Research Article

Ileal and faecal digestibility of daidzein and genistein and plasma bioavailability of these isoflavones and their bioactive metabolites in the ovariectomised rat

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Consumption of the soya isoflavones genistein and daidzein may provide protection against postmenopausal bone loss. The purpose of this study was to determine ileal and faecal digestibility of daidzein and genistein and the extent of formation of metabolites in the gastrointestinal (GI) tract in the ovariectomised rat, a model for postmenopausal bone loss. Twenty female rats were ovariectomised and fed either genistein or daidzein (0.026% of diet) for 4 wks. Genistein, daidzein and their GI-derived metabolites were quantitatively determined in plasma, urine, faeces and ileal digesta using GC/MS. Ileal and faecal digestibility of genistein (93 and 99.9%, respectively) were significantly greater than that of daidzein (32 and 77.5%, respectively). In genistein-supplemented animals, 4-ethylphenol was present in plasma in relatively high concentrations. The bioactivity of 4-ethylphenol may contribute to the physiological effects attributed to genistein consumption. The daidzein metabolite equol, was present in relatively high amounts in ileal digesta indicating substantial biotransformation of daidzein occurred in the small intestine presumably as a result of the activity of the resident microbiota. Further studies are required to determine whether 4-ethylphenol is a major metabolite of genistein in humans and the extent of biotransformation of daidzein to equol in the small intestine in humans.

Keywords: Equol / 4-Ethylphenol / Phytoestrogens / Soya

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1 Introduction

Phytoestrogens are plant-derived polyphenols capable of interacting with mammalian oestrogen receptors. Epidemiological studies suggest consumption of soya, one of the major dietary sources of phytoestrogens, is associated with a number of health benefits including reduced incidence of postmenopausal osteoporosis [1 – 3].

The metabolism of phytoestrogens is complex involving endogenous enzymes, gut microflora, as well as enterohepatic cycling. In the gastrointestinal (GI) tract, the phytoestrogens are deglycosylated and then reduced [4] whereas in the liver, oxidative metabolism of the phytoestrogens occurs [5]. Some of the metabolites of genistein and daidzein, as well as the parent phytoestrogens themselves, are bioactive [6 – 8]. Understanding the metabolism of these phytoestrogens has important consequences for understanding their physiological effects.

Although structurally similar, there are considerable differences in the processes by which the two soya phytoestrogens, genistein and daidzein, are metabolised in the mammalian GI tract. Endogenous enzymes in the GI tract of both rats and humans are incapable of reducing daidzein [9]. In contrast, the flavonoid ring of genistein is broken down by endogenous mammalian GI enzymes and 4-ethylphenol [4], a compound with known bioactivity [6, 7], is formed. The degree of metabolism of genistein to 4-ethyl-

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Abbreviations: DM, dry matter; **DMI,** DM intake; **GI,** gastrointestinal; **ND,** not detected; **OVX,** ovariectomy; ***o*-DMA,** *o*-desmethylangolensin; **SEM,** standard error of the mean

phenol and the bioavailability of this metabolite is largely unknown.

Several studies have demonstrated that colonic and faecal bacteria can metabolise both genistein and daidzein [4, 10–13]. Whereas metabolism of genistein by the gut microflora results in the formation of nonbioactive metabolites, gut microfloral metabolism of daidzein results in formation of the two bioactive metabolites equol and *o*-desmethylangolensin (*o*-DMA) [9]. Although it is known that equol and *o*-DMA are produced in the colon, microbes also reside in the small intestine. The contribution of the gut microflora in the small intestine to the metabolism of phytoestrogens and particularly to the production of equol and *o*-DMA has not yet been determined.

The objectives of the present study were to establish the extent of genistein and daidzein metabolism and disappearance during passage through the small intestine (ileal digestibility) as compared to the metabolism and disappearance of the phytoestrogens through the entire digestive tract (faecal digestibility) in order to establish whether microbiota of the small intestine have a role in the metabolism of these phytoestrogens. Secondly, we sought to determine the extent of biotransformation of genistein to 4-ethylphenol and of daidzein to equol and *o*-DMA and plasma uptake of these metabolites in the ovariectomised rat model, a commonly used animal model for the study of postmenopausal bone loss.

