

Enhancement of calcium absorption and bone health by fermented soybean

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Abstract

The effects of metabolites produced during soy fermentation on calcium absorption and maintaining bone health have been studied in the mice model by measuring serum calcium and phosphorus levels, bone mineral density (BMD) and arterial calcification. The nutritional composition of different experimental diets was determined. Both bone mineral content (BMC) and the cross-sectional area of the shaft of the femur were significantly ($p < 0.01$) increased and had a much greater effect on BMD when mice were treated with experimental diet D3 (dehulled soybean fermented with *Bacillus subtilis* MTCC 2756) and diet D4 (dehulled soybean fermented with *Rhizopus oligosporus* NCIM 1215 and *B. subtilis* MTCC 2756) as compared to diet D1 (unfermented dehulled soybean). The group of mice treated with experimental diets D3 and D4 did not show calcium deposition in the arterial wall. The beneficial effects may be due

to aglycone isoflavones as well as a higher amount of vitamin K2 in experimental diets D3 and D4.

Introduction

Calcium and phosphorus are mainly found in bone and teeth, as a hydroxyapatite crystalline structure which provides rigidity and strength. Calcium is an important component for mediating vascular contraction and vasodilatation, transmission of nerve impulses, hormonal secretion and bone growth, and plays a crucial role in metabolism [1]. Some researchers have reported that the ratio of calcium to phosphorus regulates intestinal calcium absorption and bone mineralization [2, 3]. A reduction in the calcium to phosphorus ratio in the diet reduces total bone mineral storage and turnover by affecting intestinal calcium and phosphorus absorption. A marked loss of BMC but with maintenance of calcium retention could result in an increase in soft-tissue calcification, such as vascular calcification and nephrocalcinosis [4].

It has been reported that excess intake of calcium without phosphorus supplementation reduced bone mineral density (BMD) in post-menopausal women [5]. Adequate calcium and phosphorus intake is therefore recommended for healthy bone, for attaining peak bone mass and for the prevention of osteoporosis [6]. However, the health benefits to be expected from calcium depend both on

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how much calcium is consumed and also on the absorptive efficiency of the ingested calcium, because intestinal calcium absorption is influenced by many dietary factors such as phytic acid and oxalate which form insoluble and unabsorbable complexes with calcium and retard the rate of calcium absorption in the intestine [7–9]. Thus, although soybeans have a high calcium content they are considered a poor source of calcium as they contain phytic acid (the storage form of phosphorus) that chelates the calcium and retards its absorption in the intestine.

Soybean fermented with *Bacillus subtilis* and *Rhizopus oligosporus* has reduced anti-nutrients (phytic acid) and produces therapeutically active primary and secondary metabolites like phytase, γ -polyglutamic acid [10], vitamin K₂ [11] and aglycone isoflavone [12].

Degradation of phytic acid can be achieved by endogenous phytase of soybean and by phytase-producing microorganisms during fermentation. Furthermore, the degradation products consist of lower inositol phosphates and inorganic phosphates, which can be absorbed into the intestine [13, 14]. Therefore, the consumption of fermented soybean, because of the presence of bioactive substances such as polyglutamic acid and phytase MK-7, may improve the absorption of calcium from the small intestine and increase BMD.

Gamma-polyglutamic acid (γ -PGA) is a negatively charged polymer of glutamic acid and chelation of calcium by γ -PGA inhibits the precipitation of calcium salts and thereby increases calcium bioavailability in the entire small intestine [15, 16].

Vitamin K₂ is important for post-translational carboxylation of the bone matrix protein osteocalcin, which is involved in bone mineralization and Ca²⁺ ion homeostasis. It is also essential for activating matrix Gla protein, a calcification inhibitor that is expressed in vascular tissues and removes calcium deposits from arteries [17–19]. Therefore, vitamin K₂ is an essential component for maintaining bone density while simultaneously protecting against atherosclerosis in the vascular system.

In the present work, we have developed different types of soy-based foods fermented with either *B. subtilis* or *R. oligosporus* and co-culture of both of these microbes under solid state fermentation

conditions. The nutritive effects of these fermented foods fed as experimental diets to mice were compared to an experimental diet containing only unfermented soybeans. Here, we show that these fermented soy foods are unique in terms of their nutritional components, namely aglycone isoflavones, phytase, γ -PGA and menaquinones. The beneficial effects of these metabolites (in different concentrations) on calcium absorption and maintaining bone health have been studied in the mice model by measuring serum calcium and phosphorus levels, BMD and arterial calcification.

