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Resistant starch promotes equol production and inhibits tibial bone loss in ovariectomized mice treated with daidzein

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ABSTRACT

Daidzein is metabolized to equol in the gastrointestinal tract by gut microflora. Equol has greater estrogenic activity than genistein and daidzein, with its production shown to be promoted by dietary fiber. It is known that resistant starch (RS) is not absorbed in the proximal intestine and acts as dietary fiber in the colon. In this study, we investigated the combined effects of daidzein and RS intake on equol production, bone mineral density, and intestinal microflora in ovariectomized (OVX) mice. Female mice of the ddY strain, aged 8 weeks, were either sham operated (n = 6) or OVX. The OVX mice were randomly divided into 5 groups: OVX control (n = 6), OVX fed 0.1% daidzein–supplemented diet (OVX + Dz, n = 8), OVX fed 0.1% daidzein– and 12% RS–supplemented diet (OVX + Dz + RS, n = 8), OVX fed 12% RS–supplemented diet (OVX + RS, n = 8), and OVX who received daily subcutaneous administration of 17 β-estradiol (n = 6). After 6 weeks, urinary equol concentration was significantly higher in the OVX + Dz + RS group than in the OVX + Dz group. The bone mineral density of the whole tibia was higher in the OVX + Dz +RS group compared with the OVX + Dz group. The occupation ratios of *Bifidobacterium* spp in the cecal microflora in groups fed RS were significantly higher than those in the other groups. The present study demonstrated that RS may increase the bioavailability of daidzein.

1. Introduction

Osteoporosis is characterized by bone loss that increases the risk of bone fracture. One of the treatments for postmenopausal osteoporosis is hormone replacement therapy. However, this treatment is reported to have adverse effects such as induction of hormone-dependent breast and uterine cancers [1].

Soybean isoflavones have structures similar to estrogen and have a weak affinity for estrogen receptors, properties that suggest they may exhibit an estrogenic action in various tissues [2,3]. These isoflavones have received considerable attention because of their potential to prevent postmenopausal symptoms such as cardiovascular disease, osteoporosis, and hormone-dependent cancers [4-6].

Daidzein, a major soybean isoflavone, is metabolized to equol in the gastrointestinal tract by gut microflora [7]. Equol shows greater estrogenic effects than genistein and daidzein [8]. Fujioka et al [9] reported that administration of equol inhibited bone loss in ovariectomized (OVX) mice without causing notable effects in reproductive organs. Recent studies suggest that the clinical effectiveness of isoflavones may be due to their ability to produce equol in the intestine [8].

Epidemiological studies suggest that high equol producers are at lower risk of breast cancer than low equol

Conflicts of Interest: All authors have no conflicts of interest to declare.

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producers [6]. Setchell et al [8] also demonstrated that only equol producers had increased bone mineral density (BMD) in the lumbar spine after 2 years of isoflavone intervention. Wu et al [10] also reported that the positive effects of isoflavones on bone loss depended on the extent of equol production in postmenopausal Japanese women, which suggests that equol may have an important role in the beneficial effects of isoflavones. However, equol is not produced in all healthy humans. Approximately 30% to 50% of the human population produces equol, probably because of the variable presence of the aforementioned bacteria in the gastrointestinal tract [11]. Diet can determine the dominant bacterial strains present in the gastrointestinal tract, with certain dietary changes altering the bacterial profile of the intestine. Therefore, habitual dietary patterns may influence the metabolism of isoflavones and the production of equol [7,12]. Lampe et al [13] reported that the diets of equol producers were richer in dietary fibers and carbohydrates than those of nonproducers. Therefore, several studies have examined the effect of both prebiotics and probiotics on equol production in an attempt to establish the beneficial effects of isoflavones [14-17]. For example, Ohta et al [14] reported that fructo-oligosaccharides (FOS) promoted equol production and prevented bone loss in OVX rats.

Resistant starch (RS), a component of starch, is not absorbed in the proximal intestine and therefore reaches the colon [18]. Resistant starch provides a fermentable substrate for specific microflora, including *Bifidobacterium* spp, *Lactobacillus* spp, and *Bacteroides* spp [19,20]. Daidzein is metabolized to several other products, including equol and *O*-desmethylangolensin, by the same 3 species of bacteria in the colon [7,21-23]. However, the in vivo metabolic processes of these intestinal microfloras remain to be fully understood.