2 Methods and materials

2.1 Materials

Genistein (>95%) and daidzein (>96%) were purchased from LC Laboratories, USA. B-glucuronidase/arylsulphatase (G-7017) and all standards used for GC/MS analysis (except for 4-hydroxyphenyl-2-propionic acid) were purchased from Sigma–Aldrich (Munich, Germany). The standard for 4-hydroxyphenyl-2-propionic acid (98%) was purchased from Acros Organics (Geel, Belgium) (catalogue number 302680050). All other chemicals were of analytical grade and were purchased from Sigma–Aldrich.

2.2 Animals

The study was approved by the Massey University Animal Ethics Committee (approval number 05/97) and was conducted in accordance with the principles of laboratory animal care [14]. Twenty 7-month old female Sprague-Dawley rats were obtained from the small animal production unit, Massey University. All animals underwent bilateral ovariectomy (OVX) performed under general anaesthetic (isofluorane).

Animals were initially individually housed in shoebox cages. For the final 14 days of the trial, animals were transferred to individual, stainless steel wire-bottomed metabolic cages to allow for the separation and collection of urine and faeces. Throughout the trial, animals were housed in a dedicated room maintained at 22°C (± 2°C) and with a 12 h/12 h light/dark cycle.

2.3 Diets

Animals were gradually introduced to a nutritionally balanced semisynthetic maintenance diet based on AIN93M [15] over a period of two weeks prior to undergoing OVX (week 2–0). Titanium dioxide was added to the diet (5 g/kg diet) as an indigestible marker to allow quantification of digesta and faecal phytoestrogen and metabolite contents.

Following OVX, animals were assigned at random to receive a diet containing 0.026% of either genistein or daidzein ($n = 10$ *per* group). In food, isoflavones exist as both aglycones as well as glucosides. There are three forms of glycosylated isoflavones (malonyl, acetyl and underivatised glucosides) [16] which are present in different relative amounts in different soya foods [17]. The rate of deconjugation and intestinal absorption differs for each form [18]. For this initial study into the sites of intestinal metabolism of genistein and daidzein, the aglycone forms of the isoflavones were chosen for use as these represent the isoflavones in their simplest chemical form. Samples of the diets were frozen (−20°C) and finely ground before chemical analysis. The ingredient compositions are given in Table 1.

Table 1. Ingredient composition of the experimental diets

	Percentage of diet	
	Daidzein	Genistein
Cornstarch	59.174	59.174
Sodium caseinate	14	14
Sucrose	6	6
Cellulose	5	5
AIN93M[a)] vitamin mix	5	5
AIN93M[a)] mineral mix (excluding calcium)	5	5
Calcium carbonate	1.3	1.3
Corn oil	4	4
Titanium dioxide	0.5	0.5
Genistein	0	0.026
Daidzein	0.026	0

a) AIN-93M = American Society for Nutritional Sciences (formerly American Institute of Nutrition) rodent maintenance diet.

2.4 Feeding regimen

De-ionised water was freely available to the animals at all times. For the initial 10 days following OVX, animals had unrestricted access to their respective diets. After the post-surgery recovery period (10 days), animals were trained to consume one meal *per* day. During this period, animals had unrestricted access to their respective diets for 3 h/day (07–

10 h), after which time food was removed. Two weeks following OVX, animals were placed in metabolic cages for a 1 wk acclimatisation period followed by a 6-day period of faeces and urine collection. The design of the metabolic cages was such that coprophagy was prevented.

2.5 Metabolic balance and sample collection

Rat body weight was recorded immediately prior to and following the sample collection period. All faeces and urine were collected separately on a daily basis and daily food intake was recorded. Hydrochloric acid (1 M) was added to the faeces and urine collection tubes (1 mL *per* tube) prior to sample collection to prevent continued microbial degradation of the isoflavones and metabolites in these samples postexcretion. Excreta were frozen (−20°C) and faecal samples were subsequently freeze-dried and stored frozen (−20°C).

