

### METABOLISM AND METABOLIC STUDIES

## Effects of dietary fibre and tea catechin, ingredients of the Japanese diet, on equol production and bone mineral density in isoflavone-treated ovariectomised mice

Yuko Tousen<sup>1,2</sup>, Mariko Uehara<sup>3</sup>, Marlena Cathorina Kruger<sup>2</sup> and Yoshiko Ishimi<sup>1\*</sup>

<sup>1</sup>Department of Food Function and Labeling, National Institute of Health and Nutrition, Tokyo, Japan

<sup>2</sup>Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand

<sup>3</sup>Department of Nutritional Science, Faculty of Applied Bioscience, Tokyo University of Agriculture, Tokyo, Japan

(Received 18 November 2011 – Final revision received 4 August 2012 – Accepted 8 August 2012)

*Journal of Nutritional Science* (2012), vol. 1, e13, page 1 of 9

doi:10.1017/jns.2012.14

#### Abstract

Equol is a metabolite of the isoflavone daidzein (Dz) and is produced by the bacterial microflora in the distal intestine and colon. Some epidemiological studies have reported an association between increased equol production and intakes of green tea or dietary fibre, which are ingredients of the standard Japanese diet. We examined the effects of a diet supplemented with Dz and tea catechin or dietary fibre on equol production and bone mineral density in ovariectomised (OVX) mice. Female mice of the ddY strain were either sham operated or OVX. OVX mice were fed a control diet, a 0.1 % Dz-supplemented diet or a 0.1 % Dz diet supplemented with one of the food components commonly consumed in the Japanese diet. The mice were given 1 % tea catechin (w/w) as part of the diet in Expt 1 or 5 % polydextrose (PD) and 5 % raffinose (Raf) (w/w) as part of the diet in Expt 2. Catechin reduced serum equol levels and attenuated the beneficial effect of Dz on femoral bone loss. The soluble dietary fibres PD and Raf stimulated equol production, and enhanced the bone-protective effects of Dz on femoral bone. These results suggest that dietary fibre, in particular, PD, may alter the bioavailability of isoflavones and prevent osteopenia in OVX mice.

**Key words:** Soya isoflavones; Equol; Japanese diet; Dietary fibre

The recent growing interest in health and diet has led to an increased focus on soya foods and their functional components, e.g. isoflavones. Soyabean isoflavones have biochemical structures similar to those of oestrogen and have a weak affinity for the oestrogen receptor<sup>(1)</sup>. Therefore, they have received much attention for the prevention of postmenopausal disorders such as osteoporosis<sup>(2)</sup> and CVD<sup>(3)</sup>. Postmenopausal osteoporosis is a critical disorder involving high bone turnover and bone loss attributed to oestrogen deficiency in women. One of the treatments for osteoporosis is hormone replacement therapy. However, hormone replacement therapy is not the most common treatment option

used because its use can result in adverse effects such as the development of hormone-dependent breast and uterine cancers<sup>(4)</sup>. Therefore, the use of soyabean isoflavones has received considerable attention as an alternative to hormone replacement therapy. The risk for adverse effects of isoflavone treatment appears to be lower than that of hormone replacement therapy<sup>(5)</sup>.

Recent studies suggest that the clinical effectiveness of isoflavones is due to their ability to produce equol<sup>(6)</sup>. Equol is a metabolite of the isoflavone daidzein (Dz) and is produced by the bacterial microflora in the distal intestine and colon; the affinity of equol for the oestrogen receptor is stronger

**Abbreviations:** BMD, bone mineral density; Dz, daidzein; EGCG, epigallocatechin gallate; OVX, ovariectomised; PD, polydextrose; Raf, raffinose.

\* **Corresponding author:** Yoshiko Ishimi, fax +81-3-3205-6549, email [ishimi@nih.go.jp](mailto:ishimi@nih.go.jp)



than that of Dz<sup>(6)</sup>. Fujioka *et al.* reported that administration of equol inhibited femoral bone loss in ovariectomised (OVX) mice without causing notable effects on reproductive organs<sup>(7)</sup>. Furthermore, we have previously demonstrated that soya products may be more effective in maintaining bone density in our clinical study of equol-producing individuals<sup>(8)</sup>.

Intestinal bacteria play an essential role in Dz metabolism, and specific equol-producing bacteria are required<sup>(9)</sup>. Clinical trials and animal models have provided evidence for a potentially protective role for isoflavones in postmenopausal osteoporosis. However, such findings have not been at all times consistent between human and animal models. One of the possible reasons for the inconsistencies between rodent studies and clinical trials could be because of differences in isoflavone metabolism. Most studies suggest that animals have the capacity to produce equol<sup>(10,11)</sup>, but not all human subjects are able to produce it. In Asia, 50–60 % of the adult population produced equol when given soya foods that contained isoflavones<sup>(12,13)</sup>. This percentage is significantly higher than the reported 25–30 % frequency of equol producers in Western countries<sup>(14)</sup>. The reasons for these differences are unclear, but habitual dietary patterns may influence the metabolism of isoflavones and the production of equol<sup>(15)</sup>. Some epidemiological studies have reported positive associations between equol production and intake of green tea or dietary fibre<sup>(15–17)</sup>. These foods and dietary pattern seem to be characteristic of the Japanese diet<sup>(18–20)</sup>. Okubo *et al.* reported that the traditional Japanese diet is characterised by high intakes of rice, miso soup and soya products<sup>(18)</sup>. Further, it is reported that the per capita green tea consumption in Japan is the highest in the world<sup>(21)</sup>. Green tea contains the catechin (–)-epigallocatechin gallate (EGCG). Moreover, Eshak *et al.* reported that dietary fibre intake, especially fruit and cereal fibre, may reduce the risk of mortality from CHD among Japanese people<sup>(22)</sup>.

There are several types of dietary fibre. In the present study, two types of dietary fibre were chosen on the basis of the

results of our preliminary experiment examining the effects of isoflavones and several types of dietary fibre on equol production. In the preliminary experiment, polydextrose (PD) and raffinose (Raf) effectively promoted equol production (Y. Tousein *et al.*, unpublished results). Raf is an indigestible oligosaccharide that occurs naturally in soya, many vegetables and fruits, and acts as a growth factor for *Bifidobacterium* species<sup>(23)</sup>. Another type of dietary fibre, PD, a synthetic indigestible glucose, has stimulatory effects on intestinal bacteria<sup>(24)</sup>. PD was approved as a principal ingredient of the Food for Specified Health Uses, which is allowed by the Japanese government to have statements on labels regarding beneficial effects on health<sup>(25)</sup>. The label statement for PD is that it helps increase bifidobacteria and thus helps maintain a healthful gastrointestinal condition. The Food for Specified Health Uses with PD is available as soft drinks, etc.