Materials and methods

Soybeans, microorganisms and chemicals

The soybean variety Kh-09 Bragg was received as a gift sample from the Pulse Laboratory of the Indian Agricultural Research Institute, New Delhi, India. *Rhizopus oligosporus* NCIM 1215 was collected from the National Collection of Industrial Microbes, National Chemical Laboratory, Pune, India. A fungal culture was maintained on potato dextrose agar (PDA) medium and sub-cultured every 30 days. *Bacillus subtilis* MTCC 2756 was procured from the Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India and maintained on nutrient agar medium with sub-culturing every 30 days.

Soy isoflavones (daidzin, genistin and glycitin) and polyglutamic acid were from Sigma-Aldrich Chemicals, Bangalore, India. Phytic acid, β -D-glucopyranoside and p-nitrophenyl pyruvate were from MP Biomedicals, Santa Ana, CA, USA and menaquinone-7 was from Medley Pharmaceuticals, Mumbai, India. All other media and chemicals were purchased from HiMedia, Mumbai, India and Merck, Mumbai, India.

Preparation of experimental diets

Soybeans were washed, soaked for 15 h at 25°C, dehulled and autoclaved at 121°C for 15 min, cooled to 37°C and subjected to solid-state fermentation with different microbes (*R. oligosporus* and *B. subtilis*) in separate and combined fermentation processes for 48 h at 37°C to generate different products with different nutritive values. Freshly

fermented soybeans (2 g) were ground, mixed with 10 ml of water and centrifuged at 3000 rpm for 15 min. The supernatant was stored at 4°C until used in experimental diets.

The unfermented dehulled soybeans were prepared as above except for omission of the inoculation and fermentation steps.

The diets were as follows:

- Diet D1: unfermented dehulled soybean
- Diet D2: dehulled soybean fermented only with *R. oligosporus* NCIM 1215
- Diet D3: dehulled soybean fermented only with *B. subtilis* MTCC 2756
- Diet D4: dehulled soybean fermented with both *R. oligosporus* NCIM 1215 and *B. subtilis* MTCC 2756.

The compositions of the different experimental diets are given in Table 1. All experimental diets were supplemented with calcium (130 mg/kg).

Nutritional analysis of experimental diets

All fermented and unfermented soybean diets were analysed for nutritional content.

Soy isoflavones were extracted using 80% acidic acetonitrile [20]. High-performance thin layer chromatography (HPTLC) was performed using mobile phase toluene:ethyl acetate:formic acid:acetic acid in the ratio 1:8:1:0.5, v/v/v/v. Ultraviolet detection was performed densitometrically at the maximum absorbance wavelength, 260 nm [21]. Aglycone isoflavone concentration was calculated according to the formula described by Prasad and Shah [22]. Phytase was determined according to the method of Kim and Lei [23] and γ -PGA was analysed according to method de-

scribed by Zeng *et al.* [24]. MK-7 was analysed according to the method of Kapoor and Panda [25].

Mice and experimental diet treatments

Adult male Swiss albino mice weighing 25–30 g were obtained from the Central Animal House, Jamia Hamdard, New Delhi. The study was approved by the animal ethics committee of Jamia Hamdard (934/2011), and the mice were maintained in accordance with the ethical guidelines for the care and handling of laboratory animals. Prior to the beginning of the study, animals were acclimatized for 7 days in a room maintained at 23±2°C under a 12 h light–dark cycle.

The mice were given food and water ad libitum throughout the study. Mice were divided into seven dietary groups (groups I–VII) containing eight mice each and simultaneously received oral doses of the following solutions for a period of 4 weeks:

- Group I: water+0.5% carboxymethylcellulose (CMC)
- Group II: CaCO₃
- Group III: diet D1+CaCO₃
- Group IV: diet D2+CaCO₃
- Group V: diet D3+CaCO₃
- Group VI: diet D4+CaCO₃
- Group VII: γ -PGA (10 mg/kg)+CaCO₃.

