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ORIGINAL ARTICLE

Modulation of soy isoflavones bioavailability and subsequent effects on bone health in ovariectomized rats: the case for equol

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Abstract
Introduction Soy products are of particular interest because of their potential health benefits in a range of hormonal conditions, such as osteoporosis, due to their high content in phytoestrogens. Because equol, the main metabolite from soy isoflavones, is thought to be powerful, the present study was designated to evaluate the bone-sparing effects of equol by either providing the molecule through the diet or by eliciting its endogenous production by modulating intestinal microflora by short-chain fructooligosaccharides (sc-FOS) or live microbial (*Lactobacillus casei*) together with daidzein, its precursor.
Methods A comparison with daidzein and genistein was also performed. Rats (3 months old) were ovariectomised (OVX) or sham-operated (SH). Ovariectomised rats were randomly assigned to six experimental diets for 3 months: a control diet (OVX), the control diet supplemented with either genistein (G), or daidzein (D), or equol (E) at the level of 10 μg/g body weight/d. The remaining OVX rats were given daidzein at the dose of 10 μg/g body weight/d, simultaneously with short-chain FOS (Actilight®, Beghin-Meiji) (D+FOS) or *Lactobacillus casei* (Actimel, Danone) (D+L). The SH rats were given the same control diet as OVX.
Results Genistein, daidzein or equol exhibited a bone sparing effect. Indeed, total femoral bone mineral density (BMD) was significantly enhanced (compared to that of OVX rats), as was the metaphyseal compartment. Bone strength was improved by E consumption, but not by genistein or daidzein given alone. As far as the FOS diet is concerned, the addition of prebiotics significantly raised efficiency of the daidzein protective effect on both femoral BMD and mechanical properties. The effects of lactobacillus were similar, except that the increase in metaphyseal-BMD was not significant.
Conclusion In conclusion, long-term equol consumption, like genistein and daidzein, in the ovariectomized rat, provides bone sparing effects. Adding indigestible sugars, such as FOS or live microbial as *L. casei*, in the diet significantly improves daidzein protective effects on the skeleton.

Keywords Bone prevention · Equol · Isoflavones · Pre- or probiotics · Rat

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Introduction

Osteoporosis (characterized by low bone mass, micro-architectural deterioration of bone, enhanced bone fragility,

and increase fracture risk [1]) has become a major public health concern. Although suboptimal skeletal development and age-related bone loss may be contributing factors, a hormone-dependent increase in bone resorption and accelerated loss of bone mass in the first 5 or 10 years after menopause appears to be the main pathogenetic factor. For this reason, for more than a half a century (oestrogen preparations came into use in the 1930s), various endocrine agents have been prescribed to replace the cyclical ovarian hormone production lost at the time of menopause and were also recommended for osteoporosis prevention. However such a therapy is currently a source of considerable controversy and debate because of potential side effects [2–6]. Thus, increased research into alternatives and natural strategies for menopausal oestrogen deficiency is necessary for prevention and treatment of postmenopausal conditions.

In this light, soy products are of particular interest because of their potential health benefits in a range of hormonal conditions, such as atherosclerosis [7, 8], cancers [9] and postmenopausal osteoporosis [10–12], probably due to their high content in phytoestrogens. Actually, those molecules come in several forms, a major subclass being the isoflavones (IF), which are mainly found as glycosides in soybeans and in unfermented soy food [13]. When ingested, daidzin (7,4′-dihydroxyisoflavone 7-glucoside) and genistin (4′, 5, 7-trihydroxyisoflavone 7 glucoside) are hydrolyzed by β-glucosidases in the jejunum, releasing the principal bioactive aglycones, daidzein (4′, 7-dihydroxyisoflavone) and genistein (4′, 5, 7-trihydroxyisoflavone) [14–16], which are either absorbed intact by the intestine to undergo enterohepatic recycling or further metabolized by intestinal microflora such as *Lactobacilli*, *Bacteroides and Bifidobacteria*, presumably in the colon [17], into several other products, including respectively equol, O-desmethylangiolensin and p-ethyl phenol, [18, 19]. Equol is not produced in all healthy humans. However, according to Setchell's theory [20], the clinical effectiveness of soy protein may be a function of the ability to biotransform soy isoflavones to the more potent equol. However, the potential bone-sparing effect of equol has never been assessed, even though IF have been widely examined in the ovariectomised rat and mice, animal models of postmenopausal bone loss [21–27]. Actually, among those molecules, daidzein has been shown to be more efficient than genistein in preventing castration-induced bone loss in the rat [28].