Immediately following the meal on day 7 of the metabolic balance period, animals were deeply anaesthetised *via* intraperitoneal injection (25G × 5/8′ needle and 1 mL syringe) of 0.1 mL/kg body weight of acepromazine, ketamine, xylazine and sterile H_2O (2:5:1:2) and were subsequently exsanguinated by cardiac puncture (19G × 1½′ needle and 5 mL syringe). Blood was collected in EDTA-containing vacutainers and immediately centrifuged at 2000 rpm for 10 min. Plasma was stored at −80°C for later analysis. Digesta from the terminal 20 cm of the ileum immediately distal to the ileo−caecal valve were collected by flushing with 10 mL of milli Q water using a 10 mL syringe attached to a mouse gavage needle. Digesta were frozen at −20°C and subsequently freeze-dried prior to chemical analysis.

2.6 Sample extraction

Tyrosol has previously been shown to be a good internal standard for use in the analysis of phenolic compounds [19]. As tyrosol was not detected (ND) in either the diet or any of the biological samples analysed in this study (data not shown), and the chromatographic retention time of tyrosol differed sufficiently from the other compounds of interest for good resolution and separation it was chosen for use as an internal standard in this study.

2.6.1 Urine

For each animal, total urine collected over the 6-day period was pooled and filtered through Whatman no. 4 filter paper to remove any contaminating particulate matter. Samples were mixed to ensure homogeneity and isoflavones were extracted as described earlier with slight modifications [20]. Urinary creatinine concentrations were determined by the Jaffe method [21]. Urine samples were buffered to pH 5 with 1 mL of 0.125 M sodium acetate. Tyrosol (25 μmol/mg creatinine) was added as an internal standard followed by 125 μL β-glucuronidase/arylsulphate and the samples were incubated overnight at 37°C. Samples were then acidified with 5 N HCl to a pH < 2. Samples were extracted twice, firstly with 6 mL ethyl acetate and then with 3 mL diethyl ether. An excess of anhydrous Na_2SO_4 was added (approximately 2 mg) to remove any water that may have still been present in the sample. This volume was vortexed for 30 s and centrifuged for 5 min. The solution was decanted from the pellet and dried under a nitrogen stream. The dry extract was derivatised at 90°C for 30 min with 22.6 μL bis(trimethylsilyl) trifloroacetamide (BSTFA)/μmol creatinine, 4.5 μL pyridine/μmol creatinine and 4.5 μL trimethylchlorosilane (TMCS)/μmol creatinine.

2.6.2 Ileal digesta and faeces

Freeze-dried ileal digesta and faecal samples were finely ground using a Breville coffee bean grinder. Digesta (50 mg) were mixed with 1 mL 0.125 M sodium acetate buffer (pH 5). For faecal analysis, 250 mg faeces and 2 mL 0.125 M sodium acetate buffer (pH 5) were used. In both cases 100 μL of a 0.5 mg/mL tyrosol solution was added as an internal standard. Samples were deconjugated, acidified, extracted and derivatised as for the method for urine samples.

2.6.3 Plasma

Tyrosol solution (100 μL, 0.5 mg/mL) was added to 200 μL of plasma. Samples were treated with 200 μL 100% ACN, centrifuged at 3000 rpm for 5 min and the clear supernatant collected. Enzymatic deconjugation, acidification, extraction of isoflavone metabolites and derivatisation was conducted as described for urine.

2.7 GC/MS analysis of isoflavone metabolites

Coldham *et al*. (2002) proposed two pathways for the biotransformation of genistein. The end-products of one pathway being 4-ethylphenol and 1,3,5-trihydroxybenzoic acid and of the other being 4-hydroxyphenyl-2-propionic acid and 1,3,5-trihydroxybenzene [4]. Genistein, the intermediate dihydrogenistein, 4-ethylphenol, 1,3,5-trihydroxybenzoic acid, 4-hydroxyphenyl-2-propionic acid and 1,3,5-trihydroxybenzene were analysed for in samples from genistein-fed rats. Daidzein, dihydrodaidzein and the metabolites equol and *o*-DMA were analysed for in samples of daidzein-fed rats.