We hypothesized that intake of RS may alter intestinal microflora and increase equol production, and show the cooperative effects on prevention of bone loss caused by an estrogen deficiency. In this study, we examined the effects of a diet supplemented with daidzein and RS on intestinal microflora, equol production, and BMD in OVX mice.

2. Methods

2.1. Animals and chemicals

Female mice of the ddY strain, aged 8 weeks, were purchased from the Shizuoka Laboratory Animal Center. The mice were housed in individual cages in a temperature- and humidity-controlled room (23°C ± 1°C and 60% ± 5% relative humidity) with a 12-hour light/dark cycle. The mice were given free access to an AIN-93G diet with corn oil instead of soybean oil for 4 days before performing the operation [24]. The mice were either sham operated (Sham, n = 6) or OVX on the same day. The OVX mice were randomly divided into 5 groups: OVX control (OVX, n = 6); OVX fed 0.1% daidzein–supplemented diet (OVX + Dz, n = 8); OVX fed 12% RS–supplemented diet (OVX + RS, n = 8); OVX fed 0.1% daidzein– and 12% RS–supplemented diet (OVX + Dz + RS, n = 8); and OVX administered 17 β-estradiol (E2) subcutaneously (OVX + E2, n = 6). The OVX + E2 group received subcutaneous administration of E2 (0.03 μg/d) using a miniosmotic pump (Alza, Palo Alto, CA). Table 1 shows the composition of the experimental diets, which were prepared according to the AIN-93G formulation [24]. Corn oil was used to eliminate any possible contamination from isoflavones in soybean oil. Dry powdered daidzein (purity, >98%; Nagara Science, Gifu, Japan) and RS (60% RS, Himaize; National Starch and Chemical, Bridgewater, NJ) were added to the diet instead of sugar or cornstarch, respectively. The mice were pair-fed their respective diets for 42 days with free access to distilled water during this period. The daidzein concentration was chosen based on the results of previous studies. Regarding the results of our previous studies, the combination of 0.2% isoflavone conjugates (0.66 g of daidzein, 0.17 g of genistein, and 0.30 g of glycitein per kilogram diet as aglycone) and FOS treatment [14], or the combination of the 0.1% daidzein and dietary fiber treatment was effective on the bone loss (unpublished data). Although the dose of daidzein used in this study was relatively higher than other nutritional experiments, we chose it to examine the effect of RS on equol production according to our previous study [25,26].

Table 1 – Composition of the experimental diets [a]

Ingredient	Control [b]	Dz [c]	Dz + RS [d]	RS [e]
	g/kg diet			
Cornstarch	529.5	529.5	329.5	329.5
Casein	200	200	200	200
Sucrose	100	99	99	100
Corn oil	70	70	70	70
Cellulose	50	50	50	50
Mineral mixture [1]	35	35	35	35
Vitamin mixture [1]	10	10	10	10
L-Cystine	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5
tert-Butylhydroquinone	0.014	0.014	0.014	0.014
Daidzein	–	1.0	1.0	–
RS			200	200

[a] Prepared according to the AIN-93G formulation [24].
[b] Control diet.
[c] Daidzein-containing diet.
[d] Daidzein- and RS-containing diet.
[e] Resistant starch–containing diet.
[1] The compositions of mineral mixture and vitamin mixture were based on AIN-93G [24].

After 40 days of treatment, 48-hour urine and fecal samples were collected and stored at –80°C until assay. The mice were fasted overnight the day before the anatomical investigations. After 42 days of treatment, the mice were euthanized by exsanguination under anesthesia and weighed; and then blood was drawn and stored at –80°C until assay. The uterus was removed, and the wet weight was measured. The right tibia was also removed to measure BMD. All the procedures were undertaken in accordance with the National Institute of Health and Nutrition Guidelines for the Care and Use of Laboratory Animals.

