

Effects of a *Lactobacillus casei* 393 fermented milk product on bone metabolism in ovariectomised rats

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ABSTRACT

The effects of a *Lactobacillus casei* 393 fermented milk product (FMP) on bone metabolism were examined. FMP (>0.1 mg mL⁻¹) supplementation significantly increased osteoblastic MC3T3-E1 cells proliferation whereas skim milk powder supplementation did not show any positive effect up to 1 mg mL⁻¹. The FMP (1%) supplemented ovariectomised rats had increased bone weight, bone mineral density, and bone breaking force compared with control ovariectomised rat. In addition, the activity of tartrate resistant acid phosphatase, a biomarker of osteoclasts, was significantly reduced in the FMP group. Based on the results, the *L. casei* 393 FMP had a preventative effect on bone loss in ovariectomised rats.

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1. Introduction

Osteoporosis is a global public health problem. It is closely associated with the imbalance of bone metabolism; bone resorption is greater than bone formation (Christiansen, 1992).

Milk and dairy products have been considered important for bone health due to their high bioavailable calcium contents (Heaney, 2000; Wong & LaCroix, 1980). In addition to calcium, whey protein, especially its basic fraction, has been proposed as a bone protective ingredient (Takada, Kobayashi, et al., 1997). Takada, Matsuyama, et al. (1997) demonstrated that whey protein enhanced bone breaking force in ovariectomised rats, and higher bone breaking force might be related to increased bone proteins such as collagen. The active basic whey protein fraction that inhibits bone resorption has been named Milk Basic Protein (MBP). In human clinical trials, MBP showed increased bone mineral density in adult women (Aoe et al., 2001), and showed significant positive effects on blood bone formation markers in adult men (Toba et al., 2001), although these two studies have been done only with healthy adults of 20–40 years old.

As a milk protein fragment, glycomacropeptide (GMP) was found to inhibit bone loss in ovariectomised rats by increasing calcium bioavailability (Neeser et al., 2000). In addition to protein and macro protein fragments, fermented milk products can also impart beneficial effects related to bone health. Milk protein-

derived peptides can be generated during fermentation by various lactic strains. These bioactive peptides can modulate physiological functions by offering hormone-like activity based on amino acid composition and sequence (Fitzgerald & Murray, 2006).

Caseinphosphopeptide (CPP) is generated during the fermentation or digestion of milk (Chabance et al., 1998). CPP can increase calcium bioavailability by preventing the precipitation of calcium salts in the small intestine (Bennett et al., 2000). Narva, Hallen, Vaananen, and Korpela (2004) demonstrated that milk products fermented by *Lactobacillus helveticus* effectively increased bone mineral density and bone mineral content in a long term feeding study of growing rats.

The aforementioned study (Narva et al., 2004) suggests that fermented milk products, possibly containing bioactive peptides, have positive effects on bone metabolism. In a preliminary study, an active lactic strain (*Lactobacillus casei* 393) was selected among 20 tested strains, based on the proliferation activity effects it had for osteoblastic MC3T3-E1 cells. Therefore, the present study focused on evaluating the anti-osteoporotic effects of a fermented milk product in ovariectomised rats, and compared its performance with whey protein concentrate (WPC), which has been proposed as a substance suppressing bone resorption (Takada, Matsuyama, et al., 1997).

2. Materials and methods

2.1. Materials

Alpha-modified Eagle's medium (α -MEM) and foetal bovine serum were purchased from Gibco (Grand Island, NY, USA). WPC

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and skim milk powder were purchased from New Zealand Milk Products Inc. (Wellington, New Zealand) and Seoul Milk Co. (Seoul, Korea), respectively. Yeast extract and bacto peptone were obtained from Difco Lab (Livonia, MI, USA). All other chemicals were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

2.2. Production of fermented milk product by *L. casei* 393

Active *L. casei* 393 was inoculated into a reconstituted milk medium (skim milk, 100 g L⁻¹; glucose, 20 g L⁻¹; yeast extract, 3.0 g L⁻¹; bacto peptone, 3.0 g L⁻¹) and incubated at 37 °C for 24 h. The medium was centrifuged at 2000 × g for 30 min at 4 °C to obtain the supernatant, which was designated the fermented milk product (FMP). The supernatant was freeze dried after the pH was adjusted to 6.5.