An Agilent 6890N gas chromatograph system with an HP-5 capillary column (0.25 mm × 30 m × 0.25 μm) coupled to a 5973 mass spectrometer was used for the analysis. Injection volume was 1.0 μL with a split ratio of 10:1 and a helium flow rate of 1 mL/min. Injection inlet temperature was 250°C. Initial oven temperature was 120°C maintained for 2 min. Temperature was ramped at 10°C/min to 320°C where it was maintained for a further 2 min. Total run time was 24 min.

2.8 Quantification of titanium dioxide in faeces and ileal digesta

Faecal concentration of the indigestible marker titanium dioxide was determined by colourimetry following sulphuric acid digestion as previously described [22]. Due to the relatively small quantity of ileal digesta available for analysis, the concentration of titanium was determined in the digesta residue remaining after isoflavone extraction by ICP-MS (inductively coupled plasma MS) following sulphuric acid digestion. Concentration of titanium dioxide in digesta was calculated based on the respective *Mr*s of titanium and oxygen and corrected for the quantity of isoflavones extracted.

2.9 Data analysis

Ileal and faecal genistein, daidzein and metabolite concentrations with respect to genistein and daidzein intake were determined as follows:

Concentration of compound (mg/g dry matter (DM) intake (DMI)) = [compound in digesta or faeces (mg/g DM) × TiO_2 in diet (mg/g DM)]/
[TiO_2 in digesta or faeces (mg/g DM)]
where compound = genistein, daidzein or metabolite.

Ileal and faecal daidzein and genistein digestibility were determined by:

Ileal or faecal digestibility of compound (%) = ([dietary genistein or daidzein (mol/g DMI)] − [ileal or faecal genistein or daidzein and their metabolites (mol/g DMI)])/[dietary genistein or daidzein (mol/g DMI)] × 100%

2.10 Statistical analysis

Data were analysed using Minitab version 14 (Minitab, Pennsylvania, USA). Results are reported as mean ± standard error of mean (SEM). Analysis of variance using the general linear model with posthoc Tukey testing was used for comparisons between groups. A p-value of ≤ 0.05 was considered statistically significant.

3 Results

There were no statistically significant differences among the treatment groups in terms of mean daily food intakes. Average food intakes for the 6-day balance period (g/rat/day ± SE) were 10.6 (± 0.7) for the daidzein-supplemented group and 10.9 (±0.7) for genistein-supplemented rats. Rats gained body weight over the 6-day balance period. There were no statistically significant differences among groups in weight gain or final body weight. Body weight gains (g/rat/6-days ± SE) were 16.5 (±3.3) for the daidzein-supplemented group and 19.1 (±3.3) for the genistein-supplemented group. Final rat body weights (g ± SE) were 300.2 (±8.5) for the daidzein-supplemented group and 301.3 (±8.5) for the genistein-supplemented group.

Table 2. Ileal and faecal digestibility of daidzein and genistein for ovariectomised rats consuming a diet supplemented with genistein or daidzein (0.26 mg/g DMI[a])

	Daidzein	Genistein	*p*-value
Ileal digestibility (%)	32.0 (7.9)	93.0 (1.1)	<0.0001
Faecal digestibility (%)	77.5 (6.5)	99.9 (0.06)	0.004

Values are expressed as mean with SEM in brackets.
a) DMI = Dry matter intake.

3.1 Daidzein and genistein digestibility

Both the apparent ileal and faecal digestibility of daidzein was significantly lower than that of genistein ($p < 0.0001$ and $p = 0.004$, respectively) (Table 2). Ileal digestibility of genistein was 93% indicating only 7% of total dietary genistein intake passed into the colon. The majority of dietary genistein remaining in the digestive tract at the terminal ileum was in the form of the unmetabolised phytoestrogen. The genistein metabolite 4-ethylphenol was present in very small amounts in ileal digesta and accounted for only 3% of the total amount of genistein and genistein products present at the terminal ileum. Ileal digestibility of daidzein was 32%. Hence, 68% of the total dietary intake of daidzein remained in the digestive tract at the terminal ileum and therefore, passed into the colon. The daidzein metabolite equol was present in relatively large amounts in ileal digesta accounting for 62% of the total amount of daidzein and daidzein products present in ileal digesta. Equol was also the major form in which dietary daidzein was excreted in faeces. Faecal digestibility of genistein was 99.9% compared to 77.5% for daidzein. The percentage disappearances of dietary genistein and daidzein from the digestive tract are shown in Fig. 1.