2.2. Radiographic analysis of tibia

Bone mineral density of the tibia was measured by dual-energy x-ray absorptiometry (Model DCS-600EX-R; Aloka,

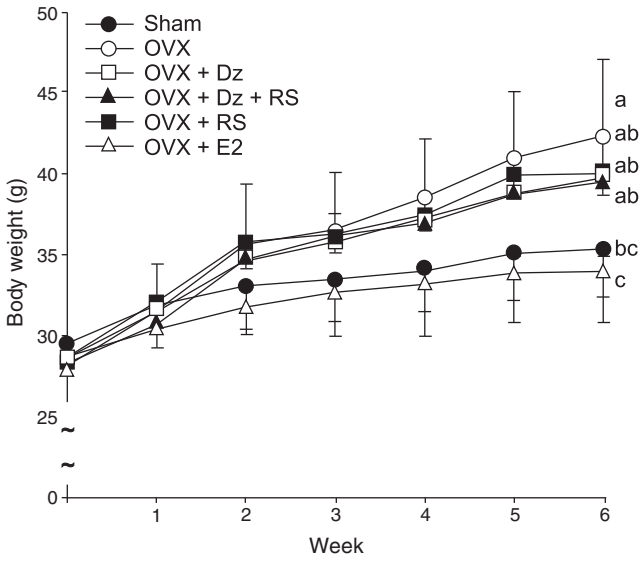

Fig. 1 – Body weight of Sham mice and OVX mice fed a control diet, a Dz-containing diet (OVX + Dz), a combination of Dz and RS diet (OVX + Dz + RS), or a RS-containing diet (OVX + RS), or receiving subcutaneous administration of E2 (0.03 μg/d) (OVX + E2) for 42 days. Values are expressed as means ± SD (n = 6-8). Body weights were analyzed using 1-factor ANOVA. Differences between treatment groups were assessed by Tukey test. Means with different letters differ significantly; $P < .05$.

Tokyo, Japan). The BMD was calculated using the bone mineral content of the measured area. The bone mineral content of the mouse tibia correlated closely with its ash weight ($r = 0.978$). The scanned area of the mouse tibia was divided into 3 equal parts: the proximal tibia, the midshaft, and the distal tibia.

2.3. *Time-resolved fluoroimmunoassay for plasma and urinary daidzein and equol*

Plasma and urinary daidzein and equol were analyzed by the time-resolved fluoroimmunoassay methods of Wang et al [27] and Brouwers et al [28], respectively. Urine and plasma were hydrolyzed by glucuronidase and sulfatase, and the plasma was extracted using diethyl ether. Plasma and urinary daidzein and equol concentrations were determined by fluorescence using a DELFIA Victor 1420 multilabel counter (PerkinElmer, Wellesley, MA). The final results = concentration (read) × 1/recovery × dilution factor (nanomoles per liter). Urine creatinine concentration was determined by enzymatic method at SRL (Tokyo, Japan). The amounts of 24-hour urinary excretion of daidzein and equol were calculated from those in 48-hour urine collection.

2.4. *DNA extraction from feces*

DNA was extracted from the fecal samples according to the method used by Nagashima et al [29, 30] with modifications. The fecal samples were suspended in a solution containing 100 mmol/L Tris-HCl (pH 9.0) and 40 mmol/L EDTA; after washing 3 times with sterile distilled water, the feces were then beaten using a FastPrep FP100A Instrument (MP Biomedicals, Santa Ana, CA). DNA was extracted from suspension using a GC series Genomic DNA whole blood kit and then purified using a Magtration 12GC system (Precision System Science, Chiba, Japan).

2.5. *Polymerase chain reaction conditions and terminal restriction fragment length polymorphism analysis*

The amplification of the fecal 16sDNA, restriction enzyme digestion, size fractionation of terminal restriction fragment (T-RFs), and terminal restriction fragment length polymorphism (T-RFLP) data analyses were according to the method

Table 2 – Concentrations of plasma and urinary daidzein and equol, and the ratio of plasma and urinary equol to daidzein concentrations in mice

	Sham*	OVX†	Dz‡	Dz + RS§	RS¶	E2#
Plasma						
Daidzein (nmol/L)	ND[c]	ND[c]	1238 ± 1329[a]	279 ± 149[b]	ND[c]	ND[c]
Equol (nmol/L)	ND[c]	ND[c]	5081 ± 1935[a]	1784 ± 765[b]	ND[c]	ND[c]
Equol to daidzein ratio	ND[c]	ND[c]	6.54 ± 3.38[a]	7.61 ± 4.65[a]	ND[c]	ND[c]
Urine						
Daidzein (μmol/24 h)	ND[c]	ND[c]	1.54 ± 0.80[a]	1.67 ± 0.20[a]	ND[c]	ND[c]
Equol (μ/24 h)	ND[c]	ND[c]	1.04 ± 0.49[b]	1.90 ± 0.70[a]	ND[c]	ND[c]
Equol to daidzein ratio	ND[c]	ND[c]	0.689 ± 0.197[b]	1.151 ± 0.426[a]	ND[c]	ND[c]

Values are expressed as means ± SD (n = 6-8). Concentrations of plasma and urinary daidzein and equol, and ratio of plasma and urinary equol to daidzein concentrations were analyzed using 1-factor ANOVA. Differences between the treatment groups were assessed by Tukey test. Means with different letters differ significantly; $P < .05$. ND indicates not detected.