Determination of calcium balance

From day 22 to day 27 of the study, all mice were placed in individual metabolic cages for the separate collection of faeces and urine. To acclimatize the mice to their new environment, they were placed in the metabolic cage 2 days before the beginning of a 4-day metabolic study for the determination of net calcium absorption. The urine and faeces of each mouse were collected simulta-

Experimental diet	PGA (g/100 g)	Vitamin K (mg/100 g)		Phytase (IU/100 g)	Phytic acid (mg/100 g)	Glycosidic isoflavones (mg/100 g)			Aglycone isoflavones (%) ^a			Calcium (mg/100 g)	Phosphorus (mg/100 g)
		MK-7	MK-4			Daidzin	Genistin	Glycitin	Daidzein	Genistein	Glycitein		
D1	–	–	–	0.891	251*	27.690	59.754	10.940	–	–	–	272	704
D2	–	–	–	9.726	92	1.313	8.434	2.955	95.25*	85.88*	72.98*	276	712
D3	0.1822	0.512	0.0017	4.077	107	6.493	12.218	1.644	76.55	79.55	84.97	278	726
D4	0.1548	0.932	0.0014	10.818*	87	3.626	9.816	1.120	86.90	83.57	89.76	284	744

* $p < 0.05$. ^aAglycone isoflavones = initial glycosidic isoflavones – residual glycosidic isoflavones / initial glycosidic isoflavones $\times 100$

Table 1 - Chemical composition of different experimental diets

neously but separately for 24 h and analyzed to determine calcium content. The food consumption of each mouse was determined on each day of the 4-day metabolic study.

Measurement of calcium and phosphorus content in urine and faeces

The faecal and urine samples of each animal were collected. The urine was acidified with 12N HCl solution and stored at -20°C until analysis. The faecal mass was dried overnight at 100°C and dried faecal samples were ash-dried at 700°C . The faecal ashes were solubilized in 6N HCl and analyzed for calcium and phosphorus content. The calcium and phosphorus content in urine and faecal samples was measured using commercially available kits (SPAN Diagnostics, Surat, India), as per the manufacturer's guidelines.

Apparent calcium absorption, the apparent calcium absorption rate, and the apparent calcium balance were calculated using following formulas:

- Apparent Ca absorption = Ca intake – faecal Ca
- Apparent Ca absorption rate (%) = $100 \times (\text{Ca absorption} / \text{Ca intake})$
- Apparent Ca balance = Ca intake – faecal Ca – urinary Ca [16].

Serum analysis

The mice were fasted overnight prior to blood collection on study days 10 and 20 from the retro-orbital venous plexus with a capillary tube for calcium analysis. Similarly, on day 30 blood was collected with a capillary tube from the retro-orbital venous plexus for biochemical measurements (calcium, phosphorus and alkaline phosphatase (ALP) activity analysis). After blood sampling, the mice were euthanized for collection of the thoracic aorta for examination of calcium deposition and of femurs for BMD and histological examinations. Blood samples were stored at room temperature at least for 30 min before the serum was separated by centrifugation at 3000 rpm for 15 min at 4°C . Supernatants (serum) were stored at -20°C until analysis. Serum calcium and phosphorus were analysed using the same method as for faecal and urine samples. Serum ALP activity was determined using a commercially available kit (SPAN Diagnostics).

Bone sample collection and analysis

The right femur was dissected out and soft tissues were removed and stored at -20°C . The femur was defrosted for 30 min before DXA (dual energy X-ray absorptiometry) scanning using a Hologic DXA machine (Discovery A model, Hologic software version 12.5; Hologic, Marlborough, MA, USA) adapted for use in small animals.

Histopathological examination

Haematoxylin-eosin staining

After decalcification in 10% buffered EDTA, left femurs were embedded in paraffin and sections were cut and stained with haematoxylin-eosin for histological analysis.

Von Kossa's method

The aorta was accessed through the left ventricle and slit open longitudinally. Then the entire length of the aorta from the base of the aortic arch up to the diaphragmatic hiatus was resected out, washed in ice-cold saline, trimmed of adventitial fat and stored in formal calcium (10% formalin, 1% calcium chloride). Mineral deposition was assessed by von Kossa staining. The aorta was fixed in 3.5% formaldehyde solution, and then treated with 5% silver nitrate solution for 30 min under ultraviolet irradiation to detect mineral deposition, and the images captured with a digital microscope (Leica Microsystems, Wetzlar, Germany).

Statistical analysis

All values of in vitro experimental results are presented as the mean \pm standard deviation (SD), while in vivo results are shown as the mean \pm standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 3.0 (GraphPad, San Diego, CA) using one-way ANOVA followed by Tukey's pairwise multiple comparison procedures. A p value of less than 0.05 was considered significant.

Results

Analysis of experimental diets

The composition of the experimental diets (unfermented and fermented soybeans) was analysed and the results (per 100 g) are presented in Table 1.