3.2 Input/output balances

By averaging genistein and daidzein intakes and excretions over a 24 h period, it is possible to estimate the extent to which the determined excretion accounted for dietary isoflavone intake. To this end, average 24 h input/output balances were calculated for genistein and daidzein (Table 3).

'Input' was calculated as the average daily dietary intake of genistein or daidzein (mol) during the 6-day balance. Total faecal and urinary excretions of genistein, daidzein and their respective metabolites during the 6-day balance were calculated based on the molar concentrations of the compounds in urine and faeces and the total volume/mass of urine or faeces excreted over the period. Mean faecal and urinary isoflavone and metabolite excretions (mol) *per* 24 h period, 'urinary and faecal outputs', were subsequently calculated. For all metabolites, metabolite formation from the parent isoflavone is on a mole *per* mole basis (*i.e.*, one mole of parent isoflavone yields one mole of metabolite).

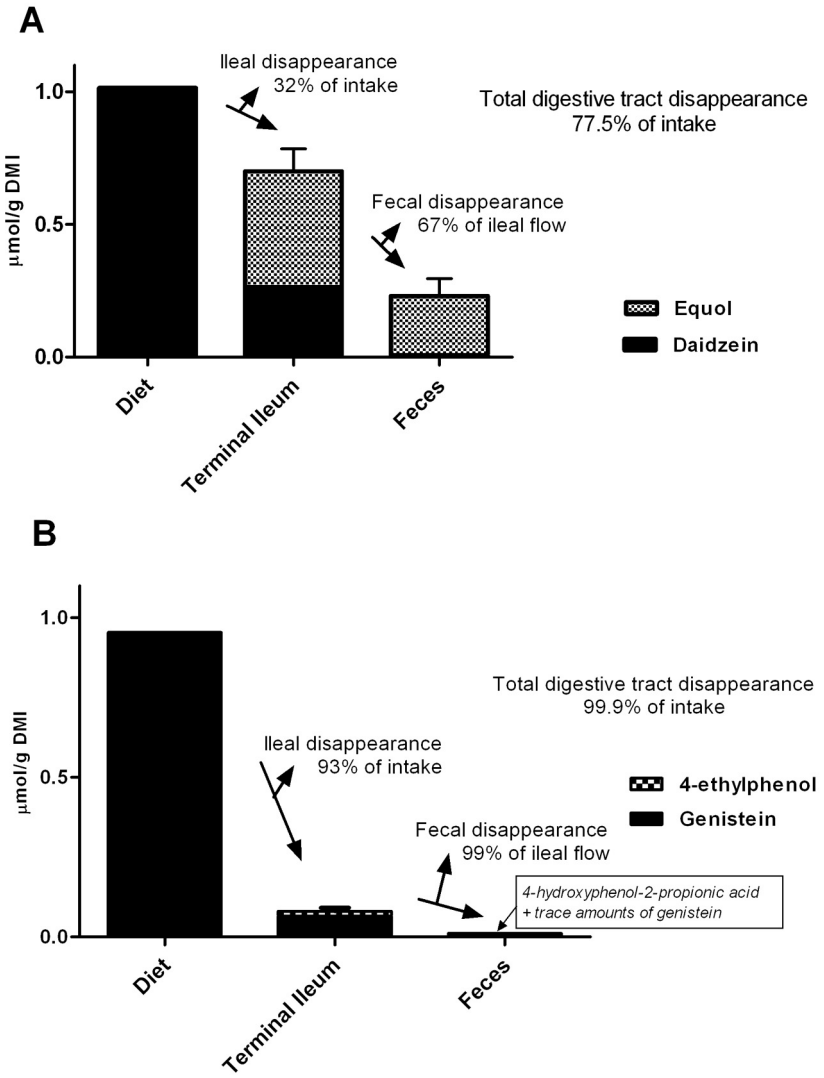

Figure 1. Diet, ileal digesta and faecal contents of unmetabolised daidzein (Fig. 1A) and genistein (Fig. 1B) and their major metabolites following dietary supplementation of ovariectomised rats. 2-Dehydro-*o*-DMA and trace amounts of dihydrodaidzein were also detected in digesta from daidzein supplemented animals (however not depicted here due to very low concentrations, refer to Table 3). The genistein metabolite 4-hydroxyphenyl-2-propionic acid was also detected in low concentrations in digesta from gen-