Besides, endogenous equol production can be induced by modulating intestinal microflora. Indeed, Uheara et al. (2002) reported that fructooligosaccharides (FOS), a mixture of indigestible and fermentable sugars [29] improves the bioavailability of daidzein in rats given IF conjugates. Moreover, the combination of dietary FOS and IF has been shown to be more efficient than either alone in the prevention of bone loss in ovariectomised mice and rat, and this was correlated with increased plasma equol levels [30, 31].

The purpose of the present study was thus to investigate in the ovariectomized rat, an animal model for postmenopausal osteoporosis, the bone-sparing effects of equol by either providing the molecule through the diet or by eliciting its endogenous production by modulating intestinal microflora by short-chain FOS (sc-FOS) or live microbial (lactic acid bacteria: *Lactobacillus casei*). A comparison with daidzein and genistein, the two main soy isoflavones, was also performed.

Materials and methods

Animals and diets

The study was conducted in accordance with current legislation on animal experiments in France. Seventy 90-day old female Wistar rats from INRA Clermont-Ferrand/Theix (St Genès-Champanelle, France) were individually housed at 21°C, on a 12 h–12 h light-dark cycles, in metabolic cages allowing separation and collection of 24 hours urine. Animals were fed a soybean-protein-free powdered semi-purified diet (Table 1) from INRA Jouy

Table 1 Composition of the soy protein-free powdered semi-purified diet

Ingredient[1]	g/kg
Casein	200
Cornstarch	660
Cellulose fiber	50
Peanut oil	25
Rapeseed oil	25
Vitamin mixture[2]	10
Mineral mixture[3]	25
DL-Methionine	3
Choline bitartrate	2

[1] Casein (Union des caséineries, Surgères, France), cornstarch (Cerestar, Saint-Maur, France), cellulose (Durieux, Marne la Vallée, France), oil (Bailly, Aulnay sous Bois, France), vitamin mixture (Roche, Neuilly sur Seine, France), mineral mixture (Prolabo, Fontenay sous Bois, France) and DL-methionine (Jerafrance, Jeufosse, France).

[2] Expressed in mg/kg of mixture : retinyl palmitate (250 IU/mg), 2000; cholecalciferol (400 UI/mg), 312; DL-α- tocopherol acetate (0.25 IU/mg), 20,000; menadione, 100; thiamine HCL, 1000; riboflavin, 1000; nicotinic acid, 4500; D-calcium pantothenate, 3000; pyridoxine HCL, 1000; inositol, 5000; D-biotin, 20; folic acid, 200; cyanocobalamin, 1.35; ascorbic acid, 10,000; p-aminobenzoic acid, 5000; choline chlorhyascorbic acid, 10,000; choline chlorhydrate, 75,000; and sucrose, finely powdered, 871.9 g.

[3] Expressed in g/kg of mixture : $CaHPO_4 \cdot 2H_2O$, 308; K_2HPO_4, 194; $CaCO_3$, 146; $MgSO_4 \cdot 7H_2O$, 109; NaCl, 168; MgO, 24.3; $FeSO_4 \cdot 7H_2O$, 20.9; $ZnSO_4 \cdot H_2O$, 12.1; $MnSO_4 \cdot H_2O$, 12.1; $CuSO_4 \cdot 5H_2O$, 0.0005; $CoCO_3$, 0.0005; Na_2SeO_3, 0.0005.

and Josas (France) for 15 days (an adaptation period). The dietary calcium intake was on the order of 0.3%, a physiological level. At 105 day of age (day 0 of the experiment), animals were intraperitonally anaesthetised with chloral hydrate (Fluka Chemie AG, Buchs, Switzerland; 80 g/l in saline solution (9 g NaCl/l); 0.4 ml/100 g body weight). Ten rats designated as controls were sham-operated (SH), while the 60 others were ovariectomized (OVX). Right after surgery, the OVX animals were randomly allocated to six diets (ten animals in each group) as follows: control diet (OVX); control diet supplemented with either genistein (G), or daidzein (D), or equol (E) at the level of 10 μg/g body weight/d. The remaining 20 OVX rats were given daidzein at the dose of 10 μg/g body weight/d, simultaneously with short-chain FOS (sc-FOS, 95%±2% FOS and 5%±2% glucose, fructose and saccharose; Actilight®, Beghin-Meiji, Neuilly-sur-Seine, France) (n=10, D+FOS) or *Lactobacillus casei* (Actimel, Danone, Paris, France) (n=10, D+L). SH rats were fed the soy protein-free powdered semi-purified diet without any additional compound.