2.3. Effect of FMP on the proliferation of osteoblastic MC3T3-E1 cells

2.3.1. Cell culture

Mouse osteoblastic MC3T3-E1 cells (America Type Culture Collection, Rockville, MD, USA) were grown at 37 °C in α -MEM medium supplemented with 10% heat-inactivated foetal bovine serum, 100 μ mL⁻¹ penicillin G, and 100 μ g mL⁻¹ streptomycin under a humidified 5% CO₂ atmosphere. The MC3T3-E1 cell line was maintained using the standard 3T3 protocol (Todaro & Green, 1963).

2.3.2. Cell viability

The MC3T3-E1 cells were seeded into 96 well plates (BD Bioscience, San Diego, CA, USA) at a density of 1 × 10⁴ cells per well. After 12 h, the growth media were exchanged to fresh serum-free media containing antibiotics supplemented with of samples (0.01, 0.1, and 1 mg mL⁻¹, as solid basis). The effects of FMP and WPC on the proliferation of MC3T3-E1 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983) after 24 h incubation. The proliferation of the MC3T3-E1 cells in response to the treatments was expressed as relative % based on absorbance of cells grown in 10% serum supplemented treatment. Values are means with their standard deviation.

2.4. Effect of FMP on ovariectomised rats

An animal study was also conducted to evaluate the anti-osteoporotic effects of the FMP in ovariectomised rats. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

2.4.1. Animals

Sixty 10-week old female Spargue-Dawley (SamTacN(SD)BR) rats were purchased from Samtako (Gyung-gi, Korea). The animals were subjected to a sham-operation (SHAM, *n* = 15) or ovariectomy (OVX, *n* = 45) on the following day. For the OVX animals, the ovaries were exposed and removed while the ovaries of the sham-operated animals were exposed but left intact. After surgery, the rats were housed in individual stainless steel cages and kept in a temperature (22 ± 2 °C) and light (12 h day–night cycle) controlled room within the small animal experimental unit (Korea University). On the day of surgery, only water was provided. A commercial rodent diet and water were provided during the recovery period (5 days). After recovery, the rats with the reduced body weights were considered as incomplete recovery and excluded from experiment. The remaining OVX animals were randomized into three groups (*n* = 13

per group), and the sham-operated animals (*n* = 13) served as controls.

2.4.2. Diets

A commercial rodent diet (National Institute of Health (NIH)-31M, Samtako, Kyung-gi, Korea) containing 18% (w/w) protein and 5.3% (w/w) fat was used as the basal diet. Each dietary treatment contained FMP or WPC (1%, w/w), respectively. An amino acid and mineral mixture was formulated based on the nutrient profile of the WPC (Table 1). The amino acid and mineral mixture was added to the control diet (SHAM and OVX) to provide a similar amino acid composition and mineral content to that of the WPC. The body weights and feed intakes of the rats were measured weekly, and the experiment was conducted for 6 weeks.

2.5. Analytical methods

2.5.1. Blood Ca and P

A spectrometry auto-analyzer, Advia 1650 chemistry system (Bayer, Fernwald, Germany), was used to determine the concentrations of Ca and inorganic P in the serum by following the manufacturer's protocols.

2.5.2. Bone physical properties

The adhering flesh and tendons were scraped off from the right femur and the femurs were dried at 80 °C for 2 days. The dry weight of each femur was then measured, after which the length between the proximal end of the intercondylar notch and the proximal limit of the base of the femoral neck was measured using a Vernier calliper. The femurs were dry-ashed in a muffle furnace at 550 °C overnight, and the ash content was determined. Their organic matter weights were calculated as the difference between the dry

Table 1

Nutrient compositions of whey protein concentrate, and amino acid (AA) and mineral mixture (g kg⁻¹) supplemented in SHAM and OVX treatments.^a

Component	Whey protein concentrate	AA and mineral mixture
Amino acids	775	775
Essential amino acid		
Histidine	17	17
Isoleucine	45	45
Leucine	84	84
Lysine	75	75
Methionine	15	15
Phenylalanine	26	26
Threonine	56	56
Tryptophan	16	16
Valine	45	45
Non-essential amino acid		
Alanine	38	–
Arginine	16	–
Aspartic acid	84	–
Cysteine	18	–
Glutamic acid	130	396
Glycine	14	–
Proline	45	–
Serine	37	–
Tyrosine	14	–
Lactose	80	80
Ca	7.75	7.5
P	2	2
Fat	100	100
Cellulose	–	35.5

