JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Antiosteoporotic Effects of *Lactobacillus*-Fermented Soy Skim Milk on Bone Mineral Density and the Microstructure of Femoral Bone in Ovariectomized Mice

Shen-Shih Chiang[†,‡] and Tzu-Ming Pan[*,†]

[†]Department of Biochemical Science and Technology, National Taiwan University, No.1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan

[‡]Department of Food Science and Biotechnology, National Chung Hsing University, No. 250 Kuokuang Road, Taichung 40227, Taiwan

ABSTRACT: Osteoporosis is a major skeletal disease associated with loss of estrogen in postmenopausal women. In this study, *Lactobacillus paracasei* subsp. *paracasei* NTU 101 (NTU 101F) and *Lactobacillus plantarum* NTU 102 (NTU 102F) were used as starters to ferment soy skim milk. This was then used as a nutritional supplement for 8 weeks to ovariectomized (OVX) mice. This study reveals that soy skim milk fermented with lactobacilli can increase the contents of aglycone isoflavones, soluble calcium, and vitamin D_3. The trabecular bone volumes and trabecular number of the distal femur in mice fed NTU 101F increased by a factor of 1.48 and 1.74 compared with the OVX group. The bone network density and thickness of the distal metaphyseal trabecular in mice fed NTU 101F and Fosamax was significantly greater than that of OVX mice. These results suggest that fermented soy skim milk can attenuate bone loss in OVX mice and lower the risk of osteoporosis.

KEYWORDS: osteoporosis, *Lactobacillus*-fermented soy skim milk, trabecular bone, ovariectomized mice

INTRODUCTION

Osteoporosis is a major skeletal disease associated with aging. It has a number of subtypes, such as senile osteoporosis and postmenopausal or menopause-related osteoporosis. One in three women over 50 years of age will suffer from osteoporotic fracture, and 1.5 million fractures occur each year as a consequence of osteoporosis in the United States.[1] This form of osteoporosis is not easy to prevent and is one of the most serious health problems in women. There are some methods used to reduce the risks of bone loss and hip-fracture complications after menopause, including the pharmacological supplementation of calcium and vitamin D or K, selective estrogen-receptor modulators and other estrogen analogues or bisphosphonates, calcitonin, hormone replacement therapy (HRT), and parathyroid hormone.[2,3] Nonpharmacological interventions include a nutritional and balanced diet, exercise, hip protectors, and orthopedic management of fractures.[2,3]

Loss of estrogen seems to be the most important mechanism causing osteoporosis. The use of ovariectomized (OVX) rat or mouse models to simulate the postmenopausal condition is well established and reproducible. These models mimic postmenopausal cancellous bone loss over relatively short periods of time.[4] Following ovariectomy, they show a biphasic loss of bone, with an initial rapid phase of bone loss up to 100 days, followed by an intermediate period of relative stabilization of cancellous bone volume at an osteopenic level.[5]

Many phytochemicals have been isolated from vegetables, fruits, spices, teas, herbs, and medicinal plants, such as flavonoids, polysaccharides, phenolic compounds, and oligopeptides.[6] Recently, the potential health benefits of soybean-based products have been widely documented. Epidemiological studies and clinical trials have shown that the soybean has a protective effect against postmenopausal symptoms, cardiovascular disease, bone-health problems, and breast, prostate, and colon cancers.[7]

In this study, we explored the potential role of dietary factors involved in modulating bone loss following estrogen deficiency by feeding *Lactobacillus*-fermented soy skim milk to OVX C57BL/6J mice and then observing the skeletal response. Specifically, we investigated the effects on reduction of bone loss in femur trabecular and cortical bone in OVX mice. We also evaluated the differences in skeletal morphology, using either a dual photon densitometer or a specially adapted microcomputed tomography (μ-CT) machine to determine the baseline bone morphology response to lactobacilli or fermented soy skim milk in OVX mice.

MATERIALS AND METHODS

Preparation of Soy Skim Milk Fermented with Lactic Acid Bacteria (LAB). The preparation of LAB-fermented soy skim milk was performed as follows. Skim milk (8%, w/v) was reconstituted with Anchor skim milk powder (Fonterra Ltd., Auckland, New Zealand). Each skim milk sample was individually treated in a water bath at 90 °C for 1 h. Nongenetically modified sugar-free soy milk was purchased from a local supermarket (Chuan Kui Yuba Factory, Taoyuan, Taiwan) and autoclaved for 15 min at 121 °C. After heat treatment and cooling of the milk to room temperature, skim milk and soy milk were mixed at a ratio of 25:75 in aseptic conditions. The best ratio of soy skim milk was according to our previous study.[8] *Lactobacillus paracasei* subsp. *paracasei* NTU 101 and *Lactobacillus plantarum* NTU 102 were inoculated in lactobacilli MRS broth and grown at 37 °C for 48 h. Thereafter, 1 mL of each of the two strain suspensions (1.0×10^8 CFU/mL) was separately inoculated into soy skim milk for fermentation at 37 °C for 24 h, lyophilized, and stored at 4 °C before use.

Received: April 6, 2011
Revised: June 14, 2011
Accepted: June 14, 2011
Published: June 14, 2011

dx.doi.org/10.1021/jf2013716 | *J. Agric. Food Chem.* 2011, 59, 7734–7742

Determination of the Proximate and Bioactive Compounds in Soy Skim Milk. Proximate compositions of soy skim milk fermented by two *Lactobacillus* strains, including moisture, crude ash, crude fat, carbohydrate content, and crude protein, were determined according to the methods of the AOAC.[9] The nitrogen factor used for crude protein calculation was 4.38.[10] The carbohydrate content was calculated by subtracting the contents of crude ash, fat, fiber, and protein from 1000 mg/g of dry matter and expressed as milligrams per gram of dry weight.

The contents of isoflavones (genistein and daidzein) were analyzed by high-performance liquid chromatography (HPLC) according to a modification of the method described by Pyo and Seong.[11] One hundred micrograms of fermented or nonfermented soy skim milk powder was dissolved in 1 mL of 80% ethanol and shaken vigorously. The isoflavones were extracted at room temperature for 20 min and centrifuged at 12000*g* for 15 min. The supernatant was then filtered through a 0.45 μm syringe filter before analysis. The HPLC system consisted of a Hitachi L-2130 pump, a Rheodyne 7161 injector, a 20 μL sample loop, a Hitachi L-2455 diode array detector, and a LiChrospher 100 RP-18 column (4.6 × 250 mm, 5 μm, Phenomenex, Torrance, CA). The mobile phase gradient was A eluent (0.05% acetic acid in water solution)/B eluent (0.05% acetic acid in acetonitrile) at 80:20 to 60:40 for 0−18 min, at 60:40 to 0:100 for 18−23 min, and held at 0:100 for 2 min before being changed to 80:20 for 25−30 min at a flow rate of 1.0 mL/min.

The vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol) contents in milk powders were measured by using the HPLC method described by Byrdwell.[12] Two grams of fermented or nonfermented soy skim milk powder was dissolved in 10 mL of dimethyl sulfoxide and shaken vigorously at 40−50 °C in a sonicator for 30 min. Then 10 mL of methanol/H_2O (1:1, v/v) was added and shaken for at least 1 min. Twenty milliliters of *n*-hexane was added to the mixture, which ws vortexed for 30 min and centrifuged at 1890*g* for 30 min. The supernatant was then filtered through a 0.45 μm syringe filter before analysis. The HPLC system consisted of a Shimadzu LC-10AT pump, a Shimadzu SPD-10A UV−vis detector at 265 nm, and a LiChrospher 100A RP-18 column (4.6 × 250 mm, 5 μm, Phenomenex). The mobile phase was methanol/acetonitrile at 10:90 for 25 min at a flow rate of 1.0 mL/min. A calibration curve was prepared using vitamin D_2 and D_3 standard, dissolved in methanol at five concentrations ranging from 0.5 to 10.0 μg/mL.