Diets were prepared by mixing the powdered genistein (Pharmaceutical Research Institute, 8 Rydygiera Street, 01-793, Warsaw, Poland), daidzein (Division of Pharmacognosy, University of Athens, Panepistimioupolis, Zografou, 15771 Athens, Greece) (without or with FOS or *L. casei*) and equol (Unité Micronutriments Reproduction, Santé, ENITA Bordeaux, France) with the soy protein-free powdered semi-purified diet. During the experiment, which lasted 3 months, sc-FOS were given in the diet at dose of 2.5% during the first week at 5%, during the 2nd week and thereafter at 7.5%, followed by a 1-month washout period. Then the same pattern of supplementation was followed for the last month. Probiotics (*L. casei*) were supplied during the whole experiment in the D+L group.

To prevent castration-induced hyperphagia, the daily amount of diet distributed to each rat was adjusted to the mean level consumed by SH rats on the previous day. Thus, food intake was constant during the entire experimental period and similar in all groups. Animals had free access to water and were weighed weekly to adjust IF doses to body weight.

Before killing (day 90), the 24-hour urines were harvested to assess deoxypyridinoline (DPD), a marker of bone resorption [32]. At necropsy, blood samples were collected by cardiac puncture into ice-cooled heparinized plastic tubes containing 200 peptidase inhibitor units of aprotinin (Iniprol, Choay, Paris, France) per ml blood, and centrifuged immediately (3,500 g for 5 min at 4°C). Plasma were then frozen at −20°C until measurements of osteocalcin, a marker for osteoblastic activity [33]. Uterine horns were removed and immediately weighed. Left and right femurs were cleaned from adjacent tissues and collected for mechanical testing and bone mineral density (BMD) measurement, respectively.

Analysis

Plasma phytoestrogen concentrations Plasma genistein, daidzein and equol concentrations were measured by ELISA [34]. The sensitivity of the method is 35, 40 and 10 nmoles for genistein, daidzein and equol, respectively. The intra and inter assay variations are 4.8 and 13.1% for genistein, 5 and 12.8% for daidzein, then 5% and 13.6%, for equol, respectively.

Bone mineral density (BMD) Bone mineral density was determined by DEXA using a Hologic QDR 4500A x-ray densitometer (Hologic, Massy, France) on the left femur (BMD), and on two subregions, one corresponding to the distal metaphyseal zone (M-BMD) which is rich in cancellous bone, and the other to the diaphyseal zone (D-BMD), mainly cortical bone, as well [35]. Results are given in g/cm^2.

Femoral mechanical testing After collection, the length of the right femur and the mean diameter of the femoral diaphysis were measured using a caliper. Because of the irregular shape of the diaphysis, the used diameter in the calculation was the mean of the greatest and the smallest diaphysis diameters. Mechanical resistance was assess by a three-point bending test. Each bone was secured on the two lower supports (diameter, 4 mm; separation, 20 mm) of the anvil of a Universal Testing Machine (Instron 4501; Instron, Canton, MA, USA). An upper cross head roller (diameter, 6 mm) was applied in front of the middle of the bone and advanced at 0.5 mm/min until rupture was automatically determined by the apparatus. Failure load (N) at rupture was recorded.

Image analysis To measure cancellous bone area in the distal femur metaphyseal zone, frontal sections were cut with a precision saw (Metaserv 2000 polisher, Buehler), ground to 10-μm sections (Leica Microsystems Nussloch GmbH, Germany), and stained with Von Kossa's reagent ($AgNO_3$ (5%), Sigma). The underlying zone to growth plate was then analyzed with an automated microscope image-analysis system, as previously described [36].

Marker for osteoblastic activity Plasma osteocalcin concentration (OC) was measured by homologous radioimmunoassay (RIA) using rat ^{125}I-labelled OC, goat anti-rat OC antibody, and donkey anti-goat second antibody (Biochemical Technologies Kit; Biochemical Technologies, Stoughton, MA, USA). The sensitivity is 0.01 nmol/l. The intra- and interassay variations are 6.8% and 8.9%, respectively.

Marker of bone resorption Deoxypyridinoline (DPD) in urine was assessed by a radioimmunoenzymatic assay using rat monoclonal anti-DPD antibody coated to the inner surface of a polystyrene tube, and ^{125}I-labelled DPD (Pyrilinks-D RIA kit, Metra Biosystems Inc., Mountain view, CA, USA). The intra- and interassay variations are 4% and 6%, respectively. Results are expressed as nmoles DPD per mmoles of creatinine [32]. The creatinine assay (Kit Bio MERIEUX SA, Marcy-l'Etoile, France) is based on a modified Jaffe's method, in which picric acid forms a coloured solution in the presence of creatinine [37].