We hypothesised that a Japanese diet characterised by the presence of tea catechin or high dietary fibre will increase equol production, and demonstrated the synergistic effects of these foods on the prevention of bone loss caused by oestrogen deficiency. We examined the effects of a diet supplemented with Dz and tea catechin or dietary fibre on equol production and bone mineral density (BMD) in OVX mice.

## Experimental methods

### Materials

In Expts 1 and 2, Dz (purity >98 %; Nagara Science Co. Ltd) was added at 1 g/kg instead of sucrose to the control diet (Table 1). Catechin (EGCG > 90 %, DSM Nutrition K. K.) at 1 g/kg was added instead of maize starch to the control diet in Expt 1. PD (Wako Pure Chemical Industries Ltd) or Raf (D (+)-Raf pentahydrate; Wako Pure Chemical Industries Ltd) at 50 g/kg was added instead of maize starch to the control diet in Expt 2.

**Table 1.** Composition of the experimental diet (g/kg diet)\*

Ingredient	Control†‡	Dz†‡	Dz + Cate†	Cate†	Dz + PD‡	PD‡	Dz + Raf‡	Raf‡
Maize starch	529.5	529.5	519.5	519.5	479.5	479.5	479.5	479.5
Casein	200	200	200	200	200	200	200	200
Sucrose	100	99	99	100	99	100	99	100
Maize oil	70	70	70	70	70	70	70	70
Cellulose	50	50	50	50	50	50	50	50
Mineral mixture*	35	35	35	35	35	35	35	35
Vitamin mixture*	10	10	10	10	10	10	10	10
L-Cystine	3	3	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
<i>tert</i> -Butylhydroquinone	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014
Daidzein	–	1.0	1.0	–	1.0	–	1.0	–
Catechin	–	–	10	10	–	–	–	–
Polydextrose	–	–	–	–	50	50	–	–
Raffinose	–	–	–	–	–	–	50	50

Control, control diet; Dz, daidzein supplemented diet; Dz + Cate, Dz and catechin-supplemented diet; Cate, Cate-supplemented diet; Dz + PD, Dz and polydextrose-supplemented diet; PD, PD-supplemented diet; Dz + Raf, Dz and raffinose-supplemented diet; Raf, Raf-supplemented diet.

\* Prepared according to the AIN-93 G formulation.

† Experiment 1.

‡ Experiment 2.



## Methods

### Experiment 1: Effects of catechin on bioavailability of daidzein in ovariectomised female mice.

Female mice of the ddY strain, aged 8 weeks, were purchased from the Shizuoka Laboratory Animal Center. They were housed in individual cages in a temperature- and humidity-controlled room ( $23 \pm 1^\circ\text{C}$  and  $60 \pm 5\%$  relative humidity) with a 12 h light–12 h dark cycle. They were given free access to an American Institute of Nutrition (AIN)-93G diet with maize oil instead of soyabean oil for 4 d before surgery<sup>(26)</sup>. Mice were either sham-operated (Sham) or OVX on the same day. The OVX mice were randomly divided into four groups ( $n$  6–8): OVX-control (OVX), OVX fed a 0.1 % Dz-supplemented diet (OVX + Dz), OVX fed a 0.1 % Dz- and 1 % catechin-supplemented diet (OVX + Dz + Cate) and OVX fed 1 % catechin-supplemented diet (OVX + Cate). Table 1 shows the composition of the experimental diets, which were prepared according to the AIN-93 G formulation<sup>(26)</sup>. Maize oil was used to eliminate any possible contamination from the isoflavones in soyabean oil. Mice were pair-fed their respective diets for 42 d with free access to distilled water during this period. After 42 d of treatment, the mice were fasted overnight and then euthanised by exsanguination under anaesthesia, weighed and blood was drawn and stored at  $-80^\circ\text{C}$  until assay. The uterus was removed, and the wet weight was measured. The right femur was also removed to measure BMD. The caecum was removed with its contents and weighed. The contents were collected, the caecal tissue was washed with saline and the weight of the contents was calculated by subtracting the tissue weight from the total weight. The pH of the caecal contents was measured with a pH meter (model B-212, Twin Compact pH meter, HORIBA, Scientific Ltd). The caecal contents were stored at  $-80^\circ\text{C}$  until analysis. All experimental procedures involving animals were approved by the National Institute of Health and Nutrition Guidelines for the Care and Use of Laboratory Animals.

### Experiment 2: Effects of dietary fibre on the bioavailability of daidzein in ovariectomised female mice.

Mice were acclimatised and either sham-operated or OVX on the same day, as in Expt 1. The mice were divided into seven groups ( $n$  6–8): sham-operated (Sham) or OVX-control (OVX), OVX fed a 0.1 % Dz-supplemented diet (OVX + Dz), OVX fed a 0.1 % Dz–5 % PD-supplemented diet (OVX + Dz + PD), OVX fed a 5 % PD-supplemented diet (OVX + PD), OVX fed a 0.1 % Dz–5 % Raf-supplemented diet (OVX + Dz + Raf) and OVX fed a 5 % Raf-supplemented diet (OVX + Raf). The mice were pair-fed their respective diets (Table 1) for 42 d with free access to distilled water during this period. After 41 d of treatment, 24-h urine was collected and stored at  $-20^\circ\text{C}$  until assay. At the end of the experiment, body weight, uterus weights, caecal weights and caecal pH were measured and the caecal contents, right femur and blood were collected as described in Expt 1.

**Radiographic analysis of the femur.** Femoral BMD was measured by dual-energy X-ray absorptiometry (DXA; Model DCS-600EX-R, Aloka Ltd). The BMD was

calculated using the bone mineral content of the measured area. The BMD of the proximal femur, midshaft and distal femur were measured. The scanned area of the mouse femur was divided into three equal parts of 5.3 mm each: the proximal femur, midshaft and distal femur. All the DXA scanning and analyses were conducted by the same researcher.