The determinations of soluble and total calcium and phosphorus elements were made employing an inductively coupled plasma atomic absorption spectrometer (Perkin-Elmer Optima model, 2100 DV) with axial viewing of the emitted radiation. For soluble calcium and phosphorus analysis, 500 μg of freeze-dried powder was diluted to a final volume of 30 mL with deionized water and filtered with 0.45 μm Millipore HVLP filter membrane (Millipore, Billerica, MA) for further analysis. Standard solutions were used for the calibration. The selection of instrumental parameters and optical wavelength was based on obtaining good sensitivity and reasonable detection limits and eliminating interferences. The spectral wavelength selections for calcium and phosphorus analysis by ICP-AES were 213.617 and 393.366 nm. A peristaltic pump was used to introduce the solutions into the ICP-AES at 1 mL/min. The dissolved samples were delivered through the pump using Tygon PVC tubing.

Ovariectomized Mouse Model on Animal Surgery and Experimental Protocol. Forty-eight female 2-month-old C57BL/6J mice were purchased from the National Laboratory Animal Center, Taipei, Taiwan. The mice were housed in polycarbonate cages and maintained under a 12 h light/dark cycle. They were given access to food (Lab Diet 5001 Rodent diet, Purina Mills LLC, St. Louis, MO) and water ad libitum for an 8 week adaptation prior to this study. At 16 weeks of age, 40 mice were ovariectomized (OVX), and 8 sham-operated mice were fed phosphate-buffered saline (group 1, SHAM). The surgical mice were anesthetized with tribromoethanol (Avertin, 250 mg/kg, ip). Two weeks after surgery, the OVX mice were divided into five groups as follows: group 2, phosphate-buffered saline (OVX); group 3, Fosamax, 0.2 mg of alendronic acid, and 8 units of colecalciferol per week (FOS); group 4, 0.1 g of freeze-dried powder of soy skim milk fermented by *L. paracasei* subsp. *paracasei* NTU 101 (NTU 101F); group 5, 0.1 g of freeze-dried powder of soy skim milk fermented by *L. plantarum* NTU 102 (NTU 102F); and group 6, 0.1 g of freeze-dried powder of nonfermented soy skim milk (NFSM).

Each group (n = 8) was subjected to daily gavaging for 8 weeks. The dosages of fermented or nonfermented milk were according to the administration of daily intake of total isoflavonse (80 mg per day) by the U.S. Food and Drug Administration. Experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC No. 98-74) of National Chung-Hsing University. From the beginning of the test, all mice were anesthetized with tribromoethanol once a month and measured for bone mineral density (BMD, g/cm^2) and bone mineral content (BMC, g/cm) using the total-body Dual Energy X-ray Absorptiometer (DEXA, Norland Corp., Cranbury, NJ). At the end of treatment, mice were euthanized by carbon dioxide inhalation, and blood samples were collected via the retro-orbital sinus. Whole blood was clotted at room temperature for 2 h, and serum was separated by centrifugation at 700*g* for 10 min and stored at −80 °C until analysis. The serum was analyzed for the following clinical biochemical measurements: liver and renal function parameters, including the alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, blood urea nitrogen (BUN), alkaline phosphatase (ALP), acid phosphatase (ACP), calcium, and phosphorus levels.

Preparation of Bone. The right and left femurs were dissected and the adherent soft tissues removed. The lengths were measured using digital calipers. Subsequently, the right femur bone specimens were placed in sealed plastic Eppendorf tubes containing 10% formalin and analyzed using the computed tomography (CT) system. The left femur bone specimens were analyzed using scanning electron microscopy (SEM).

Trabecular and Cortical Bone Assessment by μCT Imaging. The cortical and trabecular microstructure and a 3D image of the right femurs were analyzed using the SkyScan1076 in vivo μCT system (Skyscan, Kontich, Belgium). It was operated at 50 kV and 200 μA at 0.4° of a rotation step, using a 0.5 mm Al filter and 9 μm/pixel scan resolution. The voxel size was 9 × 9 × 9 μm^3. For bone mineral density (BMD) analysis, it was operated at 50 kV and 200 μA at 1° of a rotation step, using a 0.5 mm Al filter and 35 μm/pixel scan resolution. The voxel size was 35 × 35 × 35 μm^3. Cross sections were reconstructed using NRecon cone-beam algorithm software (Skyscan). Files were imported into CTAn software (Skyscan) for 3D analysis and image generation. BMD for each femur was measured by CTAn, which was calibrated using phantoms with known BMD (0.25−0.75 g/cm^3).

The region of interest (ROI) of trabecular bone was defined using 50 slices approximately 0.4 mm away from the growth plate of the distal femur. The ROI of femoral cortical geometry was assessed for slices located at the diaphyses of the midfemur. The trabecular morphometric parameters were analyzed by imaging and measured using the CTAn software. The position of trabecular bone commencing approximately 0.4 mm from the growth-plate level was selected in the direction of the metaphysic and extended from this position for an additional 1.5 mm. Separation of the trabecular bone was performed using a freehand drawing tool for delineating complex ROIs. Furthermore, 3D and 2D morphometric parameters were calculated for the trabecular selected ROIs. Trabecular bone volume (BV/TV) was calculated using bone volume (BV) and total tissue volume (TV). Mean trabecular thickness (Tb.Th) was determined from the local thickness. Trabecular separation (Tb.Sp) and trabecular number (Tb.N) were estimated using the plate model. Trabecular bone pattern factor (TBPf) is the ratio of convex to concave structures on the trabecular surface. Tb.N, Tb.Sp, and TBPf

Table 1. Contents of Approximate Components and Daidzein, Genistein, Ergocalciferol, Cholecalciferol, Calcium, and Phosphorous in Nonfermented, *Lactobacillus paracasei* subsp. *paracasei* NTU 101-Fermented, or *Lactobacillus plantarum* NTU 102-Fermented Soy Skim Milk[a]

composition/sample	NTU 101 F	NTU 102 F	NFSM
powder (mg/mL)	75.9 ± 3.1	77.4 ± 4.3	80.5 ± 0.8
Lactobacillus strain (CFU/g)	3.0×10^{11}	3.9×10^{11}	ND[b]
water (%)	9.6 ± 0.4*	8.6 ± 0.2*	6.8 ± 0.1
crude fat (%)	17.1 ± 0.6*	16.7 ± 0.4*	13.6 ± 0.0
crude protein (%)	41.8 ± 1.9	42.9 ± 0.8	42.5 ± 0.2
ash (%)	5.7 ± 0.8	5.8 ± 0.0	5.7 ± 0.1
total carbohydrate (%)	25.8 ± 3.0*	26.0 ± 1.1*	31.4 ± 0.4
daidzein (μg/mL)	85.6 ± 3.5*	96.8 ± 5.4*	11.0 ± 0.1
genistein (μg/mL)	70.8 ± 2.9*	88.1 ± 4.9*	10.4 ± 0.1
soluble phosphorus (mg/g)	1.2 ± 0.1*	1.1 ± 0.1*	1.1 ± 0.1
soluble calcium (mg/g)	1.4 ± 0.1*	1.3 ± 0.1*	0.6 ± 0.0
total phosphorus (mg/g)	8.3 ± 0.2	8.3 ± 0.1	8.0 ± 0.1
total calcium (mg/g)	4.8 ± 0.2	4.8 ± 0.1	4.6 ± 0.4
ergocalciferol (vitamin D_2) (μg/g)	14.6 ± 2.0*	33.2 ± 8.4*	21.5 ± 3.6
cholecalciferol (vitamin D_3) (μg/g)	65.3 ± 6.2*	42.9 ± 2.9*	29.7 ± 1.4