Statistical analysis

Results are expressed as means ± SEM. All data have been analysed using the Graphpad Instat software (Microsoft, San Diego, CA, USA). ANOVA was first performed to test for any significant differences among groups. When significant ($P<0.05$), the Student-Newman-Keul's multiple comparisons test was applied to determine the specific differences between means. Parametric ANOVA was performed when data were sampled from populations with equal variance. If not, nonparametric methods were selected. Then, a Kruskall-Wallis test was first carried out. If it indicated a significant difference among groups ($P<0.05$), the Mann-Whitney U test was used to determine specific differences. The level of significance was set at $P<0.05$ for all statistical tests.

Results

Body weight

During the experimental period (day 1 to day 90), body weight (g) consistently increased in each group (Fig. 1).

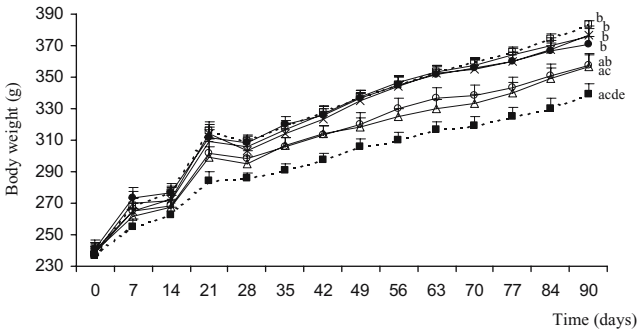

Fig. 1 Body weight changes in sham-operated (SH, ■) and ovariectomized rats (OVX, □) supplemented with genistein (G, ×), daidzein (D, ○), daidzein with FOS (D+FOS, ●), daidzein with lactobacillus (D+L, +) or equol (E, Δ). Values are means (for 10 rats) with their standard errors represented by vertical bars. [a]$p<0.05$ *vs.* OVX; [b]$p<0.001$ *vs.* SH; [c]$p<0.05$ *vs.* G, [d]$p<0.05$ *vs.* D+FOS; [e]$p<0.001$ *vs.* D+L

Table 2 Uterine weight measured in sham-operated rats (SH) and ovariectomized rats (OVX) supplemented with genistein (OVX G), daidzein (OVX D), daidzein+Fructooligosaccharides (OVX D+FOS), daidzein+Lactobacillus (OVX D+L) and equol (OVX E)

Experimental group	Number of animals	Uterine weight (g/Kg body weight)	
		Mean	SE
SH	10	0.62[b]	0.04
OVX	10	0.09[a]	0.003
OVX G	10	0.10[a]	0.01
OVX D	10	0.10[a]	0.01
OVX D+FOS	10	0.10[a]	0.005
OVX D+L	10	0.10[a]	0.004
OVX E	10	0.11[ab]	0.01

[a] $P<0.0001$ mean values significantly different from SH,
[b] $P<0.01$ mean values significantly different from OVX.
Results are expressed as mean values ± standard error to the mean (SE)

However, although consuming similar amounts of food, because of pair feeding, body weight was significantly greater in OVX than in SH by the third week of experiment and this trend was still evident on day 90 (OVX 383±3 *vs.* SH 339±6; $P<0.001$). This weight gain after ovariectomy was still evident in animals receiving the diet supplemented with genistein or daidzein given together with FOS or *L. casei.* Animals under daidzein or equol had a similar pattern than SH.

Uterine weight

As indicated in Table 2, a marked atrophy of uterine horns was induced by castration (**SH** 0.62±0.04, **OVX** 0.09± 0.003 g uterus/kg body weight; $P<0.0001$). IF consumption did not induce a significant change in the uterine weight, except for equol (0.11±0.01 $p<0.01$ *vs.* OVX).

Table 3 Plasma genistein, daidzein, equol and daidzein + equol concentrations (ng/ml) measured in sham-operated rats (SH) and ovariectomized rats (OVX) supplmented with genistein (OVX G), daidzein (OVX D), daidzein+fructooligosaccharides (OVX D+FOS), daidzein+Lactobacillus (OVX D+L) and equol (OVX E) on day 90

Experimental group	Genistein	Daidzein	Equol	Daidzein+ equol
SH	nd	nd	nd	
OVX	nd	nd	nd	
OVX G	109±24.08	nd	nd	
OVX D	nd	140.7±53.6	677.8±103.5	454.7±86.6
OVX D+FOS	nd	229.1±60.1	336±86.4	300.4±53.5
OVX D+L	nd	190.9±38.4	492.8±72.3	341.8±54.0
OVX E	nd	nd	2041±183.7	

Results are expressed as mean values ± standard error to the mean (SE).