### Time-resolved fluoroimmunoassay for plasma and urinary daidzein and equol.

Plasma and urinary Dz and equol were analysed by the time-resolved fluoroimmunoassay methods of Wang *et al.* and Brouwers *et al.*, respectively<sup>(27,28)</sup>. Urine and plasma were hydrolysed by glucuronidase and sulphatase after the plasma was extracted using ether. Plasma and urinary Dz and equol concentrations were determined by fluorescence using a DELFIA Victor 1420 multilabel counter (Perkin Elmer Co. Ltd). The final results = concentration (read)  $\times$  1/recovery  $\times$  dilution factor (nmol/l).

**$\beta$ -Glucosidase activity.** Activity of  $\beta$ -glucosidase was indicated as the rate of release of *p*-nitrophenol from *p*-nitrophenylglucoside. The reaction mixture contained 0.1 ml of a 5 mmol/l substrate solution and 0.2 ml of a 1:20 (v/v) dilution of the caecal sample in 0.1 M-phosphate buffer at pH 6.4. The reaction mixture was incubated for 30 min at  $37^\circ\text{C}$ , and the *p*-nitrophenol concentration was measured spectrophotometrically at 400 nm after the addition of 1.6 ml of 0.25 M-sodium carbonate. Enzyme activity was expressed as mmol product hydrolysed per whole caecal contents in 30 min.

### Statistical analysis

The data are expressed as mean values with their standard errors of the difference between means. The significance of differences in BMD was determined by one-factor ANOVA and Fisher's protected least significant difference test (SPSS version 11.0; SPSS Inc.). Body weight was used as a covariate in the analysis of BMD to adjust for possible confounding effects. The remaining data were analysed using ANOVA. The differences between the treatment groups were assessed by Tukey's test. A *P* value less than 0.05 was considered statistically significant.

## Results

### Experiment 1

Initial body weights of all groups of mice did not differ significantly from each other (data not shown). Final body weights were significantly lower in the OVX + Dz + Cate and OVX + Cate groups (29.9 (SE 0.7) and 30.5 (SE 0.7) g, respectively) than in the Sham, OVX and OVX + Dz groups (35.1 (SE 1.5), 37.0 (SE 0.9) and 34.7 (SE 0.7) g, respectively). The uterine weight was lower in OVX mice than in sham-operated mice, and treatment with Dz or catechin did not affect the uterine weight in OVX mice. Plasma Dz concentrations were significantly increased by Dz feeding in the OVX + Dz



and the OVX + Dz + Cate groups (0.927 (SE 0.031), 0.863 (SE 0.025)  $\mu\text{mol/l}$ , respectively). Plasma equol was detected in the OVX + Dz group (9.67 (SE 1.17)  $\mu\text{mol/l}$ ), but it was not detected in the OVX + Dz + Cate group.

The wet weight of caecal contents was significantly higher in the OVX + Cate group (0.867 (SE 0.118) g/100 g body weight) than in the OVX group (0.448 (SE 0.074) g/100 g body weight), and tended to be higher in the OVX + Dz + Cate group (0.608 (SE 0.092) g/100 g body weight) than in the OVX group ( $P = 0.276$ ). There were no significant differences in caecal pH and  $\beta$ -glucosidase activity between all groups (data not shown).

Femoral BMD was significantly lower in OVX mice than in sham mice (Table 2), and Dz treatment inhibited femoral bone loss. However, the femoral BMD in the OVX + Dz + Cate group was lower than that in the OVX + Dz group. The inhibition of bone loss by Dz was attenuated by treatment with catechin in OVX mice.

## Experiment 2

The initial and final body weights of all groups of mice did not differ significantly from each other (data not shown). Treatment with Dz, PD and/or Raf did not affect the uterine weight in OVX mice. The plasma Dz concentration was significantly higher in the OVX + Dz group than in the other groups (Fig. 1(A)). The plasma equol concentrations were higher in the OVX + Dz + Raf and OVX + Dz + PD groups (4.67 (SE 0.89) and 4.93 (SE 0.49)  $\mu\text{mol/l}$ , respectively) than in the OVX + Dz group (0.28 (SE 0.54)  $\mu\text{mol/l}$ ) (Fig. 1(B)). The urinary Dz and equol concentrations in Dz-fed groups were significantly higher than in the other groups (Fig. 2(A) and (B)). The ratio of urinary equol to Dz concentration tended to be higher in the OVX + Dz + Raf group than in the OVX + Dz group ( $P = 0.124$ ), and the ratio was significantly higher in the OVX + Dz + PD group than in the OVX + Dz group ( $P = 0.07$ ). Similar results were obtained for urinary equol concentrations (Fig. 2(C)).

Wet weights of the caecal contents in the OVX + Dz + PD and OVX + Dz + Raf groups were higher than those in the Sham, OVX, OVX + Dz and OVX + PD groups (Table 3). Caecal pH was lower in the OVX + Dz + PD group than in the other groups (Table 3). Caecal  $\beta$ -glucosidase activity was significantly higher in the OVX + Dz + PD group than in

the other groups (Table 3). The wet weights of the caecal contents were elevated in mice fed Dz and Raf, but caecal pH and caecal  $\beta$ -glucosidase activity were not affected by these additives.

Femoral BMD of the whole and each of the three parts was significantly lower in the OVX group than in the Sham group ( $P < 0.05$ ) (Fig. 3(A)–(D)). The Dz treatment groups showed inhibited femoral bone loss compared with the OVX, OVX + PD and OVX + Raf groups. Treatment with PD or Raf alone did not affect femoral BMD. The BMD of the whole, proximal, middle and distal regions of the femur in the OVX + Dz + PD and OVX + Dz + Raf groups was significantly higher than that in the OVX group (Fig. 3(A)–(D)). Furthermore, the BMD of the whole, middle and distal femur was significantly higher in the OVX + Dz + PD group than in the OVX + Dz group (Fig. 3(A), (C) and (D)), and tended to be higher in the OVX + Dz + Raf group than in the OVX + Dz group (Fig. 3(A), (C) and (D)). The BMD of the distal femur was significantly higher in the OVX + Dz + PD group than in the OVX + Dz + Raf group ( $P = 0.038$ ) (Fig. 3(D)).

## Discussion

In the present study, we examined the effects of two of the ingredients that form part of the Japanese diet, catechin and dietary fibre, on equol production and BMD in Dz-treated OVX mice. Dietary fibre stimulated equol production and enhanced the bone-protective effects of Dz on femoral bone. On the other hand, catechin reduced serum equol levels and suppressed the beneficial effect of Dz on femoral bone loss. The data suggest that the bioavailability of isoflavones may be altered by dietary fibre, in particular, PD.