* Sham-operated (Sham) mice fed a control diet.
† Ovariectomized mice fed a control diet.
‡ Ovariectomized mice fed a Dz-containing diet.
§ Ovariectomized mice fed a combination of Dz and RS diet.
¶ Ovariectomized mice fed an RS-containing diet.
\# Ovariectomized mice administered E2 (0.03 μg/d) subcutaneously.

used by Nagashima et al [30] with modifications. Briefly, polymerase chain reaction was performed using the total fecal DNA and the primers of 5′carboxy-fluorescein–labeled 516f and 1510r. The resulting 16S rDNA amplicons were treated for 3 hours at 55°C with 10 U of *Bs*/I (5′-CCNNNNN|NNGG-3′) (New England BioLabs, Ipswich, MA). The fluorescent-labeled T-RFs produced by digestion with *Bs*/I were analyzed by electrophoresis on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) in GeneScan mode (the injection time was 30 seconds; the run time was 40 minutes).

2.6. Clone library analysis

Clone library analysis was according to the method used by Nagashima et al [30] with modifications. The length and peak areas of the T-RFs were determined, and a phylogenetic tree was constructed using Gene Maths software (Applied Maths, Sint-Martens-Latem, Belgium). The fingerprint profiles generated by T-RFLP were compared using the Pearson correlation coefficient. The Dice similarity matrix was used to construct a dendrogram of the T-RFLP fingerprint profiles.

2.7. Statistics

The data were expressed as mean ± SD. The significance of differences in BMDs was determined by 1-factor analysis of covariance and Fisher protected least significant difference test (SPSS version 11.0; SPSS, Chicago, IL). Body weight was used as a covariate in the analysis of BMD to adjust for possible confounding effects. The remaining data were analyzed using analysis of variance (ANOVA). Differences between the treatment groups were assessed by Tukey test. Differences were considered significant at $P < .05$. The relationship between the ratio of urinary equol to daidzein concentration and whole tibia BMD was evaluated by linear regression analysis.

3. Results

3.1. Body and tissue weights

The 6 groups of mice in the study had similar initial mean body weight (Fig. 1.). After 42 days of treatment, the OVX group had significantly higher body weight compared with the Sham