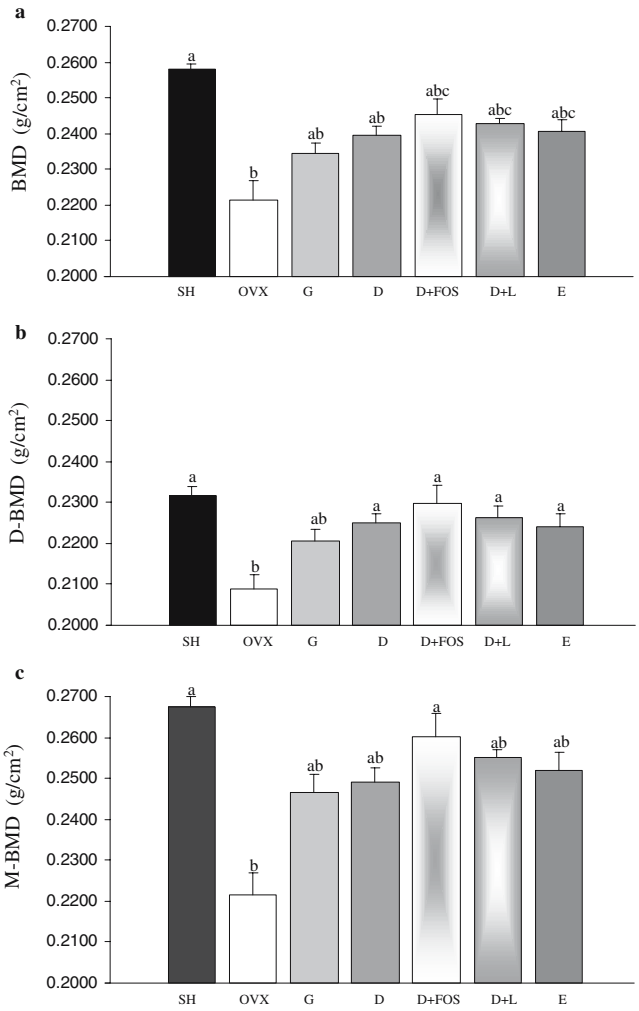

Fig. 2 (**a**) Total (BMD), (**b**) diaphyseal (D-BMD) and (**c**) metaphyseal (M-BMD) femoral bone mineral densities measured in sham-operated (SH) and in ovariectomized rats (OVX) supplemented with genistein (G), daidzein (D), daidzein with FOS (D+FOS), daidzein with Lactobacillus (D+L) or equol (E). Values are means ± SEM. $^{a}p<0.05$ *vs.* OVX; $^{b}p<0.001$ *vs.* SH; $^{c}p<0.05$ *vs.* G

Plasma IF levels

Because the pair-feeding procedure was based on the lowest consumption (the one from SH animals), every treatment was fully eaten, equol like the other isoflavones. As shown in Table 3, plasma genistein, daidzein and equol concentrations on day 91 allowed to validate experimental diet consumption. Indeed, genistein was detectable only when rats were given the supplemented diet with genistein. In the same way, daidzein was only found in those animals receiving the daidzein regimen. However, in this case, equol was also found, whenever daidzein was given alone or together with pre- or probiotics, thus confirming that it is formed from daidzein and not genistein. Among those three experimental groups, equol levels were not significantly different. The plasma sum of daidzein + equol was found to be similar, as well. As far as the equol diet is concerned, once orally absorbed, equol appeared to be able to cross the intestinal barrier without being catabolized, though it is difficult to assess the efficiency of absorption.

Bone parameters

BMD values (g/cm^2) of the total femur and its diaphyseal and metaphyseal subregions measured at the end of the experiment are shown in Fig. 2 (a,b and c respectively). As previously shown, castration induced a decrease in total BMD (0.2215±0.0052 *vs.* 0.2579±0.0017 in SH, $P<0.05$). This osteopenia was at least partially prevented by phytoestrogens consumption. Nevertheless, protection was achieved on diaphysis by all treatments except for genistein (BMD values insignificantly different from those measured in SH rats).