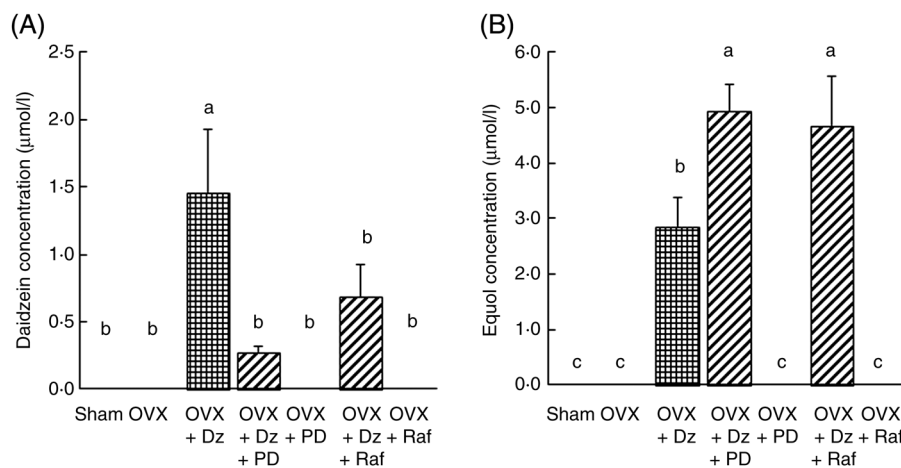
## Experiment 1

In our study, the wet weight of caecal contents in the catechin feeding group was higher than that in the OVX group, but there were no significant differences in caecal pH and  $\beta$ -glucosidase activity between all groups (data not shown). Furthermore, a significant decrease in the concentration of serum equol was observed in these groups. It has been reported that the polyphenol EGCG in green tea has therapeutic effects on colon diseases<sup>(29–32)</sup>. Shirakami *et al.* reported

**Table 2.** Bone mineral density (BMD) of the femur obtained from the mice in Expt 1 (Mean values with their standard errors)

	Sham		OVX		OVX + Dz		OVX + Dz + Cate		OVX + Cate	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Whole femur BMD (mg/cm <sup>2</sup> )	40.1 <sup>a</sup>	1.1	33.9 <sup>c,d</sup>	0.7	37.9 <sup>b</sup>	0.8	32.8 <sup>d</sup>	0.3	35.1 <sup>c</sup>	0.9
Proximal region (mg/cm <sup>2</sup> )	44.1 <sup>a</sup>	0.7	40.2 <sup>b</sup>	0.7	44.0 <sup>a</sup>	1.1	38.1 <sup>b</sup>	0.8	39.7 <sup>b</sup>	1.1
Middle region (mg/cm <sup>2</sup> )	35.1 <sup>a</sup>	0.8	31.2 <sup>b,c</sup>	0.7	34.6 <sup>a,b</sup>	1.0	30.7 <sup>c</sup>	0.3	33.2 <sup>b</sup>	1.0
Distal region (mg/cm <sup>2</sup> )	47.4 <sup>a</sup>	1.9	34.9 <sup>c</sup>	0.3	40.5 <sup>b</sup>	1.3	35.5 <sup>c</sup>	0.3	36.8 <sup>c</sup>	1.1

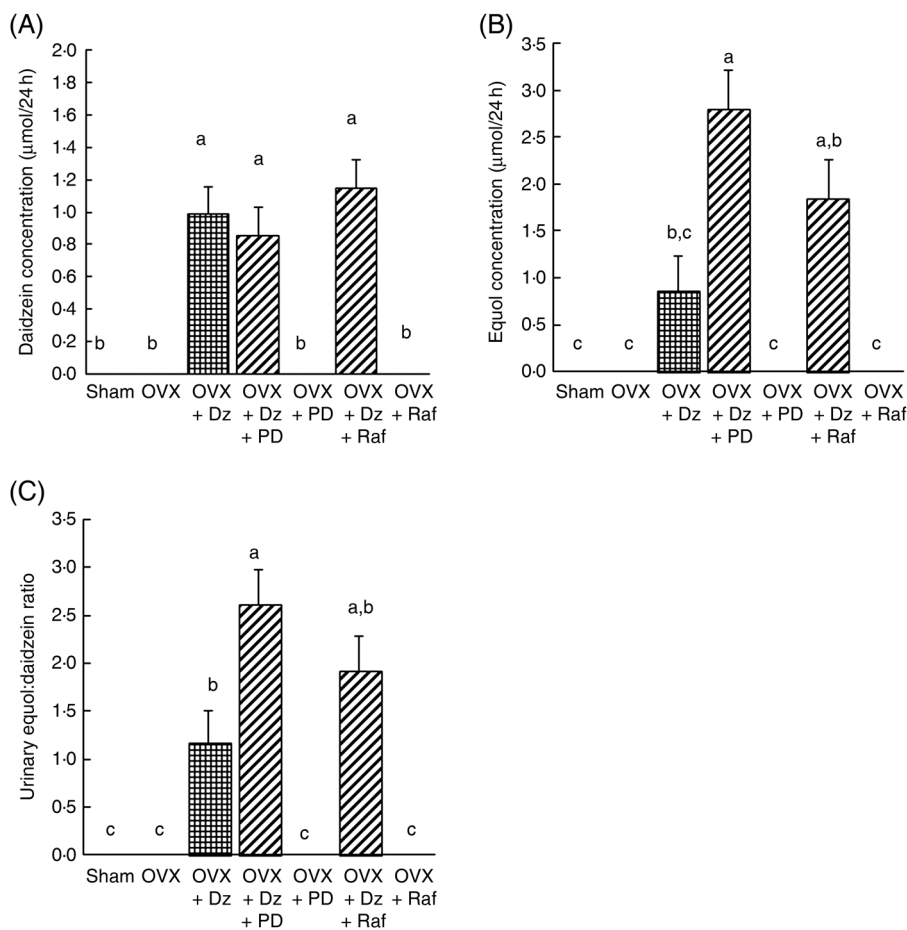
Sham, sham-operated mice fed the control diet; OVX, ovariectomised mice fed the control diet; OVX + Dz, OVX mice fed the 0.1% daidzein-supplemented diet; OVX + Dz + Cate, OVX mice fed a combination of 0.1% Dz and 1% catechin-supplemented diet; OVX + Cate, OVX mice fed the 1% Cate-supplemented diet.  
<sup>a,b,c,d</sup> Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).