^a Abbreviations are: SHAM, amino acid and mineral mixture supplementation with no ovariectomy; OVX, amino acid and mineral supplementation with ovariectomy. Non-essential amino acids in the amino acid and mineral mixture were substituted with glutamic acid based on weight. The fat source was corn oil.

weight and the ash weight. Dry-ashed femur was digested using the method described by Kelly, Siobhan, and Kevin (2003). The Ca and P concentrations of the ashed femurs were determined using inductively coupled plasma–optical emission spectrometry (ICP–OES, Varian, Palo Alto, CA, USA).

2.5.3. Dual energy X-ray spectrometry (DEXA) scans

The bone mineral density (BMD) and bone mineral content (BMC) of the dried left femur were measured by dual energy X-ray absorptiometry (DEXA, Norland, USA), using the laboratory animal mode. The beam energy was 22 keV, and the scanning speed was 20 mm s⁻¹ with a 0.5 × 1.0 mm resolution.

2.5.4. Bone strength

The breaking force of the femoral diaphysis (the centre of the femur) was measured with a Texture analyzer (Stable micro system, USA) using three-point bending method (Takada, Matsuyama, et al., 1997). The dried left femur from each treatment were placed in a jig constructor for a three-point bending test, the space of the jig constructor was 1.0 cm.

2.5.5. Bone alkaline phosphatase and tartrate resistant acid phosphatase activity

The left tibias were stored at -70 °C and then used to determine bone alkaline phosphatase (B-ALP) and tartrate resistant acid phosphatase (TRAP) activities. The proximal epiphysis of the left tibia was removed using an RTX™ rotary tool with a heavy duty cut-off disk (Black & Decker, Townson, MO, USA). For protein extraction, approximately 200 mg of the ground proximal epiphysis was placed into a 10 mL tube, and 2 mL of 0.15 M NaCl containing 3 mM NaHCO₃ was added. Homogenization (Ultra Turrax T-25, Ika Works, Wilmigton, NC, USA) was conducted three times in ice with 15 s bursts and 10 s rests. The samples were centrifuged at 1000 × g for 20 min at 4 °C. The B-ALP and TRAP activities were measured using the methods described by Janckila, Takahashi, Sun, and Yam (2001).

2.6. Statistical analysis

All data are presented as means ± SEM. Statistical analysis was performed using the General Linear Model procedure of SAS (1998) with the following model:

$$Y_i = \mu + S_i + e_i$$

where Y_i is the dependent variable, μ the overall mean, S the effect of the dietary treatment, and e_i is the residual error.

A p -value of less than 0.05 was considered significant. The differences among treatments were tested by Duncan's new multiple range test (Duncan, 1955), and the standard errors of means were calculated from the replicate treatments.

3. Results and discussion

3.1. Effect of FMP on the proliferation of osteoblastic MC3T3-E1 cells

The effects of WPC, FMP, and SMP (skim milk powder) on MC3T3-E1 cell proliferation were determined by the MTT assay. As shown in Fig. 1, FMP supplementation increased cell populations in a dose-dependent manner ($P < 0.05$). The addition of 0.01 mg mL⁻¹ FMP to the serum-free media had no effect on MC3T3-E1 cell proliferation while cell numbers were significantly increased by supplementing at concentrations higher than 0.01 mg mL⁻¹. On the other hand, SMP supplementation did not have any significant effect on cell proliferation regardless of the supplementation level.