Daidzein and equol demonstrated a higher protective effect than genistein on both total and diaphyseal femur. Actually, the bone sparing effect of equol on the whole femur was even higher than that of daidzein. In the equol experimental group. BMD values reached similar levels than that of rats given daidzein together with pre- or probiotics.

The positive effect of daidzein was even exacerbated by addition of FOS. In this case BMD was not significantly different from that measured in SH animals, on both diaphyseal and metaphyseal levels. *L. casei* improved its potency as well on the whole femur.

Femoral size was similar in all groups (average length 36.2±0.9 mm, mean diameter 4.4±0.2 mm).

As far as biomechanical properties are concerned, no significant difference was demonstrated between groups, except for D+FOS and E animals which had more resistant bones than OVX (D+FOS 114.93±4.46; E 116.29±4.54 *vs.* OVX 102.6±3.93, $P<0.05$) (Fig. 3).

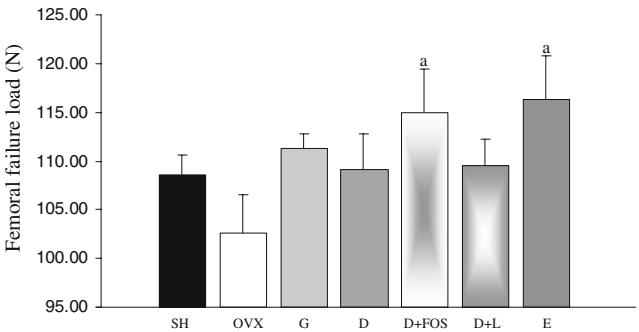

Fig. 3 Femoral failure load measured in sham-operated (SH) and in ovariectomized rats (OVX) supplemented with genistein (G), daidzein (D), daidzein with FOS (D+FOS), daidzein with Lactobacillus (D+L) or equol (E). Values are means ± SEM. $^{a}p<0.05$ *vs.* OVX

Markers for bone metabolism

Ovariectomy induced an increase in plasma OC concentrations. This acceleration of bone turn over was not prevented by isoflavones consumption (Fig. 4a).

Regarding bone resorption, urinary DPD excretion at day 90 (nmol/mmol creatinine) was also significantly greater in OVX (121±11; $P<0.05$) than in control rats (77±8; $P<0.05$). This physiological process was not significantly corrected by any diet.

This lack of effect on those parameters could be explained by the fact that modulation of metabolic process after such treatments could have reached a steady state level, with those markers only reflecting punctual bone turnover.

Image analysis

The photomicrographs of histological slides used for image analysis showed distinct variations of cancellous bone area in the distal femur metaphysis (Fig. 5). Osteopenia was associated with castration as shown by a reduced cancellous bone area and scarcity of trabeculae (Fig. 5). Isoflavones treatment was able to partially prevent the decrease of both parameters and even improved the trabecular thickness.

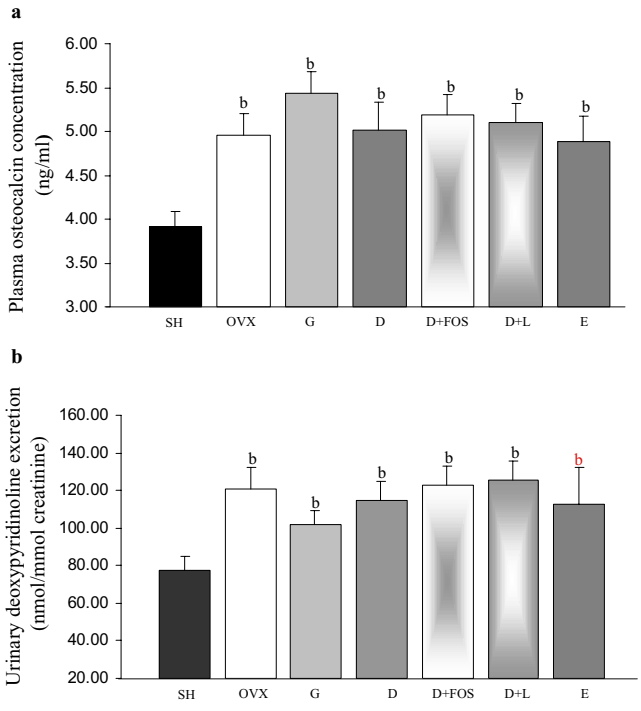

Fig. 4 (**a**) Plasma osteocalcin concentration and (**b**) urinary deoxypyridinolin excretion in sham-operated (SH) and in ovariectomized rats (OVX) supplemented with genistein (G), daidzein (D), daidzein with FOS (D+FOS), daidzein with Lactobacillus (D+L) or equol (E). Values are means ± SEM. $^{b}p<0.05$ *vs.* SH