Experimental diet D1

(unfermented soybean; low phytase, without PGA)

Analysis showed that unfermented soybeans contained phytase 0.891 IU/100 g and glycosidic isoflavones (daidzin 27.69±2.91 mg/100 g, genistin 59.75±1.85 mg/100 g and glycitin 10.941±2.24 mg/100 g). Genistin showed the highest content followed by daidzin and glycitin as seen in Table 1. Diet D1 contained a significantly ($p<0.05$) higher amount of phytic acid (215 mg/100 g) compared to the other experimental diets.

Experimental diet D2

(fermented soybean; high phytase, without PGA)

Soybeans fermented with *R. oligosporus* contained 9.726% phytase and significantly ($p<0.05$) higher amounts of aglycone isoflavones (95.25% daidzein, 85.88% genistein and 72.98% glycitein) than other experimental diets.

Experimental diet D3

(fermented soybean; low phytase and high PGA)

Soybeans fermented with *B. subtilis* contained 0.182% PGA, 4.04% phytase, 0.514% total MKs (MK-4 and MK-7) and bioactive aglycone isoflavones (76.55% daidzein, 79.55% genistein and 84.97% glycitein) as shown in Table 1.

Experimental diet D4

(fermented soybean; high phytase and high PGA)

The co-culture fermented soybeans contained metabolites such as 0.154% PGA, 10.81% phytase, 0.933% total MKs (MK-4 and MK-7) and aglycone isoflavones (86.90% daidzein, 83.57% genistein and 89.76% glycitein).

Phytase content was significantly ($p<0.05$) higher in experimental diet D4 (fermented with a co-culture of *R. oligosporus* NCIM 1215 and *B. subtilis* MTCC 2756), followed by experimental diet D2 (fermented with *R. oligosporus* NCIM

1215) and experimental diet D3 (fermented with *B. subtilis* MTCC 2756), as shown in Table 1. There was an increase in the content of aglycone isoflavones in experimental diets D2, D3 and D4 as a result of solid-state fermentation with *B. subtilis* and *R. oligosporus*, while the content of glycosidic isoflavones was decreased in these three diets. A reduction in phytic acid content to 155 mg/100 g was achieved by soaking, with further reductions to 92 mg/100 g after fermentation with *R. oligosporus* (experimental diet D2) and to 107 mg/100 g after fermentation with *B. subtilis* (experimental diet D3). Phytic acid content was reduced to 87 mg/100 g in experimental diet D4 (co-culture diet).

Bone turnover markers (serum ALP level)

The differences in serum ALP activity between the groups treated with the different experimental diets are shown in Fig. 1. ALP activity was significantly ($p<0.01$) different in group II (positive control) compared to the control group. ALP activity was significantly ($p<0.01$) increased in group IV (high phytase, but no PGA) compared to group III (low phytase, but no PGA). ALP activity in group VII was significantly ($p<0.05$) increased compared to group II (positive control). A significant ($p<0.001$) rise in serum ALP activity was observed in groups V and VI treated with experimental diet D3 and diet D4, respectively, compared to group III treated with experimental diet D1 (unfermented soybeans).

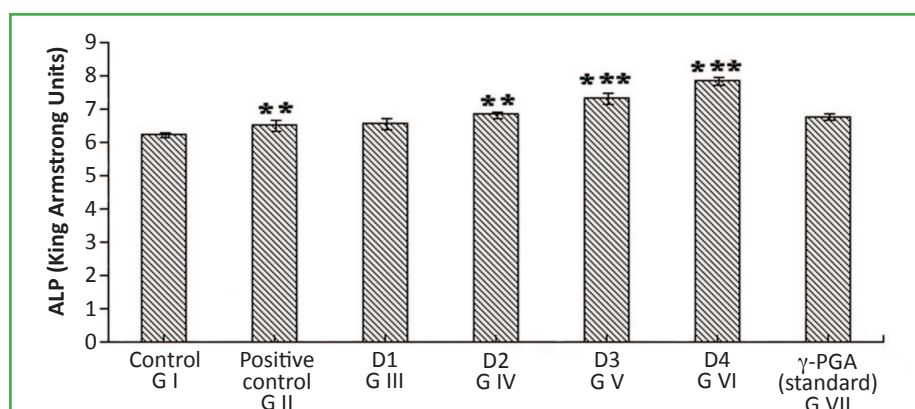


Figure 1 - Serum alkaline phosphatase (ALP) level in animals in group I (control), group II (positive control), group III (treated with experimental diet D1), group IV (treated with experimental diet D2), group V (treated with experimental diet D3), group VI (treated with experimental diet D4) and group VII (treated with γ -PGA). ALP activity was significantly different in group II compared to group I ($p<0.01$), in group IV compared to group III ($p<0.01$), in group VII compared to group II ($p<0.05$), and in both groups V and VI compared to group III ($p<0.001$). G = group

Serum calcium and phosphorus content

Serum calcium levels on days 10, 20 and 30 of treatment with the experimental diets are shown in Fig. 2, while the effects of diets on serum phosphorus are shown in Table 2.