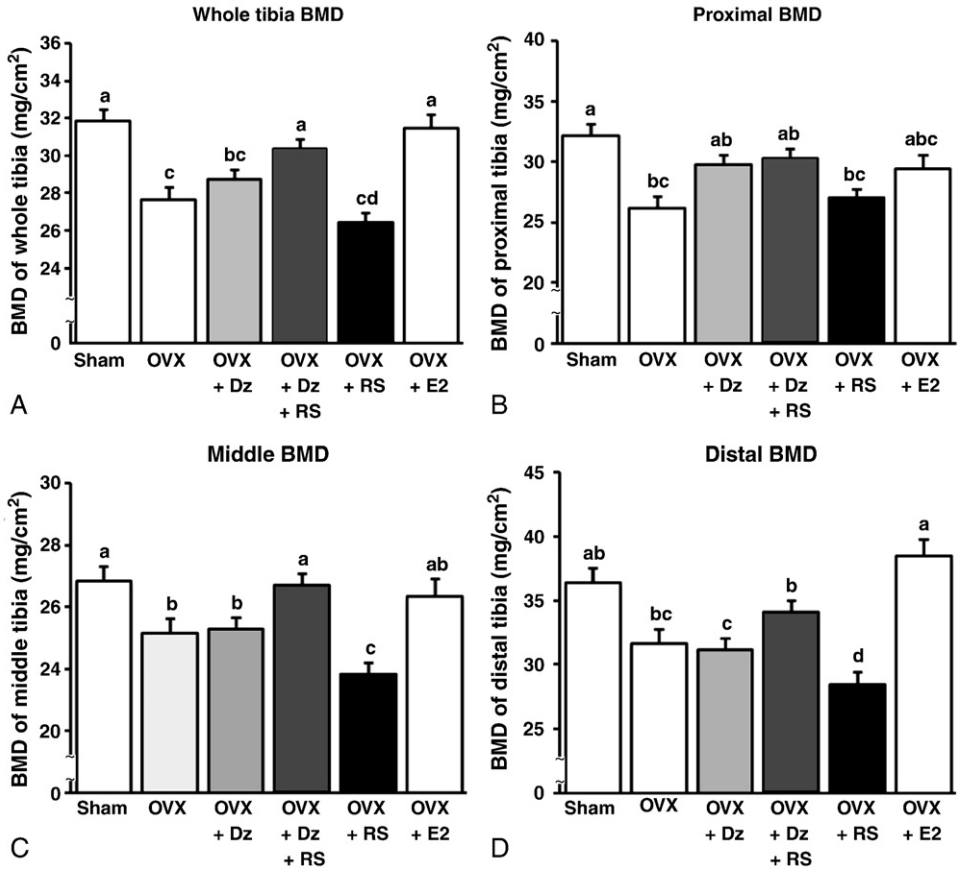

Fig. 2 – Bone mineral density of the tibia was obtained from Sham mice and OVX mice fed a control diet, a Dz-containing diet (OVX + Dz), a combination of Dz and RS diet (OVX + Dz + RS), or a RS-containing diet (OVX + RS), or receiving subcutaneous administration of E2 (0.03 μg/d) (OVX + E2) for 42 days. Bone mineral density was measured by dual-energy x-ray absorptiometry analysis. A, Whole tibia BMD. B, Proximal BMD. C, Middle BMD. D, Distal BMD. Values are expressed as means ± SD (n = 6-8). Significant differences in BMD were determined by 1-factor analysis of covariance and Fisher protected least significant difference test. Body weight was used as a covariate in the analysis of BMD to adjust for a possible confounding effect. Means with different letters differ significantly; $P < .05$.

and OVX + E2 groups. There was no significant difference in body weight between the OVX, OVX + Dz, OVX + Dz + RS, and OVX + RS groups at the end of the treatment.

The relative uterine weights (per 100 g body weight) of the mice in the Sham, OVX, OVX + Dz, OVX + Dz + RS, OVX + RS, and OVX + E2 groups were 0.483 ± 0.185, 0.072 ± 0.024, 0.150 ± 0.034, 0.146 ± 0.034, 0.076 ± 0.014, and 0.583 ± 0.177 g, respectively. Uterine weights were lower in the OVX groups than in the Sham group ($P < .05$). 17 β-Estradiol was shown to inhibit uterine atrophy induced by OVX ($P < .05$). In contrast, treatment with Dz, Dz + RS, or RS did not affect uterine weight in the OVX mice.

3.2. *Concentrations of daidzein and equol in plasma and urine*

The concentrations of daidzein and equol in plasma and urine in mice treated with daidzein were significantly higher than those in untreated mice (Table 2). The plasma concentrations of daidzein and equol were higher in the OVX + Dz group than in the OVX + Dz + RS group (Table 2). However, no significant difference was observed in the ratio of equol to daidzein concentration in the plasma between the OVX + Dz and OVX + Dz+ RS groups. No difference was observed in urinary daidzein concentration between the OVX + Dz and OVX + Dz+ RS groups. Urinary equol concentration and the ratio of urinary equol to daidzein concentration were significantly higher in the OVX + Dz + RS group compared with those in the OVX + Dz group (Table 2).

3.3. *BMD of the tibia*

The BMDs of the whole, proximal, middle, and distal regions of the tibia in OVX mice were significantly lower than those in the Sham group ($P < .05$) (Fig. 2). Daidzein treatment inhibited bone loss in the whole tibia and proximal region of the tibia, but did not affect BMD in the middle and distal regions of the tibia. Treatment with RS alone did not affect BMD in the tibia of OVX mice. The BMDs of the whole, proximal, and middle regions of the tibia in the OVX + Dz + RS group were significantly higher than those in the OVX group. The BMDs of the whole, middle, and distal tibia were also higher in the OVX + Dz +RS group than in the OVX + Dz group (Fig. 2A, C, and D). Treatment with E2 maintained the BMD in the 4 regions of the tibia in OVX mice (Fig. 2). There was no significant difference in BMD in the whole, proximal, and distal femur between the OVX + Dz + RS group and the OVX + Dz group (data not shown). However, the results in femur trended to be the same as those in the tibia. Especially, distal femur BMD in the OVX + Dz + RS group tended to be higher compared with that in the OVX + Dz group ($P = .161$).