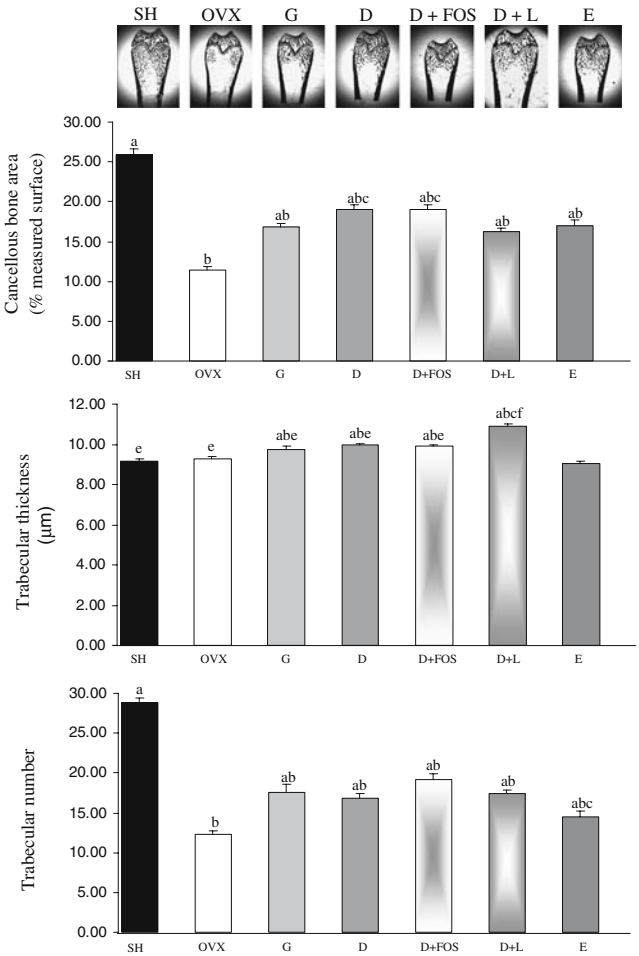

Fig. 5 Photomicrographs (X12.5) of histological slides used for image analysis, cancellous bone area, trabecular thickness and trabecular number in the distal femur metaphyseal zone from sham-operated (SH) and ovariectomized rats (OVX) supplemented with genistein (G), daidzein (D), daidzein with FOS (D+FOS), daidzein with Lactobacillus (D+L) or with equol (E). Values are means ± SEM. $^{a}p<0.001$ *vs.* OVX; $^{b}p<0.05$ *vs.* SH; $^{c}p<0.05$ *vs.* G; $^{e}p<0.05$ *vs.* D+L; $^{f}p<0.05$ *vs.* E

Regarding cancellous bone area, diets providing D or D+FOS were the most efficient. The effect of equol consumption was less evident. on those parameters. Consequently the major target for equol would be the level of mineralisation (BMD data) rather than architecture modulation () A trabeculae thickening was elicited by the combination D+L.

Discussion

Menopause is characterized by an initial period of rapid bone loss followed by a slower rate of bone waste [38, 39]. Ovariectomized rats are classically used as an animal model for postmenopausal bone loss [21]. Actually, the characteristics of skeletal physiology in the rat model share similarities with those of early postmenopausal

women in many respects, including an increased rate of bone turnover with resorption exceeding formation, greater loss of cancellous than cortical bone, and similar skeletal response to plant therapy such as soy isoflavones [26, 40].

In human and in rats, the aglycone forms of isoflavones (daidzein and genistein) are degraded by gut microflora to a series of metabolic products, in particularly equol, a bacterial degradation of daidzein. Daidzein was reported to be more bioavailable than genistin in humans [41] and rats [42]. Actually these scientists suggest that the higher bone sparing activity provided by daidzein could probably explained by daidzein conversion into equol, the major phenolic compound found in urine, blood and bile of rats maintained on isoflavones diet [43]. However, the in vivo effect of equol given orally has never been compared to that of genistein or daidzein. On the other hand, prebiotics, modulators of the gut flora, affect bioavailability of phytoestrogens and therefore improve their absorption [44, 45]. Such a modulation of the bone sparing effect of isoflavones by intestinal microflora has not been investigated with probiotics. The present study reports the potential preventive effects of genistein, daidzein, with or without prebiotics (fructooligosaccharides) or probiotics (*Lactobacillus casei*), and equol, given orally and separately at equal dose, and compared their effects on ovariectomy-induced bone loss in adults rats.