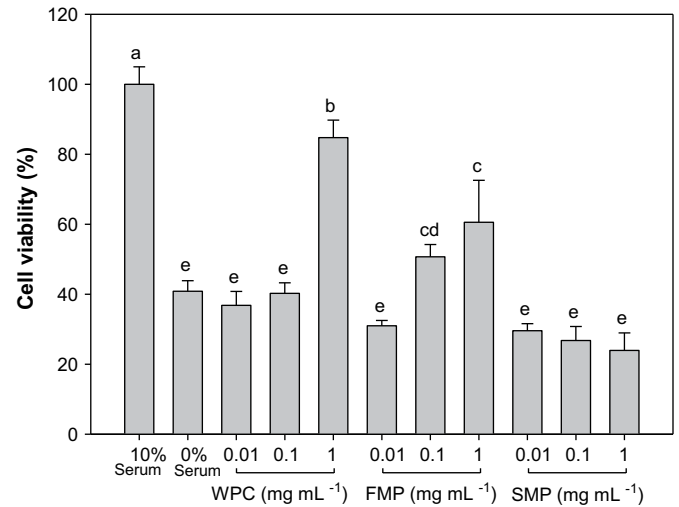


Fig. 1. Effects of whey protein concentrate (WPC), fermented milk product (FMP), and skim milk powder (SMP) on the proliferation of osteoblastic MC3T3-E1 cells.

In the case of WPC, a significant proliferation stimulating effect was observed at the 1 mg mL⁻¹ level only.

Takada, Aoe, and Kumegawa (1996) examined the effect of whey protein on the proliferation and differentiation of osteoblastic MC3T3-E1 cells. The addition of whey protein stimulated cell growth and enhanced the incorporation of hydroxyproline. Although the active components of the *L. casei* 393 FMP were not characterized in the present study, its supplementation resulted in significant increases in MC3T3-E1 cell proliferation. Based on the results of the cell cultures, the effects of the FMP and WPC on bone metabolism were further examined using ovariectomised rats.

3.2. Effect of fermented milk product on bone metabolism in ovariectomised rats

The growth performance of the rats is presented in Table 2. There were no differences in the initial body weights of the rats. At the end of the experiment, the mean body weight of the SHAM group was significantly lower than the mean body weights of the other groups ($P < 0.05$). However, there were no significant differences among the body weights of the OVX groups (OVX, FMP, and WPC) by the different dietary treatments. Ovariectomy increased daily feed intake throughout the experiment ($P < 0.05$). This increased feed intake affected body weight gain and subsequently increased the body weights of the rats in the ovariectomised groups. In addition, the supplementations of WPC and FMP did not have any significant effects on feed efficiency, respectively.

Table 2

Initial and final body weight, body weight gain, feed intake, and feed efficiency of rats fed according to different experimental diets for 6 weeks^a.

	SHAM	OVX	WPC	FMP	SEM
Initial weight (g)	249	245	245	255	2
Final weight (g)	303b	357a	351a	347a	4
Body weight gain (g d ⁻¹)	1.3b	2.7a	2.5a	2.2a	0.2
Feed intake (g d ⁻¹)	16.3b	19.1a	19.7a	19.9a	0.3
Feed efficiency (%)	7.9b	14.1a	12.6a	11.1a	0.8

^a Abbreviations are: SHAM, amino acid and mineral mixture supplementation with no ovariectomy; OVX, amino acid and mineral supplementation with ovariectomy; WPC, whey protein concentrate supplementation with ovariectomy; FMP, fermented milk product supplementation with ovariectomy; SEM, standard error of the mean. All dietary supplementations were 1% of the basal diet weight. The effects of the supplementations were analyzed using General Linear Model followed by Duncan's new multiple test ($n = 13$). The different letters in the same row indicate significant differences ($P < 0.05$).

Table 3
Physical properties of femurs of rats fed different experimental diets for 6 weeks^a.

	SHAM	OVX	WPC	FMP	SEM
Length (mm)	34.3	33.4	34.1	34.5	0.5
Dry weight (mg)	466a	417c	446b	471a	6
Ash weight (mg)	290a	253c	275b	295a	4
Organic weight (mg)	175a	163c	170b	174a	2
Ash (%)	62.3a	60.8c	61.7b	62.3a	0.2
Ash per organic weight	1.65a	1.55c	1.61b	1.66a	0.02
Ca (mg g ⁻¹ of dry bone)	260a	252b	252b	263a	1
P (mg g ⁻¹ of dry bone)	143a	137b	138b	143a	1
Ca (mg g ⁻¹ of bone ash)	417	414	409	419	2
P (mg g ⁻¹ of bone ash)	229	225	225	230	1

^a Abbreviations are: SHAM, amino acid and mineral mixture supplementation with no ovariectomy; OVX, amino acid and mineral supplementation with ovariectomy; WPC, whey protein concentrate supplementation with ovariectomy; FMP, fermented milk product supplementation with ovariectomy; SEM, standard error of the mean. All dietary supplementations were 1% of the basal diet weight. The effects of the supplements were analyzed using General Linear Model followed by Duncan's new multiple test ($n = 13$). The different letters in the same row indicate the significant differences ($P < 0.05$).