3.4. *Linear regression analysis of the ratio of urinary equol to daidzein concentration and BMD of the whole tibia*

Linear regression analysis demonstrated a significant positive correlation ($r = 0.647$, $P < .001$) between the ratio of urinary equol to daidzein concentration and BMD of the whole tibia.

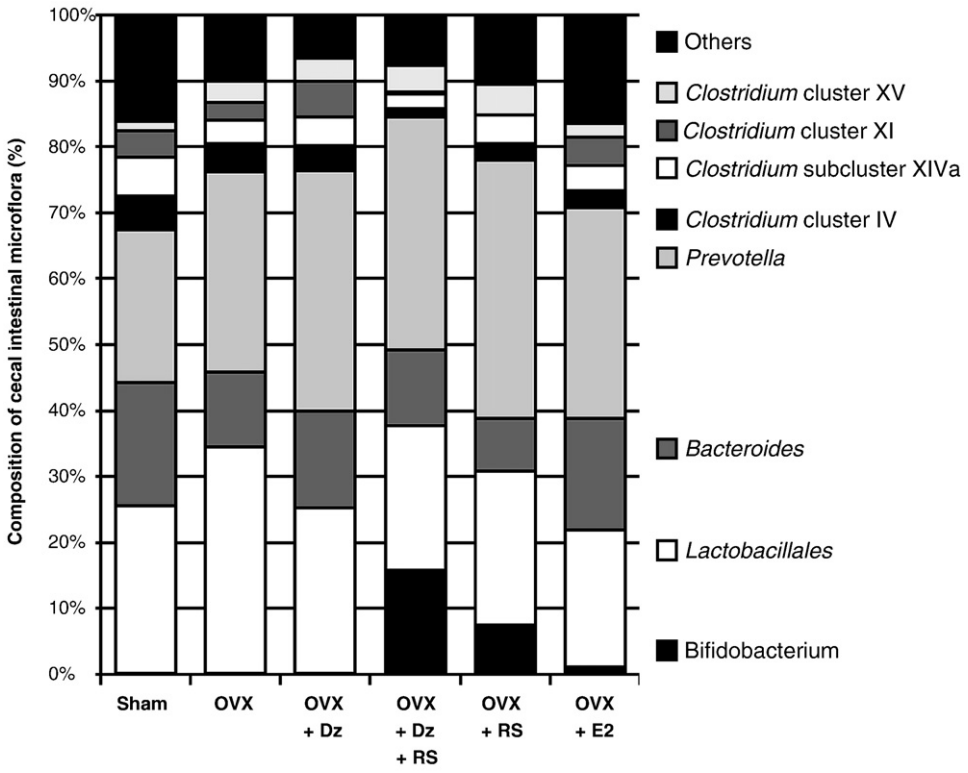

Fig. 3 – Composition of cecal intestinal microflora in Sham mice and OVX mice fed either a control diet, a Dz-containing diet (OVX + Dz), a combination of Dz and RS diet (OVX + Dz + RS), or a RS-containing diet (OVX + RS), or receiving subcutaneous administration of E2 (0.03 μg/d) (OVX + E2) for 42 days. Values are expressed as means ± SD (n = 6-8). The composition of fecal intestinal microflora was analyzed using 1-factor ANOVA. Differences between treatment groups were assessed by Tukey test ($P < .05$).

3.5. *Cecal microflora*

It has been confirmed that human intestinal microorganisms predominantly consisted of the members approximately 9 phylogenetic bacterial groups and that these bacterial groups can be distinguished by the T-RFLP system developed by Nagashima et al [29,30]. As shown in Fig. 3, cecal flora changed in the groups fed RS. The occupation ratios of *Bifidobacterium* spp in the OVX + Dz + RS and OVX + RS groups were 15.7% ± 5.08% and 7.44% ± 2.70%, respectively. These ratios were significantly higher than those in the Sham, OVX, OVX+ Dz, and OVX + E2 groups, which were 0.09% ± 0.17%, 0.13% ± 0.26%, 0.23% ± 0.46%, and 1.16% ± 0.67%, respectively. The occupation ratio of *Bifidobacterium* spp in the OVX + Dz + RS group was significantly higher than that in the OVX + RS group.