**Fig. 1.** Plasma concentrations of daidzein (Dz) (A) and equol (B) in mice in Expt 2. Sham, sham-operated mice fed the control diet; OVX, ovariectomised mice fed the control diet; OVX + Dz, OVX mice fed the 0.1 % Dz-supplemented diet; OVX + Dz + PD, OVX mice fed a combination of 0.1 % Dz and 5 % polydextrose-supplemented diet; OVX + PD, OVX mice fed the 5 % polydextrose-supplemented diet; OVX + Dz + Raf, OVX mice fed a combination of 0.1 % Dz and 5 % raffinose-supplemented diet; OVX + Raf, OVX mice fed the 5 % Raf-supplemented diet. Values are means, with their standard errors represented by vertical bars. <sup>a,b,c</sup> Mean values with unlike letters were significantly different ( $P < 0.05$ ).

that drinking water containing EGCG was beneficial to the suppression of cancer development in the inflamed colon in mice<sup>(30)</sup>. Additionally, the ameliorative effect of EGCG on

inflammatory bowel disease has been observed in rats<sup>(31)</sup>. These studies adopted drinking water or diet containing 0.01–0.20 % EGCG. In our study, supplementation of



**Fig. 2.** Concentrations of urinary daidzein (Dz) (A) and equol (B) and the ratio of urinary equol:daidzein concentrations (C) in mice in Expt 2. Sham, sham-operated mice fed the control diet; OVX, ovariectomised mice fed the control diet; OVX + Dz, OVX mice fed the 0.1 % daidzein-supplemented diet; OVX + Dz + PD, OVX mice fed a combination of 0.1 % Dz and 5 % polydextrose-supplemented diet; OVX + PD, OVX mice fed 5 % polydextrose-supplemented diet; OVX + Dz + Raf, OVX mice fed a combination of 0.1 % Dz and 5 % raffinose-supplemented diet; OVX + Raf, OVX mice fed 5 % Raf-supplemented diet. Values are means, with their standard errors represented by vertical bars. <sup>a,b,c</sup> Mean values with unlike letters were significantly different ( $P < 0.05$ ).



**Table 3.** Wet weights of the caecal content, pH and  $\beta$ -glucosidase activity in the mice in Expt 2 (Mean values with their standard errors)

	Sham		OVX		OVX + Dz		OVX + Dz + PD		OVX + PD		OVX + Dz + Raf		OVX + Raf	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Wet weight of the caecal contents*	0.154 <sup>c,d</sup>	0.015	0.123 <sup>d</sup>	0.020	0.180 <sup>c,d</sup>	0.017	0.292 <sup>a</sup>	0.013	0.164 <sup>c,d</sup>	0.024	0.248 <sup>a,b</sup>	0.029	0.202 <sup>b,c</sup>	0.028
Caecal pH	7.87 <sup>a</sup>	0.07	7.68 <sup>a,b</sup>	0.11	7.77 <sup>a,b</sup>	0.08	6.91 <sup>c</sup>	0.16	7.40 <sup>b</sup>	0.05	7.57 <sup>a,b</sup>	0.04	7.70 <sup>a,b</sup>	0.07
Caecal $\beta$ -glucosidase activity†	0.199 <sup>b</sup>	0.035	0.369 <sup>b</sup>	0.096	0.256 <sup>b</sup>	0.065	4.763 <sup>a</sup>	1.830	1.016 <sup>b</sup>	0.362	0.493 <sup>b</sup>	0.162	0.201 <sup>b</sup>	0.021

Sham, sham-operated mice fed the control diet; OVX, ovariectomised mice fed the control diet; OVX + Dz, OVX mice fed 0.1% daidzein-supplemented diet; OVX + Dz + PD, OVX mice fed a combination of 0.1% Dz and 5% polydextrose-supplemented diet; OVX + PD, OVX mice fed 5% PD-supplemented diet; OVX + Dz + Raf, OVX mice fed a combination of 0.1% Dz and 5% raffinose-supplemented diet; OVX + Raf, OVX mice fed 5% Raf-supplemented diet.

\* g/100 g body weight.

† Hydrolysed product (mmol)/whole caecal contents per 30 min.

Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).

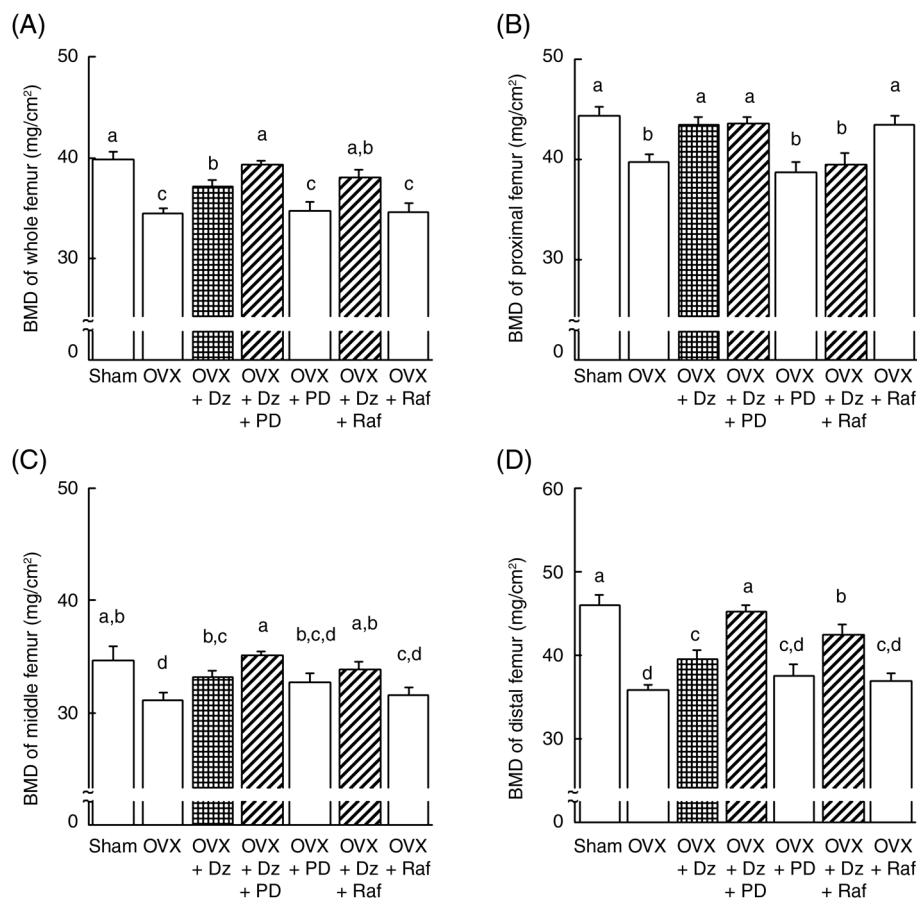
catechin at 1% of the diet resulted in negative effects reducing the equol production in the intestine. Catechin is one of the polyphenol compounds; polyphenols are potential modulators of digestive fermentation<sup>(33,34)</sup>. Molan *et al.* reported that orally administering rats with green tea extract for 6 d resulted in a slight increase in the numbers of caecal lactobacilli and bifidobacteria but decreased the numbers of *Bacteroides* and *Clostridia* significantly<sup>(35)</sup>. Zdunczyk *et al.* reported that supplementation with 0.3% polyphenol extracts significantly increased the caecal content weight<sup>(33)</sup>, but did not measure caecal bacteria. The effect of dietary flavonoids on intestinal electrolyte transport is not well established. Available data have suggested that these compounds are a diarrhoeal<sup>(36,37)</sup> or an anti-diarrhoeal agent<sup>(38)</sup>. In our study, moisture content of caecal samples was not measured; further investigations including those of water absorption in the intestine are needed.