Results demonstrated that by day 30, the serum calcium level was significantly ($p<0.05$) increased in group III (fed with experimental diet D1), group IV (fed with experimental diet D2) and group VI animals (fed with experimental diet D4) compared to group II (positive control) animals. In group V animals (fed with experimental diet D3), the serum calcium level was significantly ($p<0.01$) higher than in group II animals. Group III animals had significant ($p<0.01$) higher serum

phosphorus levels, while group VII animals had significantly ($p<0.01$) lower serum phosphorus levels compared to control mice (group I).

Apparent calcium absorption, apparent calcium absorption ratio and apparent calcium balance

The effects of the experimental diets supplemented with calcium on apparent calcium absorption, apparent calcium absorption ratio and apparent calcium balance are shown in Table 3. In the calcium balance experiment, faecal mass and urine volume were not influenced by diet treatment, but calcium content decreased in the group treated with PGA-containing diets. These results show that the high PGA-containing diets D3 and D4 significantly ($p<0.01$) increased apparent calcium absorption compared to the control. This indicates higher calcium retention and increased apparent calcium balance, BMC and calcium content in the bone of groups V and VI animals.

BMC and BMD

The effect of different experimental diets together with calcium supplements on the femoral shaft cross-sectional area, BMC and BMD of experimental animals is shown in Table 4. The femoral shaft cross-sectional area, BMC and BMD were significantly ($p<0.05$) different in group II (positive control) compared to control group mice. Both BMC and the femoral shaft cross-sectional area were significantly ($p<0.01$) increased and had a much greater effect on BMD when mice were treated with experimental diet D3 (group V) and diet D4 (group VI), as compared to unfermented soybean diet

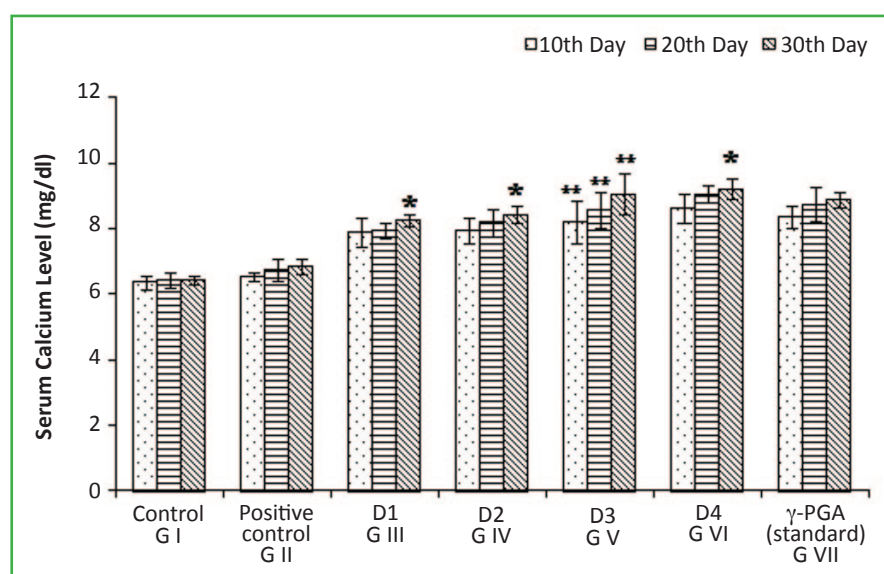


Figure 2 - Serum calcium levels in animals in group I (control), group II (positive control), group III (treated with experimental diet D1), group IV (treated with experimental diet D2), group V (treated with experimental diet D3), group VI (treated with experimental diet D4) and group VII (treated with γ -PGA) on the 10th, 20th and 30th days of treatment with experimental diets. Serum calcium level was significantly ($p<0.05$) increased in animals in groups III, IV and VI and significantly ($p<0.01$) higher in group V animals compared to group II animals. G = group