The bacteria belonging to *Clostridium* cluster XI was not detected in the OVX + RS group, whereas the occupation ratio in the OVX + Dz group was 5.42% ± 2.60%. The ratio of *Clostridium* cluster XI in the OVX + Dz +RS group was 0.27% ± 0.54%, which was slightly lower than that in the OVX + Dz group. There were no statistically significant differences in the occupation ratios of bacteria belonging to the *Clostridium* cluster XI between the Sham, OVX, OVX + Dz + RS, OVX + RS, and OVX + E2 groups (4.00% ± 2.36%, 2.77% ± 0.33%, 0.27% ± 0.54%, 0.00% ± 0%, and 4.32% ± 4.65%, respectively). The occupation ratios of bacteria belonging to the *Clostridium* cluster XV in the OVX + Dz + RS and OVX + RS groups were significantly higher than those in the Sham and OVX + E2 groups and tended to be higher than those in the OVX and OVX + Dz groups.

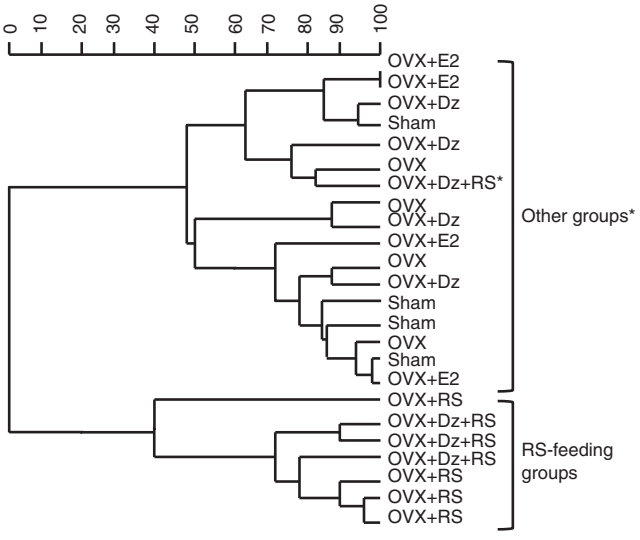

Fig. 4 – Cluster analysis of intestinal microflora based on T-RFLP analysis. The intestinal microflora in the Sham mice and OVX mice fed a control diet, a Dz-containing diet (OVX + Dz), a combination of Dz and RS diet (OVX + Dz + RS), or a RS-containing diet (OVX + RS), or receiving subcutaneous administration of E2 (0.03 μg/d) (OVX + E2) for 42 days. The T-RFLP was compared using Pearson correlation coefficient. The Dice similarity matrix was used to construct a dendrogram of the T-RFLP fingerprint profiles. *Except for a mouse in the OVX + Dz + RS group.

We performed a cluster analysis of the intestinal microflora based on T-RFLP analysis (Fig. 4.). The dendrogram shows 2 large clusters: one consisting of mice fed RS (ie, OVX + Dz + RS and OVX + RS groups) and the other consisting of mice in the other groups.

4. Discussion

In the present study, the OVX mice whose diets were supplemented with daidzein or a combination of daidzein and RS were shown to maintain their bone mass compared with the BMDs of the tibia of OVX mice that were significantly lower than those in Sham mice. In particular, mice fed Dz + RS maintained their BMD more effectively than mice fed Dz alone (Fig. 2). Although it is known that RS improves cecal calcium and magnesium absorption in rats [31,32], a diet supplemented with RS alone did not maintain BMD in OVX mice in our study. These results are similar to those of a previous report by Ohta et al and Mathey et al [14,33] in which diets supplemented with FOS, soy isoflavones conjugates, or a combination of the 2 compounds efficiently prevented the decrease in BMD in OVX mice.

Several studies have shown that diets supplemented with soy isoflavones and prebiotics increase equol production [14], thereby inhibiting bone loss induced by estrogen deficiency in animals [14,34,35]. However, no studies have examined the effects of the combination of daidzein and RS on intestinal microflora and equol production. The present study demonstrated that diets supplemented with daidzein and RS had the ability to increase equol production in the intestines by modifying the composition of intestinal microflora and that those were more efficient than either alone in inhibiting bone loss in OVX mice.

Equol, a metabolite of daidzein, is formed by intestinal microflora in humans and animals. Diet can substantially modify certain bacterial populations [7,12] as well as intestinal microflora. Furthermore, it is known that diet may influence metabolism of isoflavones and production of equol [13]. Several investigators have analyzed whether consumption of prebiotics or probiotics enhances equol production in animals and humans [14-17].