Furthermore, in our study, the dietary 1% catechin level was higher than in previous reports<sup>(29–32)</sup>. Hara *et al.* also reported that a diet supplemented with 0.2% tea polyphenols significantly decreases the total bacteria level in pigs<sup>(39)</sup>. Equol is metabolised by intestinal bacteria; thus, a decrease in the abundance of bacteria in the intestine may lead to a decrease in equol production. These results suggested that a large amount of catechin may alter the intestinal environment and depress equol production in the intestine.

The catechin in green tea, (–)-EGCG, increases the osteogenic function of mesenchymal stem cells<sup>(40)</sup>, and animal studies have strongly suggested that green tea prevents bone loss<sup>(41,42)</sup>. In contrast with previous reports, we found that the inhibition of bone loss by Dz was attenuated by catechin treatment (Table 2). The discrepancy between previous reports and the present study may be due to a different dosage of catechin. Other experiments examining the effects of catechin on animal models adopted drinking water containing 0.1 or 0.5% green tea catechin<sup>(41,42)</sup>. Shen *et al.* reported that 0.5% catechin supplemented in drinking water of rats attenuated trabecular and cortical bone loss by increasing bone formation<sup>(42)</sup>. In contrast, Iwaniec *et al.* reported that bone mineral content and volumetric BMD were reduced by green tea extract consumption in growing male mice<sup>(43)</sup>. In that study, the diets contained green tea extract at 1 or 2% of the diet. Thus, a high dose of catechin may reduce bone mass and/or accelerate bone loss. Further studies are needed to confirm the effects of catechin extracts on bone metabolism.

### Experiment 2

Our results showed that PD had a greater effect on equol production and on the enteric environment such as pH and weight of the caecal contents (Table 3). The urinary equol concentration and its ratio to Dz concentration were significantly higher in the OVX + Dz + PD group than in the OVX + Dz group in Expt 2 (Fig. 2(B) and (C)). In the present study, acidification of caecal content was more pronounced with the Dz- and PD-containing diet, compared with PD alone (Table 3). The data were consistent with a previous report that feeding of rats with 0.3% flavonoid and 5% inulin, a kind of dietary fibre added to the diet, induced significant enlargement of the



**Fig. 3.** Femoral bone mineral density (BMD) was obtained from mice in Expt 2. Whole femur BMD (A), proximal femur BMD (B), middle femur BMD (C) and distal femur BMD (D). Sham, sham-operated mice fed the control diet; OVX, ovariectomised mice fed the control diet; OVX + Dz, OVX mice fed 0.1 % daidzein-supplemented diet; OVX + Dz + PD, OVX mice fed a combination of 0.1 % Dz and 5 % polydextrose-supplemented diet; OVX + PD, OVX mice fed 5 % polydextrose-supplemented diet; OVX + Dz + Raf, OVX mice fed a combination of 0.1 % Dz and 5 % raffinose-supplemented diet; OVX + Raf, OVX mice fed 5 % Raf-supplemented diet. Values are means, with their standard errors represented by vertical bars. <sup>a,b,c,d</sup> Mean values with unlike letters were significantly different ( $P < 0.05$ ).

caecum compared with rats fed the control diet. The greatest enlargement of caecal contents was observed in rats fed a flavonoid-containing diet<sup>(33,34)</sup>. Since Dz is a kind of flavonoid, it is possible that it induces synergistic effects with PD on the enteric environment. These results suggest that the combination of dietary fibres, either PD or Raf, and Dz enhances not only the metabolism of isoflavone conjugates to aglycone but also the metabolism of aglycone to equol from Dz. Further studies are required to confirm the effects of each type of dietary fibre on equol production.

The ability to produce equol depends on the presence of certain intestinal microflora; thus, the enteric environment has been known to affect equol production<sup>(44)</sup>. We measured  $\beta$ -glucosidase activity in the caecal contents and found an increase in enzymatic activity in mice fed PD and Dz (Table 3). The activity of  $\beta$ -glucosidase in the caecal contents is an indicator of the activity of the intestinal enzymes hydrolysing the glycoside bond of isoflavone conjugates and this leads to stimulation of the intestinal absorption of isoflavone aglycones. So  $\beta$ -glucosidase activity has a critical role in isoflavone metabolism. Ohta *et al.* reported that fructo-oligosaccharide increases  $\beta$ -glucosidase activity in mice<sup>(10)</sup>. PD produces SCFA in the process of fermentation and has a stimulatory effect on colonic *Lactobacillus* and

*Bifidobacterium* species similar to fructo-oligosaccharides<sup>(24,45)</sup>. Jie *et al.* reported that ingestion of PD increases the faecal weight and faecal abundance of *Lactobacillus* and *Bifidobacterium* species but decreases faecal pH in Chinese subjects<sup>(24)</sup>. Similarly, Raf produces SCFA and increases the faecal populations of *Bifidobacterium* and *Lactobacillus* species<sup>(46)</sup>. It is not clear how bacterial species produce equol in the intestine. *Lactobacillus* and *Bifidobacterium* species have been identified to play a role in the metabolism of Dz to equol<sup>(9,44)</sup>. We have previously reported that a diet supplemented with Dz and resistant starch, which is not absorbed in the intestine, increases the equol production to occupation ratio of *Bifidobacterium* species to that of the intestinal microflora in mice<sup>(11)</sup>. Although we did not measure the composition of the intestinal microflora, it is possible that *Lactobacillus* and/or *Bifidobacterium* species increased in the present study.