Group	Dietary treatment	Serum P (mg/dl)	Faecal P (mg/day)	Urine P (mg/day)
I	Control	10.15±0.14	20.23±0.11	19.46±0.27
II	Positive control	10.36±0.10	21.49±0.48	18.66±0.30
III	D1	11.22±0.15**	28.65±0.48**	45.603±0.24***
IV	D2	11.73±0.19	26.64±0.33	40.51±0.21
V	D3	10.51±0.25*	25.39±0.34*	35.68±0.38***
VI	D4	11.45±0.16	23.233±0.38	34.18±1.72
VII	γ -PGA (standard)	9.56±0.25**	26.436±0.52**	18.88±0.31

All values are expressed as mean±SEM (n=8)

* $p<0.05$, vs. group I (control) ** $p<0.01$, vs. group I (control) *** $p<0.001$, vs. group I (control)

Table 2 - Effect of experimental diets on serum, faecal and urine phosphorus (P) levels

D1. Group VI treated with diet D4 showed a significant ($p < 0.05$) increase in BMC and BMD compared to the other experimental diets. There was also a significant ($p < 0.001$) increase in the BMC and BMD

of the group VII animals treated with γ -PGA when compared to the control group mice. These findings indicate that prolonged intake of diets D3 and D4 with calcium supplementation can improve the BMC and BMD of mice.

Histological examination of bone and aorta

The results of histopathological examination of the bone and arteries from treated and untreated animals are shown in Fig. 3. Atherosclerotic plaque formation was examined by staining the surface of the aorta with Oil Red O. Our hypothesis is that higher phosphorous intake can increase calcium deposition. Surprisingly, however, diets D3 and D4 significantly suppressed the development of atherosclerotic plaque compared to diets D1 and D2 (Fig. 3C1,D1). Normal coronary artery (control group) showed an intact intima (Fig. 3A1). The mice fed with only calcium (positive control) showed discontinuous endothelium with some minor changes in the surrounding cardiac tissues, and minor calcium deposition was observed (Fig. 3B1). The groups treated with experimental diets D3 and D4 had a continuous endothelial layer, and no calcium deposition was found in the artery lumen (Fig. 3E1,F1).

Discussion

This study has provided the first experimental evidence of the effects of an unfermented soy diet (D1) and fermented soy diets (D2, D3 and D4) on calcium absorption, bone

Group	Dietary treatment	Faecal Ca (mg/day)	Urine Ca (mg/day)	Apparent Ca absorption (mg/day)	Apparent Ca absorption ratio (%)	Apparent Ca balance (mg/day)
I	Control	14.63±1.12	0.758±0.0081	16.09±1.54	52.90±0.18	15.146±0.95
II	Positive control	16.69±0.98	0.824±0.007	17.84±1.77	51.93±1.32	16.95±1.34
III	D1	15.28±0.87	0.767±0.0206	19.32±2.12	56.46±2.04	19.06±1.67
IV	D2	14.97±1.32	0.758±0.0121	20.82±1.67	57.35±2.15	18.94±0.92
V	D3	13.28±1.21	0.664±0.0166	21.89±2.17**	62.68±1.43**	21.04±1.78**
VI	D4	13.35±1.56	0.624±0.00721	21.71±1.43**	61.95±0.98**	21.37±1.28**
VII	γ -PGA (standard)	13.78±1.72	0.556±0.0250	21.57±0.90	60.74±1.12	20.56±1.84

All values are expressed as the mean±SEM (n=8)
** $p < 0.01$, vs. group I (control)

Table 3 - Effect of experimental diets on faecal calcium (Ca), urine Ca, apparent Ca absorption, apparent Ca absorption ratio and apparent Ca balance

health and arterial calcification. In this study, fermented diets containing several bioactive substances such as menaquinones, aglycone isoflavones, polyglutamic acid and phytase in different proportions (experimental diets D2, D3 and D4) were examined. Our results have shown that fermented soybean diets D2, D3 and D4 along with calcium supplementation improve serum calcium level, ALP activity, BMC and BMD. We speculate that these results are influenced by the synergistic effects of several bioactive metabolites that are created during fermentation with microbes (*B. subtilis* and *R. oligosporus*) individually (diets D2 and D3) or together (diet D4). Soybean naturally contains glycosidic isoflavones as a major component; after fermentation with *B. subtilis* [26] and *R. oligosporus* [27], the glycosidic form is converted into the aglycone form via microbial β -glucosidase enzyme activity. The bioavailability of aglycone isoflavone in humans was greater than that of the corresponding glycosidic form [28, 29]. Soy isoflavones were also reported to enhance calcium absorption in