[a] Values are expressed as the mean ± SD ($n = 3$). Asterisks indicate that the difference between the fermented milk and the NFSM milk group value is statistically significant (*, $p < 0.01$). NTU 101F, *L. paracasei* subsp. *paracasei* NTU 101-fermented soy skim milk; NTU 102F, *L. plantarum* NTU 102-fermented soy skim milk; NFSM, nonfermented soy skim milk. [b] Not detected.

were measured according to the parallel-plate model.[13] The structure model index (SMI) is a parameter that has been described as the characteristic form of trabecular bone in terms of its plate-like or rod-like nature.[14] The degree of anisotropy (DA) measures the orientation of the trabecular within the ROI. Increasing values of DA indicate increasing alignment in one direction relative to other directions.[15]

SEM Observation. The distal part of the femur was trimmed in the sagittal plane and treated with a 5% sodium hypochlorite solution to expose the trabecular bone. After dehydration with acetone, air-drying, mounting on stubs, and coating with gold/palladium using a Hitachi E101 ion sputter (Hitachi Ltd., Tokyo, Japan), the bones were examined using a FEI Inspect S SEM (FEI Co., Hillsboro, OR). The endosteal cutting surfaces were processed for SEM observation. Two micrographs at the final magnifications of ×80 and ×130 were obtained for each mouse.

Statistical Analysis. The statistical significance of the femoral and biochemical effects was determined by one-way analysis of variance (ANOVA) using a Statistical Analysis System (2008, *SAS/STAT User's Guide*, ver. 9.1.3. SAS Institute, Inc., Cary, NC), followed by ANOVA with Duncan's test. Differences with $p < 0.05$ and <0.01 between the test groups and the control group were considered to be statistically significant. Values are expressed as the mean ± standard deviation (SD) with eight animals and three milk samples. Pearson's correlation coefficient was used to calculate the linear relationship between two variables. Stepwise multiple linear regression analyses were used to adjust for potential covariates.

RESULTS

Proximate Analyses of Lyophilized Soy Skim Milk Powders. Table 1 shows the approximate components and contents of isoflavones (daidzein and genistein), total phosphorus and calcium, ergocalciferol, and cholecalciferol in fermented or nonfermented soy skim milk powders. Recoveries and crude protein and ash contents of fermented or nonfermented

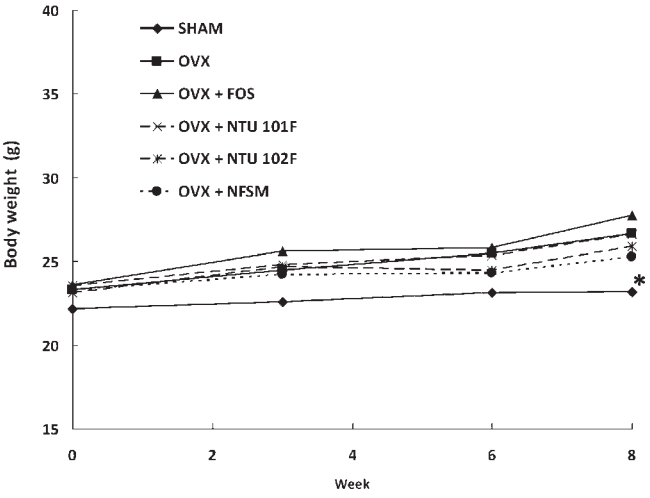

Figure 1. Changes in body weight during the administration period. SHAM, sham-operated and PBS group; OVX, ovariectomized and PBS group; OVX + FOS, ovariectomized and Fosamax group; OVX + NTU 101F, ovariectomized and *L. paracasei* subsp. *paracasei* NTU 101-fermented soy skim milk; OVX + NTU 102F, ovariectomized and *L. plantarum* NTU 102-fermented soy skim milk; OVX + NFSM, ovariectomized and nonfermented soy skim milk. Data are expressed as the mean ± SD ($n = 8$). Asterisks indicate that the difference between the mean and the OVX group value is statistically significant (*, $p < 0.01$).

lyophilized powders displayed no significant differences among all samples. The water and crude fat contents of two fermented powders were higher than those of nonfermented milk, whereas the total carbohydrate contents of fermented milks were significantly lower than those of nonfermented milk. The contents of two isoflavone aglycone isomers (genistein and daidzein) were determined. Fermented milk from NTU 101 and NTU 102 had higher amounts of these isoflavones than nonfermented milk, and daidzein content (85.6−96.8 μg/mL) was greater than that of genistein (70.8−88.1 μg/mL).

In particular, the levels of soluble calcium and cholecalciferol (vitamin D_3) in NTU 101-fermented soy skim milk powders (1.4 mg/g and 65.3 μg/g, respectively) were significantly higher than in NTU 102-fermented (1.3 mg/g and 42.9 μg/g) and nonfermented milks (0.6 mg/g and 29.7 μg/g).

Body Weights and Serum Biochemical Parameters. The final body weights of all OVX mice were found to be significantly higher than those of the SHAM group (Figure 1). Table 2 illustrates how the liver parameters of ALT and AST in NTU 101-fermented and NTU 102-fermented groups' mice sera were lower than that in other OVX groups. The renal function parameters of creatinine and blood urea nitrogen in mice fed fermented or nonfermented soy skim milk groups were significantly lower than those of the SHAM and OVX groups (Table 2). The results of alkaline and acid phosphatase activities, levels of serum calcium and phosphorus, and serum lipid parameters are shown in Table 2. The activities of ACP and levels of calcium showed no significant difference among all groups. The activity of ALP was highest in the SHAM group and lowest in the OVX + FOS groups. The phosphorus levels in groups fed milk powders were significantly lower than those of other groups.

Effects of *Lactobacillus*-Fermented Soy Skim Milk on Femur Bone BMD and Trabecular Parameters. Results of the femur BMD and parameters of trabecular structure estimated by

Table 2. Liver and Renal Parameters in OVX C57BL/6J Mice Sera after 8 Weeks of Oral Administration of either *L. paracasei* subsp. *paracasei* NTU 101- or *L. plantarum* NTU 102-Fermented or Nonfermented Soy Skim Milk

	serum parameters[a]							
treatment[b]	ALT (U/L)	AST (U/L)	creatinine (mg/dL)	BUN (mg/dL)	Ca^{2+} (mg/dL)	P^{3-} (mg/dL)	ALP (U/L)	ACP (U/L)
SHAM	39.7 ± 2.9	21.3 ± 2.7	0.29 ± 0.03	30.9 ± 3.4*	11.7 ± 0.3	14.3 ± 1.5	104.7 ± 14.6*	8.23 ± 0.55
OVX	48.6 ± 5.8	26.3 ± 3.9	0.28 ± 0.04	22.0 ± 2.9	11.3 ± 0.4	12.8 ± 1.2	91.7 ± 11.1	7.56 ± 0.67
OVX + FOS	46.3 ± 10.0	24.5 ± 6.3	0.20 ± 0.05*	21.0 ± 4.1	11.3 ± 0.3	11.2 ± 0.8	57.2 ± 15.5*	7.70 ± 0.71
OVX + NTU 101F	39.0 ± 4.2*	21.3 ± 2.8	0.24 ± 0.05*	24.4 ± 3.7	11.2 ± 0.4	9.0 ± 0.7*	85.9 ± 9.0	7.83 ± 0.75
OVX + NTU 102F	36.1 ± 1.7*	22.1 ± 2.6	0.21 ± 0.03*	25.5 ± 2.3	11.2 ± 0.3	7.7 ± 1.1*	86.4 ± 11.5	7.43 ± 0.86
OVX + NFSM	47.1 ± 7.1	29.6 ± 13.4	0.18 ± 0.06*	24.2 ± 4.6	11.1 ± 0.3	7.7 ± 1.0*	96.3 ± 27.2	7.01 ± 1.35