Mora-Gutierrez, Farrell, Attaie, Mcwhinney, and Wang (2007) reported that highly bioavailable Ca contained in feed could increase blood Ca levels, and thereby result in increased bone mineralization in rats (Mora-Gutierrez et al., 2007). However, there were no differences in the blood Ca and P levels of the OVX and SHAM groups (Data not shown). Blood Ca and P contents do not usually vary because they are under the precise control of hormone systems such as parathyroid hormone and calcitonin. Similar to the result found in this study, Takada, Matsuyama, et al. (1997) did not find differences in Ca retention and serum Ca levels between ovariectomised rats fed low Ca diets containing 1% whey protein and a control diet group. In addition, Toba, Takada, Tanaka, and Aoe (1999) reported that in growing rats, the Ca source (CaCO₃ versus whey Ca) did not affect Ca absorption but milk component such as lactose and milk protein did. Therefore, the unchanged blood Ca and P levels observed in the present study might suggest there were no differences in the bioavailabilities of Ca and P among the diets.

The physical properties of the femurs are shown in Table 3. Femoral length was not changed by ovariectomy or by supplementation, but femoral dry weight decreased in response to ovariectomy (466 versus 417 mg, $P < 0.05$). The femoral weights of the rats in the WPC and FMP groups were significantly higher than those of the rats in the OVX group ($P < 0.05$). However, the mean femoral weight of the WPC group was lower than the weights of the

SHAM and FMP groups, respectively ($P < 0.05$). In addition, the ash and organic matter weights of the femurs were reduced by ovariectomy ($P < 0.05$). However, these decreases were lessened by the dietary supplementations of WPC and FMP ($P < 0.05$), respectively, and there were no differences in the ash or inorganic matter weights of the SHAM and FMP groups.

The concentrations of two primary minerals, Ca and P, were significantly different in the dried femurs by FMP supplementation ($P < 0.05$). The FMP fed group had significantly higher Ca and P contents than the OVX group ($P < 0.05$), and maintained the same levels as the SHAM group. Although Kruger et al. (2006) reported that the organic weight of the femur was not changed by ovariectomy, the ovariectomised rats in the present study showed decreased organic and inorganic matter weights. Based on previous studies, feeding of whey protein (Takada, Matsuyama, et al., 1997) or milk basic protein (Kato et al., 2000) increases the organic matter in bone, especially collagen specific amino acids such as proline and hydroxyproline, without changes of Ca and P content. In terms of primary mineral deposition, FMP supplementation demonstrated a better Ca and P deposition in femur as compared with WPC.

The bone mineral content (BMC) and bone mineral density (BMD) were also determined (Fig. 2). Ovariectomy resulted in significantly decreased BMC and BMD (SHAM versus OVX). The BMD of the OVX group presented the lowest value, indicating decreased mineral deposition. This reduction in BMC (or BMD) was significantly improved by the supplementations of FMP and WPC, respectively. The BMC of the FMP group was not different from that of the SHAM group. This result was consistent with the mineral content of the femurs (Table 3).

The physical strength of the femur was evaluated using a texture analyzer (Fig. 3). The loading of mechanical stress on bone causes both elastic and plastic deformations under test conditions. Elastic deformation occurs in the initial stage of the stress versus strain curve, and no permanent damage is exerted on the bone. As the mechanical stress increases, the bone is subject to plastic deformation, which results in permanent damage (Turner & Burr, 1993). Although the clear contributions of elastic and plastic deformation could not be determined, the breaking force of the OVX group was significantly lower than that of the SHAM group ($P < 0.05$). However, the bone breaking forces of the WPC and FMP groups were not different from that of the SHAM group ($P > 0.05$).