We were able to show that OVX mice whose diets were supplemented with Dz or a combination of Dz and PD or Raf maintained their femoral BMD compared with OVX mice (Fig. 3). In particular, mice fed Dz + PD maintained their BMD more effectively than mice fed Dz + Raf. Although PD and Raf improve caecal Ca absorption in rats<sup>(47,48)</sup>, a diet supplemented with PD or Raf alone did not maintain the BMD in OVX mice in our study. These results



are similar to those of our previous study in which diets supplemented with resistant starch and Dz efficiently prevented BMD decrease in OVX mice<sup>(11)</sup>. In the present study, the synergistic effects may be credited with the ability of dietary fibre to enhance equol production.

The reported plasma equol levels for the two experiments differ. The plasma equol concentrations in the OVX + Dz group in Expt 1 and those in Expt 2 were 9.67 (SE 1.17) and 2.85 (SE 1.51)  $\mu\text{mol/l}$ , respectively. The plasma equol concentration in the OVX + Dz group in Expt 1 was approximately 3-fold higher than that in the OVX + Dz group in Expt 2. As the reason for this difference, serum equol concentration is influenced by the timing of blood collection and changes substantially<sup>(14)</sup>. Therefore, equol may have reached peak concentrations in plasma and may have been excreted in the urine. Poulsen *et al.* reported that 49.1 % of Dz and its metabolites, including equol, were excreted in the urine and faeces of rats within 24 h<sup>(49)</sup>. However, Setchell & Cole reported that expressing the product–precursor relationship as the concentration ratio of equol to Dz in urine is a more reliable indicator of the amount of Dz converted to equol<sup>(14)</sup> (Fig. 2(C)).

### Conclusions

This study demonstrated the effects of components from a typical Japanese diet (isoflavones, tea catechin or dietary fibre) on equol production and bone metabolism in OVX mice. The dietary fibre increased equol production and inhibited bone loss in OVX mice. This effect was greater than that of Dz alone for preventing bone loss in Dz-treated OVX mice. In contrast, catechin reduced serum equol levels and suppressed the beneficial effect of Dz on femoral bone loss. These results suggest that additional studies should be carried out to determine whether a combination of isoflavones and dietary fibre is useful in maintaining the bone health of postmenopausal women.

### Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the Strategic Japanese (Science and Technology Agency)–New Zealand (Ministry of Science and Innovation) Cooperative Program on Functional Food. The authors declare no conflicts of interest. All the authors have contributed to the preparation of the paper and agree with the submitted manuscript content. Y. I., U. M. and Y. T. designed the studies. Y. I. and Y. T. performed the studies. Y. T. analysed the samples and data and drafted the manuscript. M. K. advised on the research and the manuscript.

### References

- Schmitt E, Dekant W & Stopper H (2001) Assaying the estrogenicity of phytoestrogens in cells of different estrogen sensitive tissues. *Toxicol In Vitro* **15**, 433–439.
- Taku K, Melby MK, Takebayashi J, *et al.* (2010) Effect of soy isoflavone extract supplements on bone mineral density in menopausal women: meta-analysis of randomized controlled trials. *Asia Pac J Clin Nutr* **19**, 33–42.
- Villa P, Costantini B, Suriano R, *et al.* (2009) The differential effect of the phytoestrogen genistein on cardiovascular risk factors in postmenopausal women: relationship with the metabolic status. *J Clin Endocrinol Metab* **94**, 552–558.
- Beral V, Bull D & Reeves G (2005) Endometrial cancer and hormone-replacement therapy in the Million Women Study. *Lancet* **365**, 1543–1551.
- Messina MJ (2002) Soy foods and soybean isoflavones and menopausal health. *Nutr Clin Care* **5**, 272–282.
- Lampe JW (2009) Is equol the key to the efficacy of soy foods? *Am J Clin Nutr* **89**, 1664S–1667S.
- Fujioka M, Uehara M, Wu J, *et al.* (2004) Equol, a metabolite of daidzein, inhibits bone loss in ovariectomized mice. *J Nutr* **134**, 2623–2627.
- Wu J, Oka J, Ezaki J, *et al.* (2007) Possible role of equol status in the effects of isoflavone on bone and fat mass in postmenopausal Japanese women: a double-blind, randomized, controlled trial. *Menopause* **14**, 866–874.
- Atkinson C, Frankenfeld CL & Lampe JW (2005) Gut bacterial metabolism of the soy isoflavone daidzein: exploring the relevance to human health. *Exp Biol Med (Maywood)* **230**, 155–170.
- Ohta A, Uehara M, Sakai K, *et al.* (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.
- Tousen Y, Abe F, Ishida T, *et al.* (2011) Resistant starch promotes equol production and inhibits tibial bone loss in ovariectomized mice treated with daidzein. *Metabolism* **60**, 1425–1432.
- Arai Y, Uehara M, Sato Y, *et al.* (2000) Comparison of isoflavones among dietary intake, plasma concentration and urinary excretion for accurate estimation of phytoestrogen intake. *J Epidemiol* **10**, 127–135.
- 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.
- Setchell KD & Cole SJ (2006) Method of defining equol-producer status and its frequency among vegetarians. *J Nutr* **136**, 2188–2193.
- Lampe JW, Karr SC, Hutchins AM, *et al.* (1998) Urinary equol excretion with a soy challenge: influence of habitual diet. *Proc Soc Exp Biol Med* **217**, 335–339.
- Rowland IR, Wiseman H, Sanders TA, *et al.* (2000) Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora. *Nutr Cancer* **36**, 27–32.
- Miyanaga N, Akaza H, Takashima N, *et al.* (2003) Higher consumption of green tea may enhance equol production. *Asian Pac J Cancer Prev* **4**, 297–301.
- Okubo H, Sasaki S, Murakami K, *et al.* (2008) Three major dietary patterns are all independently related to the risk of obesity among 3760 Japanese women aged 18–20 years. *Int J Obes (Lond)* **32**, 541–549.
- Okubo H, Murakami K, Sasaki S, *et al.* (2010) Relative validity of dietary patterns derived from a self-administered diet history questionnaire using factor analysis among Japanese adults. *Public Health Nutr* **13**, 1080–1089.
- The Ministry of Health, Labour and Welfare in Japan (2010) The Results of National Health and Nutrition Examination Survey in 2008. <http://www.mhlw.go.jp/bunya/kenkou/eiyou09/01.html> (In Japanese).
- International Tea Committee (2004) *Annual Bulletin of Statistics*. London: International Tea Committee.
- Eshak ES, Iso H, Date CS, *et al.* (2010) JACC study group. Dietary fiber intake is associated with reduced risk of mortality from cardiovascular disease among Japanese men and women. *J Nutr* **140**, 1445–1453.