[a] Each value is expressed as the mean ± SD (n = 8). Asterisks indicate that the difference between the mean and the OVX group value is statistically significant (* $p < 0.01$). [b] SHAM, sham-operated and PBS group; OVX, ovariectomized and PBS group; OVX + FOS, ovariectomized and Fosamax group; OVX + NTU 101F, ovariectomized and *L. paracasei* subsp. *paracasei* NTU 101-fermented soy skim milk; OVX + NTU 102F, ovariectomized and *L. plantarum* NTU 102-fermented soy skim milk; OVX + NFSM, ovariectomized and nonfermented soy skim milk; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; ALP, alkaline phosphatase; ACP, acid phosphatase.

μCT are shown in Figure 2. Figure 2A shows the 3D reconstruction of micro-CT imaging results from CTAn and CTvol software analysis. Figure 2A presents the trabecular parameter from 1.5 mm thick sections of trabecular bone in the distal metaphysic femur.

Femur BMD, Tb.Th, and SMI values showed no significant differences among the six treatment groups ($p > 0.05$, Figure 2B, D,H). Analysis of data from the trabecular bone of OVX mice given FOS, NTU 101F, NTU 102F, and NFSM shows an increase in BV/TV by factors of 1.38, 1.55, 1.45, and 1.38, respectively, when compared with the data from the OVX group not receiving these (Figure 2C). Tb.N in OVX mice fed NTU 101F and NTU 102F milks were significantly higher than OVX control values and similar to that of the SHAM group ($p < 0.01$, Figure 2E). The Tb.Sp in NTU 101F-treated and NTU 102F-treated groups and DA in FOS and NTU 101F groups were also significantly decreased ($p < 0.01$, Figure 2G,I). The Tb.Sp in the SHAM mice was significantly lower than that of mice in the OVX group ($p < 0.01$, Figure 2F). Compared with the distal femur reconstructed 3D image in Figure 2A,C,E, the BV and trabecular bone network from the SHAM, FOS, and milk-feeding groups were obviously denser than that from the OVX control group.

SEM and Micro-CT Imaging of Trabecular Bone in the Femoral Diaphysis. SEM images (×80 and ×130) of the endosteal surfaces of the femur metaphysis at the ultrastructural level are shown in Figure 3. The growth plate in SHAM was thicker than other OVX groups and showed the SHAM mice were still growing. The images of trabecular bone in the SHAM and OVX + NTU 101F groups showed significantly greater thickness and more plate form than those of the OVX group in thinner rod form.

Pearson's Correlation Coefficient in Regression Analysis. Table 3 shows the correlation coefficients between the bioactive compounds and femur trabecular parameters. There is a significant positive association between contents of soluble calcium and cholecalciferol in fermented milk and BV/TV and Tb.N in OVX mice ($p < 0.05$), but negative association related to Tb.Sp ($p < 0.1$) and Tb.Pf ($p < 0.05$). Furthermore, the coefficients of genestien and daidzein were significantly negatively associated with Tb.Sp ($p = 0.22$ and 0.17).

DISCUSSION

The pathophysiological involutional osteoporosis of estrogen deficiency has been identified in both the early accelerated and late slow phases of bone loss in postmenopausal women. The accelerated phase is most apparent during the first decade after menopause, involves a disproportionate loss of cancellous bone, and mainly mediated loss of the direct restraining effects of estrogen on bone cell function.[16] Ovariectomized mice are classically used as an animal model to simulate postmenopausal bone loss and patterns of bone loss after ovariectomy. The characteristics of bone loss in mice varied among inbred strains of mice.[17−21]

Bisphosphonates (alendronic acid, risedronic acid, ibandronic acid) are the most widely prescribed drugs in osteoporosis. They inhibit osteoclastic resorption and reduce the rate of bone turnover, thereby reducing the risk of fracture.[22,23] Weekly doses of alendronic acid have been shown to provide benefits, in terms of BMD and changes in biochemical markers, similar to those seen with daily formulations.[23] Studies of animals using high doses of bisphosphonates have also reported normal quality bone with increased strength.[22] We therefore used alendronate (Fosamax) as the positive control drug to prevent osteoporosis in this postmenopausal mice model.

Soybean isoflavones represent a class of naturally occurring plant phytochemicals characterized by a steroid-like structure and weak estrogenic activity.[24] They usually exist in glycoside form in soy foods or relative products. The glycosidic bonds may be hydrolyzed by intestinal microflora such as *Lactobacillus* and *Bifidobacterium*, resulting in the formation of active forms in the serum or urine of animals.[25,26] There have been several investigations into the antiosteoporotic effects of milk proteins, soy milk, or its isoflavone products in postmenopausal women and rodents.[27−32] The present study is the first to report on the *Lactobacillus*-fermented soy skim milk for OVX mice and also the first to investigate the active compounds in fermented milks that have antiosteoporotic effects. The effects of calcium and vitamin D on the skeleton are well established.[33−37] The biologically active form of 1,25-$(OH)_2$ vitamin D is the main regulator of calcium homeostasis and skeletal metabolism, and this is necessary for the body to remain in good health. A reduction in the supply of calcium and vitamin D will therefore have harmful effects on the skeleton.[33]

In the present study, we found that daidzein and genistein were the main isoflavones in fermented soy skim milk (NTU 101F and NTU 102F). In NFSM their levels were lower (Table 1). The isoflavones of NFSM are mainly seen in glycoside

