to human health. *J Nutr* **129**, 758S–767S.

Setchell KD & Lydeking-Olsen E (2003) Dietary phytoestrogens and their effect on bone: evidence from in vitro and in vivo, human observational, and dietary intervention studies. *Am J Clin Nutr* **78**, 593S–609S.

Song T, Lee SO, Murphy PA & Hendrich S (2003) Soy protein with or without isoflavones, soy germ and soy germ extract, and daidzein lessen plasma cholesterol levels in golden Syrian hamsters. *Exp Biol Med* **228**, 1063–1068.

Steer TE, Johnson IT, Gee JM & Gibson GR (2003) Metabolism of the soybean isoflavone glycoside genistin in vitro by human gut bacteria and the effect of prebiotics. *Br J Nutr* **90**, 635–642.

Stone-Dorshow T & Levitt MD (1987) Gaseous response to ingestion of a poorly absorbed fructo-oligosaccharide sweetener. *Am J Clin Nutr* **46**, 61–65.

Suzuki T, Hara H, Kasai T & Tomita F (1998) Effects of difructose anhydride III on calcium absorption in small and large intestines of rats. *Biosci Biotechnol Biochem* **62**, 837–841.

Tamura M, Hirayama K, Itoh K, Suzuki H & Shinohara K (2002) Effects of rice starch-isoflavone diet or potato starch-isoflavone diet on plasma isoflavone, plasma lipids, cecal enzyme activity, and composition of fecal microflora in adult mice. *J Nutr Sci Vitaminol.* **48**, 225–229.

Tamura M, Ohnishi-Kameyama M & Shinohara K (2004*a*) *Lactobacillus gasseri*: effects on mouse intestinal flora enzyme activity and isoflavonoids in the caecum and plasma. *Br J Nutr* **92**, 771–776.

Tamura A, Shiomi T, Shigematsu N, Tomita F & Hara H (2003) Evidence suggesting that difructose anhydride III is an indigestible and low fermentable sugar during the early stage after ingestion in humans. *J Nutr Sci Vitaminol* **49**, 422–427.

Tamura A, Shiomi T, Tamaki N, Shigematsu N, Tomita F & Hara H (2004*b*) Comparative effect of repeated ingestion of difructose anhydride III and palatinose on the induction of gastrointestinal symptoms in humans. *Biosci Biotechnol Biochem* **68**, 1882–1887.

Uehara M, Ohta A, Sakai K, Suzuki K, Watanabe S & Adlercreutz H (2001) Dietary fructooligosaccharides modify intestinal bioavailability of a single dose of genistein and daidzein and affect their urinary excretion and kinetics in blood of rats. *J Nutr* **131**, 787–795.

Ueno T, Uchiyama S & Kikuchi N (2002) The role of intestinal bacteria on biological effects of soy isoflavones in humans. *J Nutr* **132**, 594S.

Uesugi T, Toda T, Tsuji K & Ishida H (2001) Comparative study on reduction of bone loss and lipid metabolism abnormality in ovariectomized rats by soy isoflavones, daidzin, genistin, and glycitin. *Biol Pharm Bull* **24**, 368–372.

Wangen KE, Duncan AM, Xu X & Kurzer MS (2001) Soy isoflavones improve plasma lipids in normocholesterolemic and mildly hypercholesterolemic postmenopausal women. *Am J Clin Nutr* **73**, 225–231.

Yousef MI, Kamel KI, Esmail AM & Baghdadi HH (2004) Antioxidant activities and lipid lowering effects of isoflavone in male rabbits. *Food Chem Toxicol* **42**, 1497–1503.

Zafar TA, Weaver CM, Jones K, Moore DR 2nd & Barnes S (2004) Inulin effects on bioavailability of soy isoflavones and their calcium absorption enhancing ability. *J Agric Food Chem* **52**, 2827–2831.