23. Benno Y, Endo K, Sayama K, *et al.* (1987) Effects of raffinose intake on human fecal microflora. *Bifidobacteria Microflora* **6**, 59–63.
24. Jie Z, Bang-Yao L, Ming-Jie X, *et al.* (2000) Studies on the effects of polydextrose intake on physiologic functions in Chinese people. *Am J Clin Nutr* **72**, 1503–1509.
25. Yamada K, Sato-Mito N, Nagata J, *et al.* (2008) Health claim evidence requirements in Japan. *J Nutr* **138**, 1192S–1198S.
26. Reeves PG, Nielsen FH & Fahey GC (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition *ad hoc* writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* **123**, 1939–1951.
27. Brouwers E, L'Homme R, Al-Maharik N, *et al.* (2003) Time-resolved fluoroimmunoassay for equol in plasma and urine. *J Steroid Biochem Mol Biol* **84**, 577–588.
28. Wang GJ, Lapeck O, Hampl R, *et al.* (2000) Time-resolved fluoroimmunoassay of plasma daidzein and genistein. *Steroids* **65**, 339–348.
29. Kumar N, Shibata D, Helm J, *et al.* (2007) Green tea polyphenols in the prevention of colon cancer. *Front Biosci* **12**, 2309–2315.
30. Shirakami Y, Shimizu M, Tsurumi H, *et al.* (2008) EGCG and polyphenon E attenuate inflammation-related mouse colon carcinogenesis induced by AOM plus DDS. *Mol Med Report* **1**, 355–361.
31. Mochizuki M & Hasegawa N (2010) (–)-Epigallocatechin-3-gallate reduces experimental colon injury in rats by regulating macrophage and mast cell. *Phytother Res Suppl* **1**, S120–S122.
32. Abboud PA, Hake PW, Burroughs TJ, *et al.* (2008) Therapeutic effect of epigallocatechin-3-gallate in a mouse model of colitis. *Eur J Pharmacol* **579**, 411–417.
33. Zdunczyk Z, Juskiewicz J & Estrella I (2006) Cecal parameters of rats fed diets containing grapefruit polyphenols and inulin as single supplements or in a combination. *Nutrition* **22**, 898–904.
34. Aprikian O, Duclos V, Guyot S, *et al.* (2003) Apple pectin and a polyphenol-rich apple concentrate are more effective together than separately on cecal fermentations and plasma lipids in rats. *J Nutr* **133**, 1860–1865.
35. Molan AL, Liu Z & Tiwari R (2010) The ability of green tea to positively modulate key markers of gastrointestinal function in rats. *Phytother Res* **24**, 1614–1619.
36. Nguyen TD, Canada AT, Heintz GG, *et al.* (1991) Stimulation of secretion by the T84 colonic epithelial cell line with dietary flavonols. *Biochem Pharmacol* **41**, 1879–1886.
37. Cermak R, Follmer U & Wolfram S (1998) Dietary flavonol quercetin induces chloride secretion in rat colon. *Am J Physiol* **275**, G1166–G1172.
38. Di Carlo G, Autore G, Izzo AA, *et al.* (1993) Inhibition of intestinal motility and secretion by flavonoids in mice and rats: structure–activity relationships. *J Pharm Pharmacol* **45**, 1054–1059.
39. Hara H, Orita N, Hatano S, *et al.* (1995) Effect of tea polyphenols on fecal flora and fecal metabolic products of pigs. *J Vet Med Sci* **57**, 45–49.
40. Lin RW, Chen CH, Wang YH, *et al.* (2009) (–)-Epigallocatechin gallate inhibition of osteoclastic differentiation via NF-κB. *Biochem Biophys Res Commun* **379**, 1033–1037.
41. Shen CL, Cao JJ, Dagda RY, *et al.* (2011) Supplementation with green tea polyphenols improves bone microstructure and quality in aged, orchidectomized rats. *Calcif Tissue Int* **88**, 455–463.
42. Shen CL, Yeh JK, Cao JJ, *et al.* (2011) Green tea and bone health: evidence from laboratory studies. *Pharmacol Res* **64**, 155–161.
43. Iwaniec UT, Turner RT, Koo SI, *et al.* (2009) Consumption of green tea extract results in osteopenia in growing male mice. *J Nutr* **139**, 1914–1919.
44. Yuan JP, Wang JH & Liu X (2007) Metabolism of dietary soy isoflavones to equol by human intestinal microflora—implications for health. *Mol Nutr Food Res* **51**, 765–781.
45. Tiihonen K, Suomalainen T, Tynkkynen S, *et al.* (2008) Effect of prebiotic supplementation on a probiotic bacteria mixture: comparison between a rat model and clinical trials. *Br J Nutr* **99**, 826–831.
46. Dinoto A, Suksomcheep A, Ishizuka S, *et al.* (2006) Modulation of rat cecal microbiota by administration of raffinose and encapsulated *Bifidobacterium breve*. *Appl Environ Microbiol* **72**, 784–792.
47. Hara H, Suzuki T & Aoyama Y (2000) Ingestion of the soluble dietary fibre, polydextrose, increases calcium absorption and bone mineralization in normal and total-gastrectomized rats. *Br J Nutr* **84**, 655–661.
48. Weaver CM, Martin BR, Story JA, *et al.* (2010) Novel fibers increase bone calcium content and strength beyond efficiency of large intestine fermentation. *J Agric Food Chem* **58**, 8952–8957.
49. Poulsen RC, Loots du T, Moughan PJ, *et al.* (2009) Ileal and faecal digestibility of daidzein and genistein and plasma bioavailability of these isoflavones and their bioactive metabolites in the ovariectomised rat. *Mol Nutr Food Res* **53**, Suppl., 27–35.