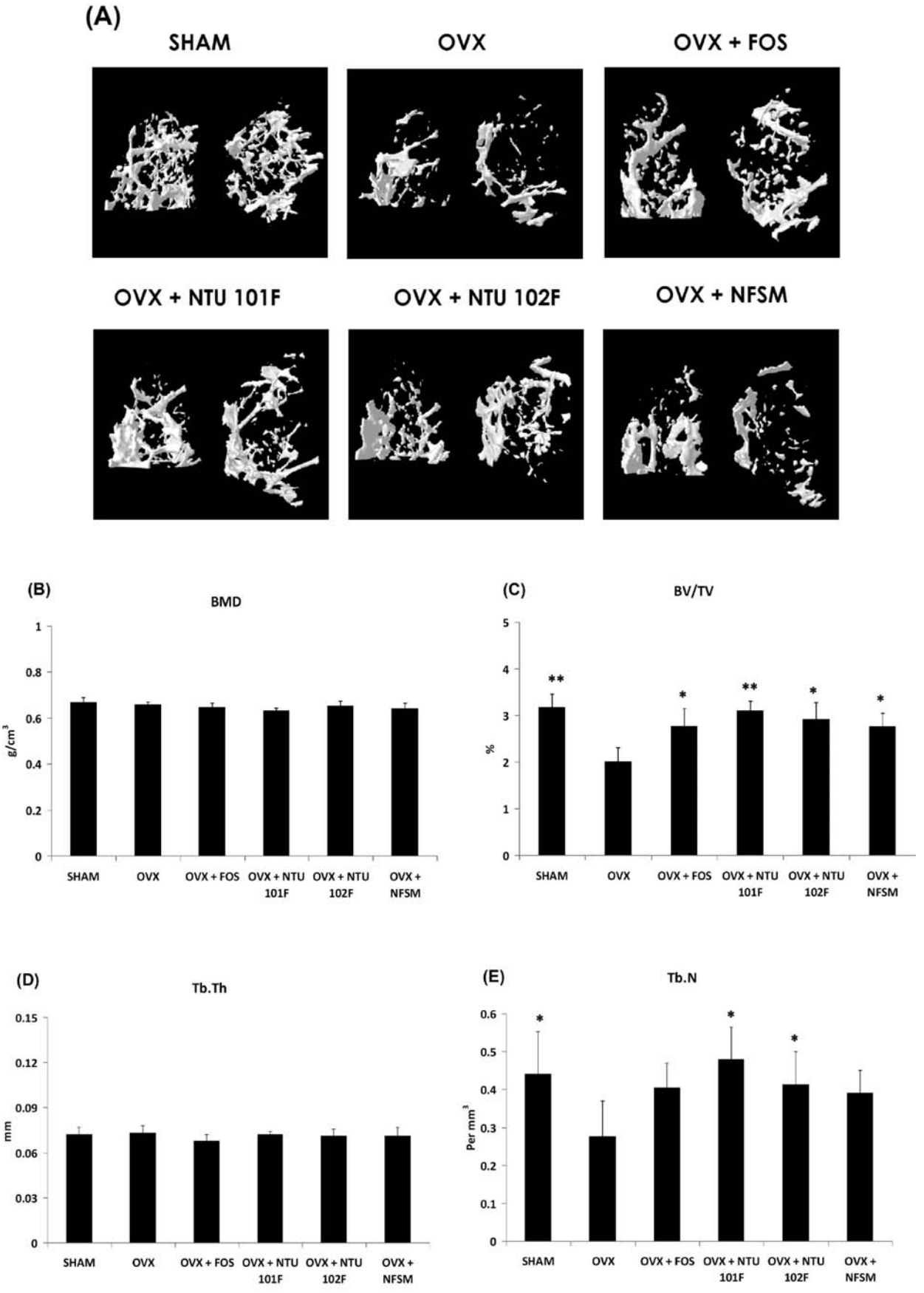

Figure 2. Continued

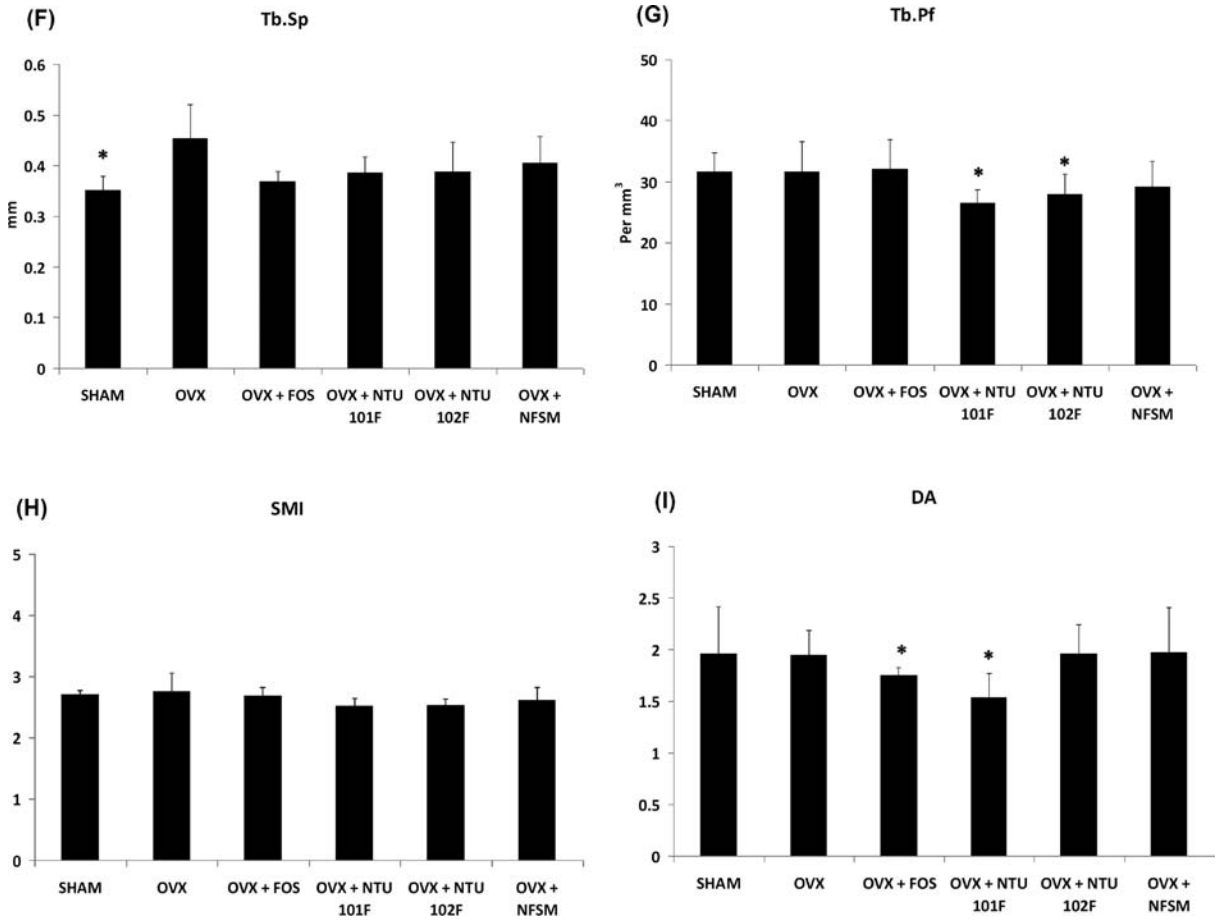

Figure 2. Three-dimensional microstructural properties of the distal femoral metaphyseal trabecular bone in C57BL/6J female ovariectomized mice after 8 weeks of oral administration of either *Lactobacillus paracasei* subsp. *paracasei* NTU 101-fermented or *Lactobacillus plantarum* NTU 102-fermented and nonfermented soy skim milk. (A) Three-dimensional tomographic rendering of a mouse femur revealing the complexity of the bone structure (images of the distal femoral metaphysis trabecular bone (1.5 mm thick images); the left-hand image shows the side view and the right-hand image the top view); (B) BMD, bone mineral density; (C) BV/TV, trabecular bone volume; (D) Tb.Th, mean trabecular thickness; (E) Tb.N, trabecular number; (F) Tb.Sp, trabecular separation; (G) TBPf, trabecular bone pattern factor; (H) SMI, structure model index; (I) DA, degree of anisotropy. SHAM, sham-operated and PBS group; OVX, ovariectomized and PBS group; OVX + FOS, ovariectomized and Fosamax group; OVX + NTU 101F, ovariectomized and *L. paracasei* subsp. *paracasei* NTU 101-fermented soy skim milk; OVX + NTU 102F, ovariectomized and *L. plantarum* NTU 102-fermented soy skim milk; OVX + NFSM, ovariectomized and nonfermented soy skim milk. Asterisks indicate that the difference between the mean and the control value is statistically significant (∗, $p < 0.01$).

form, such as daidzin and genistin. We also found that the levels of soluble calcium and vitamin D_3 in NTU 101F were significantly higher than those in NTU 102F and NFSM. This may show that levels of bioactive soluble calcium and vitamin D_3 are increased by the fermentation of lactobacilli. There was a positive relationship between these levels and prevention of bone loss.