On a mol/mol basis, 24 h urinary plus faecal excretion of genistein and its known metabolites represented 41.7% of average 24 h dietary genistein intake. Similarly, 24 h excretion of daidzein and its known metabolites represented 49.1% of average 24 h dietary daidzein intake. Excretion of both genistein and daidzein was largely through urine (Table 3).

Equol was the major metabolite of daidzein, and 4-ethylphenol was the major metabolite of genistein detected in the plasma. There was no significant difference in circulating concentrations of the unmetabolised phytoestrogen in animals consuming daidzein as compared to those consuming genistein (Table 4).

4 Discussion

The study had two primary objectives. Firstly, to determine the ileal and faecal digestibility of daidzein and genistein and secondly, to determine the extent of formation of 4-ethylphenol from genistein, and of equol and *o*-DMA from daidzein, and plasma uptake of these compounds in the ovariectomised rat model for postmenopausal bone loss.

The extent of disappearance of genistein from the digestive tract prior to the terminal ileum (ileal digestibility) was considerably greater than that of daidzein. This is likely to be a result of differences in the sites of metabolism and/or absorption of the two isoflavones. In the present study, 93% of dietary genistein disappeared from the digestive tract prior to the terminal ileum. Both 4-ethylphenol, a genistein metabolite formed by the activity of endogenous enzymes in the GI tract [4] as well as 4-hydroxyphenyl-2-propionic acid, the end-product of gut microflora-mediated genistein metabolism in humans and rats [4] were detected in ileal digesta of genistein-fed animals. Similar to previously reported findings in sheep [23], 4-ethylphenol was the main genistein metabolite detected in urine in rats in the present study and urinary excretion of 4-ethylphenol accounted for

Table 3. Mean (±SE) intake of genistein and daidzein and excretion of these isoflavones and their metabolites over 24 h

Input (diet)			Output (excretion)			
	Quantity (μmol/24 h)		Urinary excretion (μmol/24 h)	Urinary excretion as a percent of intake (Mol/Mol%)	Faecal excretion (μmol/24 h)	Faecal excretion as a percent of intake (Mol/Mol%)
Genistein	10.13 (0.390)	Genistein	0.91 (0.319)	8.34 (2.691)	Trace	–
		4-Ethylphenol	3.41 (0.98)	32.43 (8.89)	ND	–
		4-(OH)Phenyl-2-propionic acid	0.049 (0.0188)	0.46 (0.172)	0.00065 (0.000434)	0.0068 (0.00446)
		Dihydrogenistein	0.055 (0.0498)	0.46 (0.417)	ND	–
		Total	4.43	41.74	0.00065	0.0068
	Total input 10.13 μMol Total output (urine + faeces) 4.43 μMol, 43.7% of mean 24 h genistein intake					
Daidzein	11.26 (0.504)	Daidzein	0.93 (0.186)	8.53 (1.86)	0.049 (0.0241)	0.43 (0.214)
		Equol	1.97 (0.468)	17.49 (4.46)	1.34 (0.369)	12.65 (3.85)
		Dihydrodaidzein	0.56 (0.209)	5.07 (1.88)	ND	–
		2-Dehydro-*o*-DMA	0.65 (0.201)	5.83 (1.782)	ND	–
		Total	4.05	36.34	1.39	13.09
	Total input 11.26 μMol Total output (urine + faeces) 5.41 μMol, 49.1% of mean 24 h daidzein intake					

The difference between average 24 h input and average 24 h output (excretion) of genistein is 5.7 μmol (56% of intake) and represents the amount of intake unaccounted for.
The difference between average 24 h input and average 24 h output (excretion) of daidzein is 5.8 μmol (51% of intake) and represents the amount of intake unaccounted for.
ND = Not detected, *o*-DMA = *o*-desmethylangolensin.
Values are expressed as mean with SEM in bracket.