Previous studies have indicated that increased protein concentration in bone can increase the force required to break it (Kato et al., 2000; Takada, Matsuyama, et al., 1997). Takada, Matsuyama, et al. (1997) examined the relationship between Ca concentration

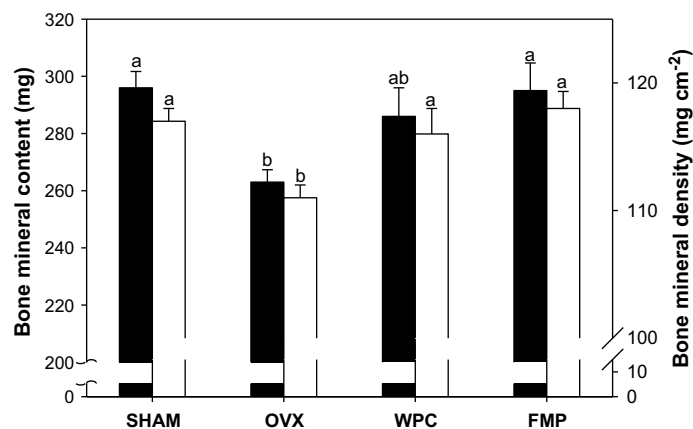


Fig. 2. Bone mineral content (solid bars) and bone mineral density (open bars) of femurs in rats fed different experimental diets for 6 weeks. Abbreviations are: SHAM, amino acid and mineral mixture supplementation with no ovariectomy; OVX, amino acid and mineral supplementation with ovariectomy; WPC, whey protein concentrate supplementation with ovariectomy; FMP, fermented milk product supplementation with ovariectomy.

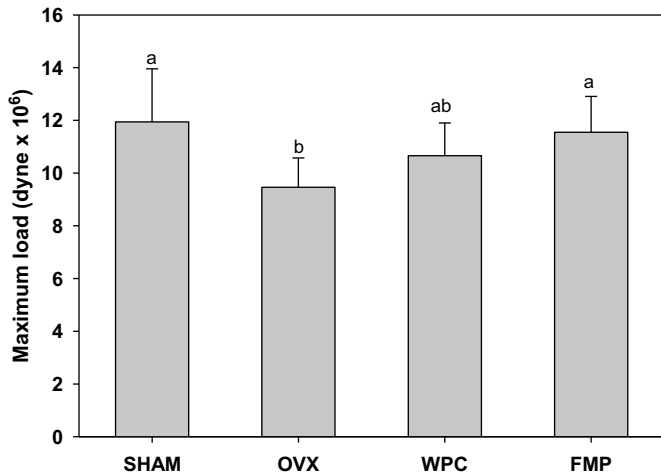


Fig. 3. Bone breaking strength of femurs in rats fed different experimental diets for 6 weeks. Abbreviations are: SHAM, amino acid and mineral mixture supplementation with no ovariectomy; OVX, amino acid and mineral supplementation with ovariectomy; WPC, whey protein concentrate supplementation with ovariectomy; FMP, fermented milk product supplementation with ovariectomy.

in the femur and bone breaking strength. They reported that the bone breaking strength increased linearly as Ca content increased. Therefore, the higher bone breaking force found in the WPC and FMP groups might have been due to increases in both their femoral organic matter and calcium contents.

Bone remodelling is a complex process that involves bone formation and bone resorption. The processes of bone remodelling are performed by specific bone cells, namely osteoblasts and osteoclasts. Bone alkaline phosphatase (ALP), which builds bone, is produced by osteoblasts (van Straalen, Sanders, Prummel, & Sanders, 1991), whereas tartrate resistant acid phosphatase (TRAP), which absorbs bone, is produced by osteoclasts (Burstone, 1959). To investigate which process was affected by dietary supplementation of WPC and FMP, respectively, the activity of each enzyme was determined. The ALP and TRAP activities of the proximal epiphysis of the femur are presented in Fig. 4. ALP activity was not changed by ovariectomy or by the dietary treatments of WPC and FMP, respectively. However, the activity of the bone resorption enzyme, TRAP, increased significantly by ovariectomy. In the ovariectomised

groups, TRAP activity was decreased by the supplementations of WPC and FMP ($P < 0.05$), respectively.