In the clinical observation in all test groups (Figure 1), we found that the body weights of the five OVX mouse groups were higher than the SHAM group. Images of dissections showed many fat tissues in the abdominal cavity of the OVX mice. Comparison of liver- and kidney-related serum biochemical parameters (in Table 2) found no direct correlation, however. By comparison with the normal values of these serum chemistry parameters in 8-week-old C57BL/6 female mice from the National Laboratory Animal Center (Taipei, Taiwan), the values of ALT, AST, creatinine, BUN, ACP, calcium, and phosphorus in treated groups were all in the normal range.

ALP activity was highest in SHAM mice and lowest in FOS mice (Table 2). This may be related to an increase in serum ALP activity at a young age or proliferative bone lesions resulting in increased osteoblastic activity in the SHAM group. We also investigated femur cortical and trabecular bone from multiple skeletal sites to interpret the microstructural changes of bone in OVX mice fed PBS, Fosamax, NFSM, NTU 101F, or NTU 102F. Evidence from administration with soy milk in OVX rats showed that the trabecular bone volume was 68% higher and trabecular thickness (Tb.Th.) thicker than the OVX group.[28] Long-term intervention with *Lactobacillus helveticus*-fermented milk in growing rats significantly increased the bone mineral density and bone mineral content compared to the skim milk.[38] Our study results showed that the NTU 101F group had the highest BV/TV and Tb.N values and the lowest Tb.Sp and DA values in the trabecular bone among all groups (Figure 2). The present results confirmed that the NTU 101F milk increased the connectivity between trabecular and diminished the DA. Lower DA values suggested a more plate-like structure, which is in accordance with enhanced bone strength. The prevention effects in bone loss by ovariectomy in the NTU 101F group were superior

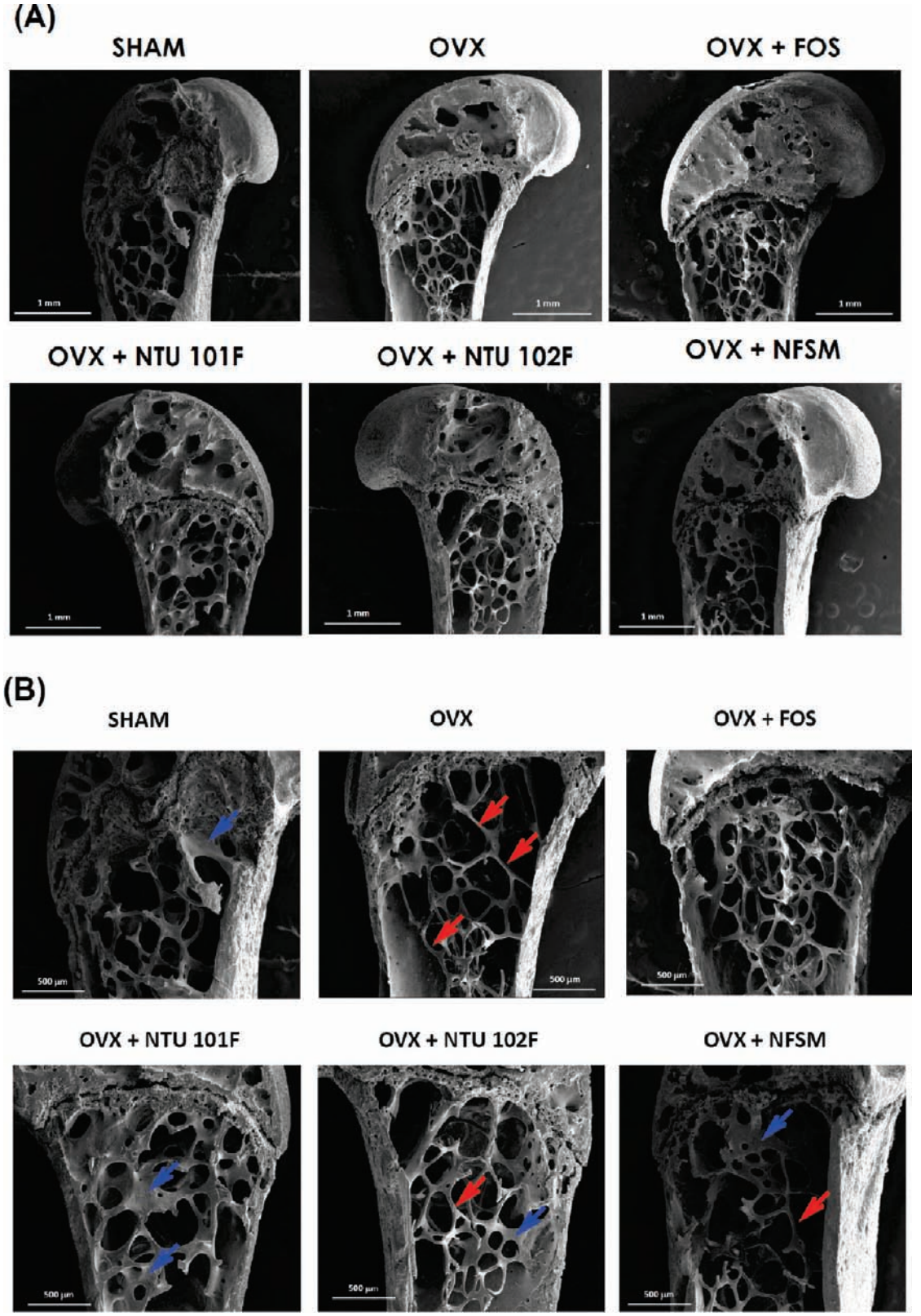

Figure 3. Electron microphotographs of the endosteal surface in the femoral metaphysis of OVX C57BL/6J mice: (A) 80×; (B) 130×. Scale bar: (A) 1 mm; (B) 500 μm. Red arrow represents the cylindrical rod form trabecular bone, and the blue arrow represents the parallel plate form trabecular bone.

to the FOS group in BV/TV, Tb.N, and Tb.Pf values. These values were the same as for the SHAM group. It was suggested that the composition of NTU 101F milk increased the volume and amount of trabecular bone, enhanced it so that it developed from rod form to plate form, and made it thicker than the clinical drug Fosamax.

Table 3. Correlation Coefficients between the Proximate Compounds in Fermented Soy Milks and Serum Parameters in Mice[a]

correlation	daidzein	S-P	S-Ca	ergocalciferol	cholecalciferol	BV/TV	Tb.N	Tb.Sp	Tb.Pf	DA
genestein	**0.995***	0.683	0.906	0.354	0.633	0.498	0.525	−0.936	−0.727	−0.330
daidzein		0.748	0.942	0.265	0.702	0.577	0.602	−0.964	−0.788	−0.416
S-P			0.928	−0.442	**0.998***	0.498	0.525	−0.936	−0.728	−0.330
S-Ca				−0.075	**0.901***	**0.818***	**0.836***	**-0.997***	**-0.949***	−0.698
ergocalciferol					−0.500	−0.634	−0.610	−0.000	0.385	0.766
cholecalciferol						**0.987***	**0.991***	−0.866	**-0.992***	−0.940
BV/TV							**0.9919****	**-0.794***	−0.572	−0.538
Tb.N								**-0.836***	−0.537	−0.536
Tb.Sp									0.026	0.227
Tb.Pf										0.441

[a] S-P, soluble phosphorus; S-Ca, soluble calcium; BV/TV, percent bone volume; Tb.N, trabecular number; Tb.Sp, trabecular separation; TBPf, trabecular pattern factor; DA, degree of anisotropy. Significant correlations are printed in bold. *, $p < 0.05$; **, $p < 0.001$.