As previously shown by Miller et al. [46], Fig. 2 indicates that, in experimental rats, ovariectomy (confirmed by uterine atrophy; Table 2) induced femoral osteopenia, in both distal metaphysis (rich in cancellous bone that is mainly involved in metabolic functions) and diaphysis (rich in cortical bone that fulfills essentially mechanical and protective functions), as shown by M- and D-BMD, respectively. Histological data performed on metaphysis followed the same pattern. Indeed the metaphyseal BMD drop was associated with a decrease in cancellous bone area (Fig. 5) and trabeculae number. However, the diaphyseal BMD reduction was not associated with an impairment of mechanical properties, as shown by the constant femoral failure load in OVX and in SH rats (Fig. 3). Moreover, as in human subjects [47], this osteopenia probably resulted from an increase in bone turnover, as indicated by higher plasma osteocalcin concentrations and urinary DPD excretion in OVX than in SH rats (Fig. 4a,b).

In our experimental conditions, soy isoflavones were orally given at equivalent amount, i.e., 10 μg/g body weight per day, a dose which has previously been shown to be efficient [28]. As previous studies [24, 28, 48], ingestion of soy isoflavones has demonstrated preventive effects on ovariectomy-induced osteopenia in rats. Actually, in the present experiment, a bone sparing effect was exhibited on metaphyseal and diaphyseal bone mineral density. Among tested phytoestrogens, equol appeared to be more potent on the whole femur than the other molecules. Furthermore, addition of pre- or probiotics exacerbated daidzein efficiency, which reached similar level than equol. These results are coherent with histological data showing that all isoflavones exert their protective effect by partially preventing the decrease of cancellous bone area and trabeculae number and by leading to their thickening (Fig. 5). A higher impact was seen on trabecular thickness in the D+L group, while FOS addition appeared to improve maintenance of the number of trabeculae (although not statistically different from other treatments).

These results suggest that the estrogenic effects of IF on prevention of bone loss are also likely to be induced in vivo by equol. Setchell et al. have shown that equol given as a single bolus oral dose (25 mg) in healthy adult, was rapidly absorbed and demonstrated similarity in its pharmacokinetics to other isoflavones, although the slower plasma clearance was striking (Cl/F=6.85 h) compared with its precursor, daidzein (Cl=17.5 L/h) [49]. Thus, regarding this molecule, only bioavailability data have been published. It is, thus, the first in vivo demonstration of the potential of equol. In our study, plasma equol levels reached at J90 after consumption were higher than those of genistein and daidzein (even when bioconversion into equol was considered (Table 3). These observations are supported by those from Brown et al., who reported that the slow clearance rate of equol contributes to the maintenance of high plasma concentrations in rats which are currently far in excess of those of daidzein or genistein in this species [50].

As reported previously, this study revealed several interesting facts concerning the effects of FOS on protective effect on bone loss and then on bioavailability of daidzein, [30, 31, 44, 51, 52]. Actually, addition of FOS to daidzein diet increased significantly metaphyseal-BMD and improved biomechanical properties (Figs. 2 and 3). Moreover, in rats fed FOS with daidzein cancellous bone area and trabeculae number were slightly enhanced, compared to data from the daidzein diet. Actually, FOS are known to increase calcium absorption in rats [53], leading to a protective effect on bone structure [52]. In fact, our experimental data demonstrating a further protection when FOS were added to daidzein could be partly explain by this phenomenon. We did performed a calcium balance study in the OVX, D and D+FOS groups. Apparent digestive utilization (%) was 61.58±9.83, 69.06±11.45 and 83.41± 1.75, respectively. Thus a trend towards a higher calcium bioavailability was seen.

The effects of lactobacillus were similar, with the exception that the increase in metaphyseal-BMD was not significantly. This *L. casei* is made of viable microorganisms that beneficially affect the host by improving the properties of the indigenous microflora. It is, however,

difficult to explain their mechanism of action because equol plasma levels were not higher than in D animals.

In conclusion, the present study shows that, like genistein and daidzein, long-term equol consumption in the ovariectomized rat provides bone sparing effects. Addition of indigestible sugars such as FOS, or live microbial as *L. casei* in the diet, significantly increases bone protection exhibited by daidzein. Consequently, a dietary combination of prebiotic and/or probiotic and isoflavones may have a potential promise for maintaining or improving bone mass of human subject.

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