L. casei 393 was selected for fermentation based on the proliferative effects their FMP had on the osteoblastic cell line, MC3T3-E1. We expected increased bone formation activity (ALP) in the FMP supplemented group; however, an increase in ALP activity was not reflected by the results. The increased TRAP activity of the OVX group suggests that bone resorption activity, mediated by osteoclasts, was increased. Kelly et al. (2003) reported that the ALP activity of demineralised bone implants was increased in growing rats fed whey protein, but TRAP activity was not changed. These conflicting results might be due to differences in the animals' growth stages and bone (femoral epiphyses) mineral contents. Sinha, Smith, and Soares (1988) compared the ALP and TRAP activities of demineralised and mineralized bone implants in young and old female rats. ALP activity was much higher in the demineralised bone implants, and TRAP activity was higher in the mineralized bone implants.

Many studies have reported that bone health can be improved by the consumption of dairy products. Increased Ca supply and bioavailability would be involved in this mechanism. Igarashi et al. (1994) reported that the supplementation of *Bifidobacterium longum* increased bone strength by increasing Ca absorption. Phosphorylated casein peptides are shown to improve calcium absorption by binding to calcium and maintaining the cation in a soluble form (Camara-Martón & AmaroLopez, 2002; Tsuchita, Sekiguchi, & Kuwata, 1993).

As a different mechanism, milk basic protein, present in milk whey, can enhance bone strength without affecting Ca absorption and retention. Kato et al. (2000) reported that the administration of milk basic protein did not result in increased femoral Ca content but resulted in increased femoral breaking strength. Here, increased bone protein (proline, hydroxyproline, and hydroxylysine) content was responsible for the increase in bone strength.

Future work will involve the identification of the active substances participating in the bone strengthening effect of the *L. casei* 393 FMP. This beneficial effect might be the result of bacterial metabolites, and more likely, peptides derived from the proteolytic activity of *L. casei* 393. Considering that the types of peptides generated during milk fermentation depend on the bacteria used (Matar, Amiot, Savoie, & Goulet, 1996), *L. casei* 393 could be useful in the production of functional foods consumed to increase bone strength.

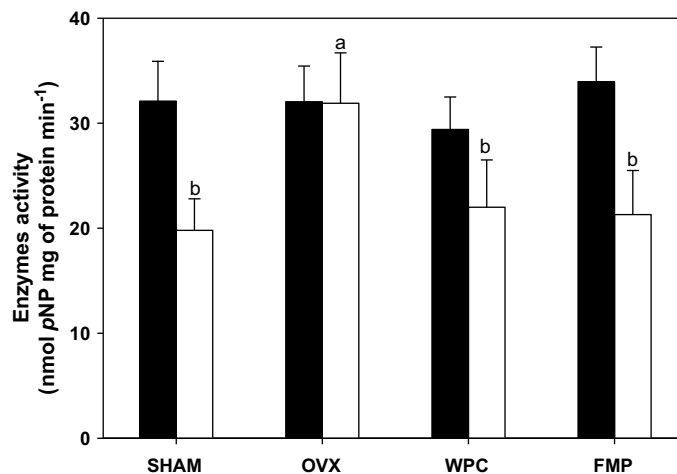


Fig. 4. Alkaline phosphatase (solid bars) and tartrate resistant acid phosphatase (open bars) activities of proximal femoral epiphyses in rats fed different experimental diets. Abbreviations are: SHAM, amino acid and mineral mixture supplementation with no ovariectomy; OVX, amino acid and mineral supplementation with ovariectomy; WPC, whey protein concentrate supplementation with ovariectomy; FMP, fermented milk product supplementation with ovariectomy.

4. Conclusions

In this study, a milk product fermented by *L. casei* 393 significantly attenuated the reduction of bone strength that occurred in response to ovariectomy. These effects were related to increased organic matter and calcium contents in the bone. FMP (1%) increased the bone weight, bone mineral density, and bone breaking force of femurs in supplemented rats as compared with OVX rats. In addition, TRAP activity was significantly reduced in the FMP group. Postmenopausal osteoporosis in humans is related to increased bone resorption by osteoclasts and thereby decreases bone strength. Based on the above results, the consumption of an *L. casei* 393 FMP could help reduce the risk of osteoporosis.

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