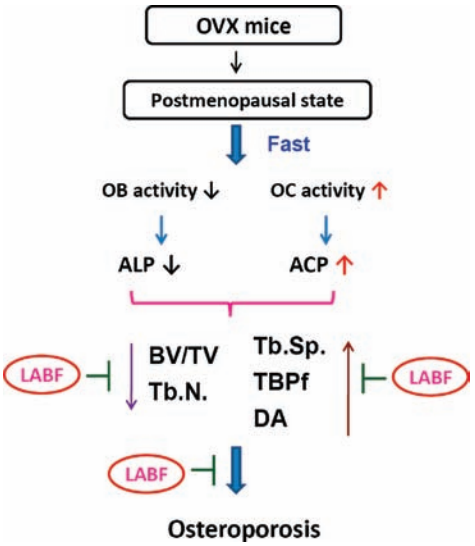

Figure 4. Hypothetical diagrams for roles of *Lactobacillus*-fermented products (LABF) on the osteoporosis pathway in OVX mice.

According to the results in Table 3, there was a positive relationship between the contents of bioactive compounds (soluble calcium and cholecalciferol) and protection effects by increasing the BV/TV and Tb.N as well as decreasing the Tb.Sp and Tb.Pf in OVX mice.

Physiological bone changes generally begin as a result of reduced estrogen levels after ovariectomy. Osteopenia increases with bone turnover rate and with resorption exceeding formation.[19] Influence of soy aglycon isoflavones (SAI) on bone-related traits and lens protein characteristics of ovariectomized rats and bioactivity performance of osteoprogenitor cells were investigated by Lien et al.[39] This in vivo study indicated that daily body weight gains in the OVX and OVX + SAI groups were greater than that of the SHAM group. Bone ash and Ca contents of the SHAM and OVX + SAI groups were higher than those of the OVX group. The in vitro study with osteoprogenitor cells revealed that cell viability, alkaline phosphatase activity, osteocalcin, and Ca contents of the SAI-supplemented group were higher than those of the unsupplemented group.[39]

In the fermented milks, daidzein is the most abundant isoflavone, and it can be further metabolized to equol, a compound with greater estrogenic activity than other isoflavones. The combination of purified daidzein and high Ca in OVX mice favorably protected against the loss of femur and vertebrae BMD and biomechanical strength at multiple skeletal sites.[40] Figures 3 and 2A shows the 2D and reconstructed 3D images from SEM and μCT, respectively. The OVX specimens evaluated in this study represent a specialized therapeutic application of *Lactobacillus*-fermented soy skim milk. These results show that the OVX process simultaneously reduced trabecular bone volume to a significant degree. Feeding with Fosamax or isoflavone-containing milk can inhibit these events. The aglycone type of isoflavones or other bioactive compounds (such as calcium and vitamin D_3) enhances the inhibitory effect. The effect of oral administration of *Lactobacillus*-fermented milk on bone mass in postmenopausal women is an area that warrants further study.

It should be noted that there were smaller increases in bone mass in the NTU 102F-treated and NFSM-treated groups than with NTU 101F, even though these milk types were also rich in isoflavones. From these results, we hypothesized that the LAB-fermented soy skim milks (LABF) may attenuate bone loss by increasing the BV/TV and Tb.N and reducing the Tb.Sp, Tb.Pf, and DA in OVX mice (Figure 4). On the basis of the efficacy of *Lactobacillus*-fermented milk, we hypothesize that the combined effects of high levels of soy isoflavone, calcium, and vitamin D would result in maximizing the potential for preventing bone loss in OVX mice.

AUTHOR INFORMATION

Corresponding Author

*Phone: +886-2-3366-4519, ext. 10. Fax: +886-2-3366-3838. E-mail: tmpan@ntu.edu.tw.

ACKNOWLEDGMENT

We thank Dr. Shu-Fen Lu and Yan-Zhen Liu, the staff of the Tawian Mouse Clinic (NSC 98-3112-B-001-041) which is funded by the National Research Program for Genomic Medicine (NRPGM) at the National Science Council (NSC) of Taiwan, for technical support in providing the μCT system. We also thank Dr. Shiang-Jiuun Chen and Ya-Chan Yang, the staff of TC5 Bio-Image Tools, Technology Commons, College of Life Science, NTU (Taiwan), for assistance with SEM in this study.

REFERENCES

(1) Parvez, T. Postmenopausal osteoporosis. *JK-Practitioner* **2004**, *11*, 281–283.