Table 4. Mean plasma (± SE) concentrations of genistein, daidzein and their known metabolites in ovariectomised rats consuming a diet supplemented with either genistein or daidzein (0.26 mg/g DMI)

Diet		Plasma concentration (nmol/L)
Genistein	Genistein	24[a)] (15)
	Dihydrogenistein	ND
	4-Ethylphenol	67 (19)
	4-Hydroxyphenyl-2-propionic acid	ND
Daidzein	Daidzein	17[a)] (13)
	Dihydrodaidzein	ND
	o-DMA	ND
	Equol	18 (4.9)

ND = Not detected.
DMI = Dry matter intake.
o-DMA = *o*-desmethylangolensin.
Values are expressed as mean with SEM in bracket.
a) The difference in plasma concentration of unmetabolised phytoestrogen between animals consuming the genistein-supplemented diet and animals consuming the daidzein-supplemented diet was not statistically significant by one-way ANOVA ($p = 0.21$).

32% of dietary genistein intake. In circulation, 4-ethylphenol was also present in high amounts relative to that of genistein in the present study. In humans, 4-ethylphenol is also a metabolite of genistein and has been detected in urine and plasma [16]. However, the extent of biotransformation of genistein to 4-ethylphenol in humans has not been determined. In bovine reproductive tissue, 4-ethylphenol has been shown to stimulate production of prostaglandin F2α [7]. Whether this effect is due to oestrogenic activity is unclear as although the stimulatory effect of 4-ethylphenol on prostaglandin synthesis was shown to be inhibited by an oestrogen receptor antagonist [6], 4-ethylphenol has been found to have a binding affinity for the oestrogen receptor of just 0.00007% relative to 17β-oestradiol [24]. Regardless of the mechanism of action of 4-ethylphenol, its ability to modify prostaglandin synthesis may have important physiological consequences as prostaglandins are important mediators in many inflammatory diseases including osteoporosis [25]. The extent of metabolism of genistein to 4-ethylphenol may be clinically relevant and the bioactivity of 4-ethylphenol should not be overlooked as a possible contributing factor to the physiological effects observed following genistein consumption.

The presence of 4-hydroxyphenyl-2-propionic acid in ileal digesta from genistein-supplemented animals indicates some microbial metabolism of genistein had occurred in the small intestine in the present study. However, given the substantial biotransformation of genistein to 4-ethylphenol and as only trace amounts of this metabolite were detected in ileal digesta and it was not detected at all in plasma, metabolism of genistein by microbiota in the small intestine may be minimal. Faecal digestibility of genistein was 99.9%. The major genistein metabolite excreted in the faeces was 4-hydroxyphenyl-2-propionic acid with only trace amounts of unmetabolised genistein detected in faeces. We did not detect 1,3,5-trihydroxybenzoic acid or 1,3,5-trihydroxybenzene in any of the biological samples

collected. Coldham *et al*. (2002) also failed to detect either of these theoretically possible genistein metabolites following gut microfloral metabolism of genistein [4]. It is therefore, likely that these proposed genistein metabolites are either not produced *in vivo* or are rapidly subjected to further metabolism.

In the present study, equol production was observed in all animals consistent with previous reports that all rats are equol-producers [26]. Presence of high amounts of equol in ileal digesta in the present study indicates that substantial biotransformation of daidzein to equol occurred prior to the terminal ileum, presumably as a result of metabolism of daidzein by microflora residing in the small intestine. This finding demonstrates that the small intestine has a major role in metabolising daidzein to equol in rats. Indeed, in the rat, the microflora of the small intestine may be more important than the colonic microflora in metabolising daidzein. Equol production has previously been demonstrated in the small intestine of swine [27] and urinary equol excretion has been observed in one human subject with an ileostomy [28]. As equol has greater oestrogenic activity than daidzein [9], but an estimated 50 – 70% of humans are incapable of producing equol [29], presumably due to a lack of daidzein-metabolising gut bacteria [30], there is interest in developing methods to increase equol production in humans. Further research is required to determine the extent of equol production in the small intestine in humans. Identifying the site of equol production has important consequences for the development of strategies to maximise daidzein biotransformation to equol.