In the present study, plasma daidzein and equol concentrations in the OVX + Dz + RS group were lower than those in the OVX + Dz group. The reason for this discrepancy is that the mice were fasted the night before being killed. Therefore, daidzein and equol may have reached peak concentrations in plasma and may have been excreted in the urine. Poulsen et al [36] reported that 49.1% of daidzein and its metabolites, including equol, were excreted in the urine and feces of rats within 24 hours. Furthermore, RS not only increased the amount of daidzein absorption, but also may have accelerated the rate of the metabolism of daidzein to equol and the excretion of those into the urine, which could be a potential explanation for the finding that the amount of urinary daidzein plus equol in the OVX + Dz + RS group was higher than that in the OVX + Dz group. Further studies, including kinetics in blood and urinary excretion, are required to confirm the effects of RS on daidzein metabolism.

On the other hand, Setchell and Cole [37] reported that expressing the product-precursor relationship as the ratio of

equol to daidzein in urine provides a more reliable indicator of the amount of daidzein converted into equol. Our results showed that urinary equol concentration and the ratio of urinary equol to daidzein concentration were significantly higher in the OVX + Dz + RS group than in the OVX + Dz group. Thus, a diet supplemented with a combination of daidzein and RS efficiently increases the urinary equol concentration. Whereas these results suggest that RS enhances equol production in rodents, Larkin et al [17,38] reported that RS supplementation with soy consumption had no effect on the equol-producing capacity in older male and postmenopausal female subjects. Originally, rodents in particular have very high plasma and urinary equol concentrations. In this manner, they can produce more equol with RS consumption. However, for humans, it might not be easy to enhance equol production, especially for nonproducers of equol. Further studies are required to confirm whether RS enhances equol production in humans.

Linear regression analysis demonstrated a significant, positive correlation between the urine equol to daidzein ratio and BMD of the whole tibia. These results suggest that a diet supplemented with daidzein and RS prevents decrease in BMD in OVX mice, partially by promoting equol production and not by enhancing mineral absorption with RS because the RS group did not inhibit bone loss due to OVX. This is the first evidence indicating that stimulation of equol production by RS may induce bone-protecting effects.

Regarding studies in humans, Wu et al [10] showed in Japanese women treated for 1 year with 75 mg/d of isoflavone conjugate that subjects who produced equol had a significantly lower percentage change in bone loss in the total hip BMD compared with nonproducers. Similarly, after a 2-year dietary intervention with isoflavones, Setchell et al [8] showed that BMD of the lumbar spine increased by 2.4% only in subjects who produced equol. In fact, studies that examined the direct effect of equol on maintenance of bone in animals have shown a direct correlation between equol and BMD [9].

On the other hand, administration of E2 inhibited uterine atrophy induced by OVX. In contrast, a diet supplemented with daidzein and RS did not affect uterine weight in OVX mice, despite inhibiting bone loss to the same degree as E2 administration.

To investigate the effect of RS on intestinal microflora, we performed a T-RFLP analysis. Although the specific equol-producing bacterial species in the intestine are not completely understood, *Lactobacillus* spp and *Bifidobacterium* spp have been identified as playing a role in the metabolism of daidzein to equol [7,11,22]. Furthermore, RS is known to increase production of short-chain fatty acids and intestinal fermentation [18]. Our results showed that the occupation ratio of *Bifidobacterium* spp and the ratio of urinary equol to daidzein concentration in the OVX + Dz +RS group were significantly higher than those in the other groups. It has been reported that metabolic activity of fecal equol production in mice may be affected by certain *Bifidobacterium* spp [39]. Moreover, in our study, the composition of cecal flora differed between the groups fed RS and the other groups. Dietary RS may affect equol production in the intestines by modifying the intestinal microflora population. Although a few equol-producing bacteria have been discovered, for example, *Lactococcus garvieae* spp, which have the ability to metabolize S-equol from daidzein-rich soy germ [40], the relevance of dietary-induced changes in gastrointestinal microflora activity to isoflavone bioavailability is poorly understood and requires further investigation.

In summary, the present study showed that a diet supplemented with daidzein and RS significantly altered the intestinal microflora and increased equol production. This effect was greater than that of daidzein alone for preventing bone loss in OVX mice. These results suggest that studies should be done to determine if intake of a combination of isoflavones with RS is useful for maintaining bone health in postmenopausal women. Further studies, including the influence of intestinal microflora, are required to confirm the overall effect of isoflavones.

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