(2) Delmas, P. D. Treatment of postmenopausal osteoporosis. *Lancet* **2002**, *359*, 2018–2026.
(3) Keen, R. Osteoporosis: strategies for prevention and management. *Best Pract. Res. Clin. Rheumatol.* **2007**, *21*, 109–122.
(4) Egermann, M.; Goldhahn, J.; Schneider, E. Animal models for fracture treatment in osteoporosis. *Osteoporos. Int.* **2005**, *16*, S129–S138.
(5) Wronski, T. J.; Dann, L. M.; Scott, K. S.; Cintrón, M. Long-term effects of ovariectomy and aging on the rat skeleton. *Calcif. Tissue Int.* **1989**, *45*, 360–366.
(6) Nishino, H.; Tokuda, H.; Satomi, Y.; Masuda, M.; Onozuka, M.; Yamaguchi, S.; Takayasu, J.; Tsuruta, J.; Takemura, M.; Ii, T.; Ichiishi, E.; Kuchide, S.; Okuda, M.; Murakoshi, M. Cancer chemoprevention by phytochemicals and their related compounds. *Asian Pac. J. Cancer Prev.* **2000**, *1*, 49–55.
(7) Setchell, K. D.; Cassidy, A. Dietary isoflavones: biological effects and relevance to human health. *J. Nutr.* **1999**, *129*, 758S–767S.
(8) Liu, C. F.; Hu, C. L.; Chiang, S. S.; Tseng, K. C.; Yu, R. C.; Pan, T. M. Beneficial preventive effects of gastric mucosal lesion for soy-skim milk fermented by lactic acid bacteria. *J. Agric. Food Chem.* **2009**, *57*, 4433–4438.
(9) AOAC. *Official Methods of Analysis*, 15th ed.; Association of Official Analytical Chemists: Washington, DC, 1990.
(10) Crisan, E. V.; Sands, A. Nutritional value. In *The Biology and Cultivation of Edible Mushrooms*; Chang, S. T., Hayes, W. A., Eds.; Academic Press: New York, 1978; pp 137−165.
(11) Pyo, Y. H.; Seong, K. S. Hypolipidemic effects of *Monascus*-fermented soybean extracts in rats fed a high-fat and -cholesterol diet. *J. Agric. Food Chem.* **2009**, *57*, 8617–8622.
(12) Byrdwell, W. C. Comparison of analysis of vitamin D_3 in foods using ultraviolet and mass spectrometric detection. *J. Agric. Food Chem.* **2009**, *57*, 2135–2146.
(13) Mellish, R. W.; O'Sullivan, M. M.; Garrahan, N. J.; Compston, J. E. Iliac crest trabecular bone mass and structure in patients with non-steroid treated rheumatoid arthritis. *Ann. Rheum. Dis.* **1987**, *46*, 830–836.
(14) Hildebrand, T.; Ruegsegger, P. Quantification of bone microarchitecture with the structure model index. *Comput. Methods Biomech. Biomed. Eng.* **1997**, *1*, 15–23.
(15) Laffosse, J. M.; Kinkpe, C.; Gomez-Brouchet, A.; Accadbled, F.; Viguier, E.; Sales de Gauzy, J.; Swider, P. Micro-computed tomography study of the subchondral bone of the vertebral endplates in a porcine model: correlations with histomorphometric parameters. *Surg. Radiol. Anat.* **2010**, *32*, 335–341.
(16) Riggs, B. L.; Khosla, S.; Melton, L. J. A unitary model for involutional osteoporosis: estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men. *J. Bone Miner. Res.* **1998**, *13*, 763–773.
(17) Turner, R. T. Mice, estrogen and postmenopausal osteoporosis. *J. Bone Miner. Res.* **1999**, *14*, 187–191.
(18) Li, C. Y.; Schaffler, M. B.; Wolde-Semait, H. T.; Hernandez, C. J.; Jepsen, K. J. Genetic background influences cortical bone response to ovariectomy. *J. Bone Miner. Res.* **2005**, *20*, 2150–2158.
(19) Bouxsein, M. L.; Myers, K. S.; Shultz, K. L.; Donahue, L. R.; Rosen, C. J.; Beamer, W. G. Ovariectomy-induced bone loss varies among inbred strains of mice. *J. Bone Miner. Res.* **2005**, *20*, 1058–1092.
(20) Iwaniec, U. T.; Yuan, D.; Power, R. A.; Wronski, T. J. Strain-dependent variations in the response of cancellous bone to ovariectomy in mice. *J. Bone Miner. Res.* **2006**, *21*, 1068–1074.
(21) Klinck, J.; Boyd, S. K. The magnitude and rate of bone loss in ovariectomized mice differs among inbred strains as determined by longitudinal in vivo micro-computed tomography. *Calcif. Tissue Int.* **2008**, *83*, 70–79.
(22) Liberman, U. A. Long-term safety of bisphosphonate therapy for osteoporosis: a review of the evidence. *Drugs Aging* **2006**, *23*, 289–298.
(23) Reginster, J. Y.; Malaise, O.; Neuprez, A.; Jouret, V. E.; Close, P. Intermittent bisphosphonate therapy in postmenopausal osteoporosis: progress to date. *Drugs Aging* **2007**, *24*, 351–359.
(24) Gennari, C. Calcitonin, bone-active isoflavones and vitamin D metabolites. *Osteoporos. Int.* **1999**, *9*, S81–S90.
(25) Xu, X.; Harris, K. S.; Wang, H. J.; Murphy, P. A.; Hendrich, S. Bioavailability of soybean isoflavones depends upon gut microflora in women. *J. Nutr.* **1995**, *125*, 2307–2315.
(26) Allred, C. D.; Twaddle, N. C.; Allred, K. F.; Goeppinger, T. S.; Churchwell, M. I.; Ju, Y. H.; Helferich, W. G.; Doerge, D. R. Soy processing affects metabolism and disposition of dietary isoflavones in ovariectomized BALB/c mice. *J. Agric. Food Chem.* **2005**, *53*, 8542–8550.
(27) Kenny, A. M.; Mangano, K. M.; Abourizk, R. H.; Bruno, R. S.; Anamani, D. E.; Kleppinger, A.; Walsh, S. J.; Prestwood, K. M.; Kerstetter, J. E. Soy proteins and isoflavones affect bone mineral density in older women: a randomized controlled trial. *Am. J. Clin. Nutr.* **2009**, *90*, 234–242.
(28) Taguchi, H.; Chen, H.; Yano, R.; Shoumura, S. Comparative effects of milk and soymilk on bone loss in adult ovariectomized osteoporosis rat. *Okajimas Folia Anat. Jpn.* **2006**, *83*, 53–59.
(29) Wang, S. F.; Jiang, Q.; Ye, Y. H.; Li, Y.; Tan, R. X. Genistein derivatives as selective estrogen receptor modulators: sonochemical synthesis and in vivo anti-osteoporotic action. *Bioorg. Med. Chem.* **2005**, *13*, 4880–4890.
(30) Ren, P.; Ji, H.; Shao, Q.; Chen, X.; Han, J.; Sun, Y. Protective effects of sodium daidzein sulfonate on trabecular bone in ovariectomized rats. *Pharmacology* **2007**, *79*, 129–136.
(31) Alekel, D. L.; Germain, A. S.; Peterson, C. T.; Hanson, K. B.; Stewart, J. W.; Toda, T. Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women. *Am. J. Clin. Nutr.* **2000**, *72*, 844–852.
(32) Kim, D. W.; Yoo, K. Y.; Lee, Y. B.; Lee, K. H.; Sohn, H. S.; Lee, S. J.; Cho, K. H.; Shin, Y. K.; Hwang, I. K.; Won, M. H.; Kim, D. W. Soy isoflavones mitigate long-term femoral and lumbar vertebral bone loss in middle-aged ovariectomized mice. *J. Med. Food* **2009**, *12*, 536–541.
(33) Peacock, M. Effects of calcium and vitamin D insufficiency on the skeleton. *Osteoporos. Int.* **1998**, *8*, S45–S51.
(34) Bischoff-Ferrari, H. A.; Shao, A.; Dawson-Hughes, B.; Hathcock, J.; Giovannucci, E.; Willett, W. C. Benefit-risk assessment of vitamin D supplementation. *Osteoporos. Int.* **2010**, *21*, 1121–1132.
(35) Isenor, J. E.; Ensom, M. H. Is there a role for therapeutic drug monitoring of vitamin D level as a surrogate marker for fracture risk? *Pharmacotherapy* **2010**, *30*, 254–264.
(36) Kärkkäinen, M.; Tuppurainen, M.; Salovaara, K.; Sandini, L.; Rikkonen, T.; Sirola, J.; Honkanen, R.; Jurvelin, J.; Alhava, E.; Kröger, H. Effect of calcium and vitamin D supplementation on bone mineral density in women aged 65−71 years: a 3-year randomized population-based trial (OSTPRE-FPS). *Osteoporos. Int.* **2010**, *21*, 2047–2055.
(37) Kröger, H.; Kärkkäinen, M.; Honkanen, R. Calcium and vitamin D in promotion of postmenopausal bone health. *Womens Health (London)* **2010**, *6*, 773–776.
(38) Narva, M.; Collin, M.; Lamberg-Allardt, C.; Kärkkäinen, M.; Poussa, T.; Vapaatalo, H.; Korpela, R. Effects of long-term intervention with *Lactobacillus helveticus*-fermented milk on bone mineral density and bone mineral content in growing rats. *Ann. Nutr. Metab.* **2004**, *48*, 228–234.
(39) Lien, T. F.; Chen, W. L.; Hsu, Y. L.; Chen, H. L.; Chiou, R. Y. Y. Influence of soy aglycon isoflavones on bone-related traits and lens protein characteristics of ovariectomized rats and bioactivity performance of osteoprogenitor cells. *J. Agric. Food Chem.* **2006**, *54*, 8027–8032.
(40) Fonseca, D.; Ward, W. E. Daidzein together with high calcium preserve bone mass and biomechanical strength at multiple sites in ovariectomized mice. *Bone* **2004**, *35*, 489–497.