Approximately 13% of dietary daidzein intake was excreted in faeces in the present study, predominately in the form of equol. As with genistein, only small amounts of unmetabolised daidzein were excreted in faeces. In humans, faecal recovery of ingested isoflavones also tends to be low with several studies reporting excretion of <2% of dietary isoflavones in the faeces over a range of different isoflavone intakes [31 – 33]. However, in one study Xu *et al*. (1995) observed higher faecal excretion of unmetabolised phytoestrogens (between 5 and 9% of ingested dose) in a subset of women, possibly due to reduced isoflavone metabolising ability in these subjects [32]. Differences between postmenopausal women and ovariectomised rats in terms of the digestibility of phytoestrogens may have important consequences in terms of the relative effectiveness of dietary phytoestrogen consumption in combating postmenopausal bone loss in humans as opposed to postOVX bone loss in rats.

In the present study, only daidzein and equol were detected in plasma of daidzein-fed rats. Although *o*-DMA was ND in plasma, this is likely to be a reflection of the relatively low concentrations of the phytoestrogens and their metabolites in plasma rather than indicative of a lack of absorption of *o*-DMA as 2-dehydro-*o*-DMA was detected in urine. As endogenous enzymes are believed incapable of synthesising *o*-DMA, the presence of 2-dehydro-*o*-DMA in urine indicates it was absorbed from the intestine. Plasma concentrations of genistein, daidzein and equol in the present study were similar to those reported in other studies in rats [34, 35]. Circulating concentrations of genistein in animals receiving the genistein-supplemented diet were not significantly different from those of daidzein in animals receiving the daidzein supplemented diet, indicating that the bioavailabilities of the unmetabolised phytoestrogens are similar. However, daidzein, genistein and equol are known to be present predominately as glucuronide and sulphate conjugates in plasma [16, 36]. Although some of these conjugates have bioactivity [37, 38], it is likely that not all conjugated forms of the isoflavones are bioactive. In the present study, all samples were deconjugated prior to analysis therefore whether the isoflavones were present in a bioactive form in the biological samples analysed was not determined.

For both genistein and daidzein, mean 24 h genistein or daidzein intake exceeded mean 24 h excretion. Neither the isoflavones nor their metabolites appear to accumulate in body tissues in substantial amounts [39]. However once absorbed, genistein, daidzein and equol are metabolised by liver microsomes [5, 40]. As the present study focussed on intestinal metabolism of the isoflavones, the metabolites formed by microsomal oxidation were not screened for in this study. It is likely that hepatic oxidation of the isoflavones accounts for at least some of the apparent 'imbalance' between excretion and intake observed in the present study. Several of the metabolites formed by hepatic oxidation of genistein and daidzein are bioactive [41] and their formation is likely to impact on the physiological effects following genistein/daidzein consumption.

In the present study, the aglycone forms of genistein and daidzein were fed to rats. The glycosylation profile of phytoestrogens influences their bioavailability [18]. The extent of intestinal metabolism and plasma bioavailability of the various genistein and daidzein glycosides is likely to differ from that of the aglycones. Further research into the effects of the glycosylation profile of genistein and daidzein on the extent of biotransformation of the phytoestrogens and the bioavailability of the isoflavones and their metabolites is required.

In conclusion, this study demonstrates that 4-ethylphenol is a major metabolite of genistein present in circulation in rats. This finding highlights the need to more closely examine the bioactivity of 4-ethylphenol and to quantify the formation of this metabolite in humans in order to fully understand the physiological impact of genistein consumption. This study also demonstrates that substantial equol production occurs in the small intestine in ovariectomised rats. Further studies are required to determine the extent of biotransformation of daidzein to equol by microbiota in the small intestine as opposed to in the colon in humans. Determining similarities and differences in the metabolism of

isoflavones in the ovariectomised rat model compared to in postmenopausal women is likely to aid in interpreting results of intervention studies designed to examine the effects of isoflavone consumption on postmenopausal and post-OVX bone loss.

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