Group	Dietary treatment	Area (cm ²)	BMC (g)	BMD (g/cm ²)
I	Control	0.27±0.030	0.017±0.002	0.058±0.004
II	Positive control	0.32±0.041*	0.019±0.001*	0.061±0.005*
III	D1	0.27±0.065	0.018±0.002	0.062±0.002
IV	D2	0.25±0.032	0.020±0.003	0.081±0.002
V	D3	0.32±0.022**	0.028±0.002**	0.087±0.001***
VI	D4	0.31±0.034**	0.029±0.005**	0.092±0.001***
VII	γ -PGA (standard)	0.32±0.053***	0.022±0.006***	0.068±0.002***

All values are expressed as mean±SEM (n=8)
* $p < 0.05$, vs. group I (control) ; ** $p < 0.01$, vs. group I (diet D1); *** $p < 0.001$, vs. group I (control)

Table 4 - Effect of experimental diets on femoral shaft cross-sectional area, bone mineral content (BMC) and bone mineral density (BMD)

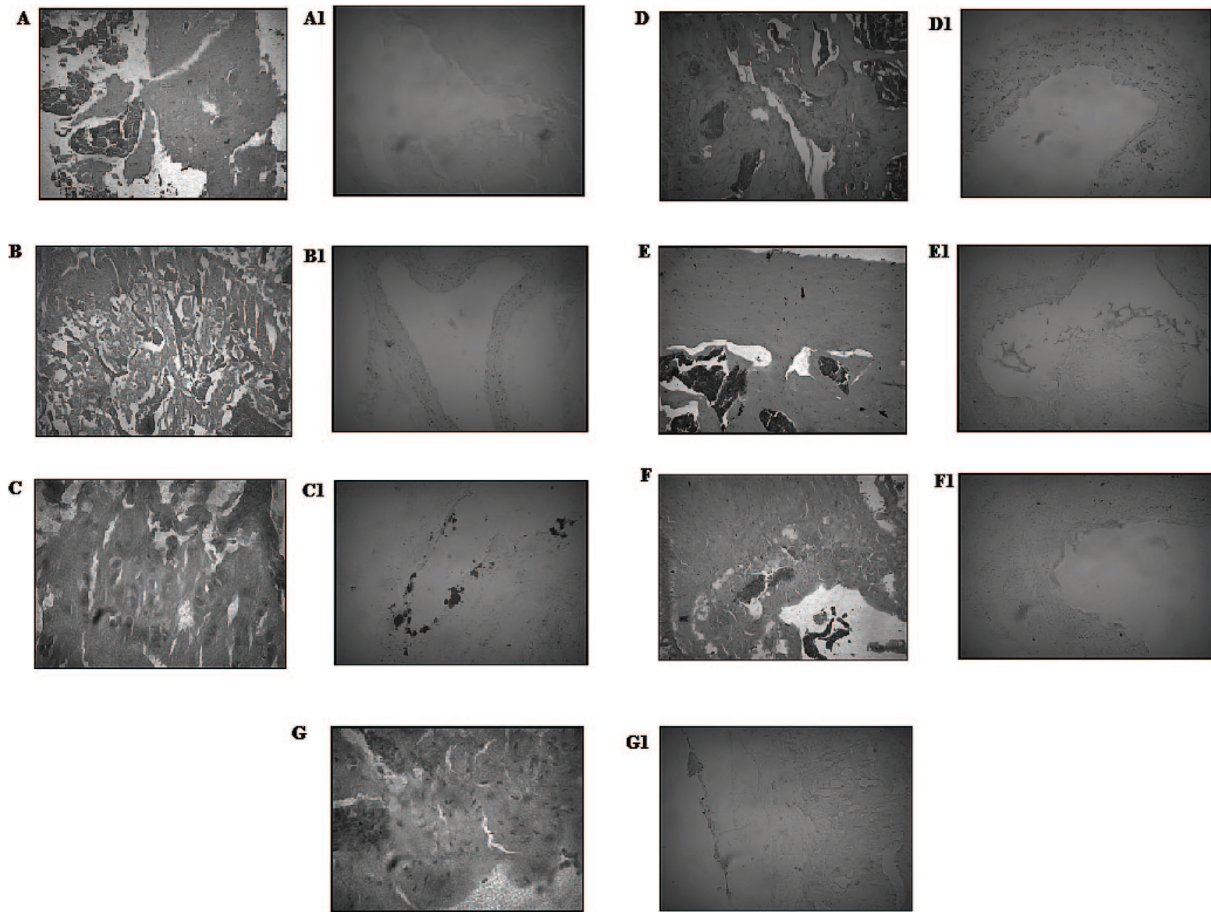


Figure 3 - Histopathological examination of haematoxylin-eosin stained left femur bones: A, B, C, D, E, F and G represent animals in groups I, II, III, IV, V, VI and VII, respectively. Von Kossa's stained aorta: A1, B1, C1, D1, E1, F1 and G1 represent animals in group I (showing normal coronary artery), II (showing minor calcium deposition), III and IV (showing development of atherosclerotic plaque), V and VI (showing continuous endothelial layer, and no calcium deposition) and VII (showing calcium deposition), respectively

rats [30]. Therefore, isoflavones may be the factor responsible for the high calcium absorption ratio in groups IV–VI observed in this study.

Among the experimental diets, the level of phytic acid was found to be reduced in experimental diet D4 because of the presence of microbial phytase, which hydrolyses phytic acid into inositol and orthophosphate. Egounlety and Aworh [31] have reported that fermentation of soybean resulted in a 30.7% decrease in phytic acid content and therefore the increase in the bioavailability of phosphorus in fermented food could be attributed to microbial activity. A reduction in phytic acid considerably increases the availability of minerals such as calcium and zinc [32]. The inhibitory effect of phytic acid on calcium absorption in humans has been described [33], and calcium absorption from low-phytate soybeans was reported to be significantly higher than that from high-phytate soy-

beans [8]. During fermentation, phytase reduces phytic acid, which could have enhanced phosphorus availability in experimental diets D2, D3 and D4. Furthermore, faecal excretion of both calcium and phosphorus was decreased in experimental diets D2, D3 and D4 compared to experimental diet D1 (unfermented soybeans). This calcium alone, however, is not usually sufficient to provide the recommended daily intake, as calcium in the diet forms insoluble and un-absorbable complexes in the intestine, which reduces intestinal calcium absorption. Consequently, strategies that increase the bioavailability of calcium or reduce the phytic acid content of major sources of dietary calcium (e.g., soybean) are helpful. Fermented soy foods with calcium supplements can provide a useful alternative source of calcium for subjects who do not consume dairy products, and the superior absorption of calcium from low-phytate soy food

may be advantageous. Fermented soy foods could be beneficial to increase calcium absorption and BMD in humans.

According to Murao [34], the molecular weight of PGA produced by *B. subtilis* ranges from 10^3 to 10^6 and differs depending on the bacterial strain and the purification procedure. The molecular weight of the PGA used for this study was considered to be within this range. Results showed that the PGA content of soybeans fermented with *B. subtilis* (diet D3) and with *B. subtilis* and *R. oligosporus* (diet D4) was 0.182% and 0.154%, respectively.

Furthermore, our results regarding the effects of different diets on bone mineralization revealed that PGA increased serum calcium level while decreasing serum phosphorus level. It also increased BMC in the femur and increased bone density. An increased serum calcium level caused by PGA and phytase in the diet could be explained by the reduced faecal and urinary calcium excretion. These findings were partially similar to those of a previous study which reported that PGA in the diet has a role in increasing the BMC and BMD of the femur in rat [16].

We have also seen that consumption of diets D3 and D4 can reduce calcium deposition in the aorta due to the presence of metabolites like vitamin K₂, which is mainly produced during fermentation of soybeans with *B. subtilis*. Gast *et al.* [19] showed that MK-7 is involved in coronary calcification reduction and could lower the risk of cardiovascular disease. MK-7 is suggested to remove calcium deposits from arteries and increase deposition in bones [35] and could be effective in the carboxylation of Gla protein, which is a major inhibitor of cardiovascular disease [19, 36]. This report supports our belief that fermented soy foods are better than unfermented soy foods. Soybeans fermented with *B. subtilis* (diet D3) and with *B. subtilis* and *R. oligosporus* (diet D4) showed the best results among the fermented soy foods studied.

In conclusion, the present study indicates that the consumption of soy-based fermented food significantly increased the bioavailability of calcium as well as bone mineralization. It is worth noting that diet D4 containing metabolites such as MK-7, PGA, phytase and isoflavones, was the

best at increasing calcium bioavailability. Thus, the present work directs attention towards the beneficial role of fermented soy containing therapeutically active metabolites which help enhance mineral bioavailability and subsequently may be useful in reducing loss of skeletal calcium and in preventing arterial calcium deposition in humans.

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Conflict of Interest

The authors declare there are no conflicts of interest.

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