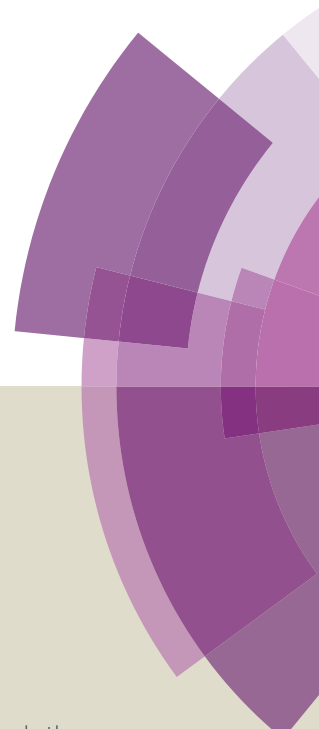
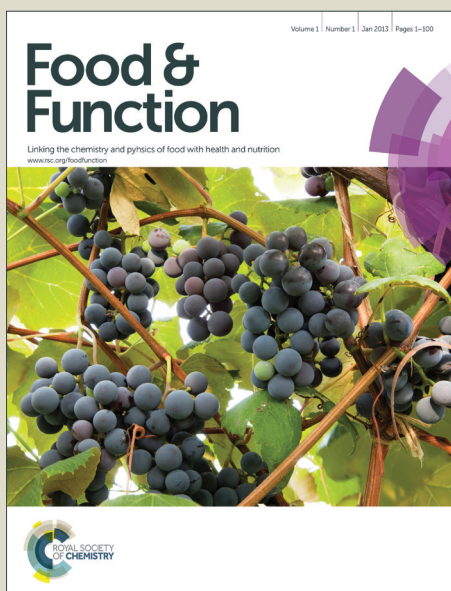


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## Effects of the amount and source of dietary protein on bone status in rats

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**ABSTRACT**

The study examined the effects of the dietary amount and source of protein on bone status in rats. 140 male Wistar rats aged 8 weeks were randomly allocated to 4 groups (n=35) fed normal-protein (NP, 10% richness), or high-protein (HP, 45% richness) diets based on whey protein (WP) or soy protein (SP) sources for 12 weeks. Plasma urea was 46% higher for the HP compared to the NP diet ( $p<0.001$ ). Urinary calcium was 65% higher for the HP compared to the NP and 60% higher for the WP compared to the SP diets (all,  $p<0.001$ ). Urinary pH was 8% more acidic in the HP compared to the NP diet ( $p<0.001$ ) and 4% in the WP compared to the SP diet ( $p<0.01$ ). Plasma osteocalcin concentration was 19% higher for the NP compared to the HP ( $p<0.05$ ) and 25% for the SP compared to the WP diets ( $p<0.01$ ). Femur ash, metaphyseal and diaphyseal cross-sectional, trabecular and cortical areas were 3% higher in the HP compared to the NP diet (all,  $p<0.05$ ). Femur diaphyseal periosteal and endocortical perimeters were also 3% higher in the HP compared to the NP diet (both,  $p<0.01$ ). Groups fed the SP diet showed 2% higher femur ash percentage, 7% higher calcium content (both,  $p<0.001$ ), and 3% higher diaphyseal cortical area and thickness (both,  $p<0.05$ ) than those fed the WP diet. Some interactions were found, such as the greater effects of SP diet on decreasing the higher plasma urea concentration promoted by the intake of the HP diet ( $p<0.001$ ). Under adequate Ca intake, HP diets could better maintain bone properties than NP diets, even with increasing some acidity markers, which could be reduced by the intake of SP sources.

**Keywords:** High-protein, soy-protein, whey-protein, bone, acidity, rats.

## 1. INTRODUCTION

The impact of the amount and source of protein on bone health is still a matter of debate. The use of high protein (HP) diets has gained popularity in the last decade, especially among people interested on losing weight<sup>1</sup>. Although there is currently no evidence that HP intake per se would be detrimental for bone mass and strength, in theory, excessive animal protein consumption is acidogenic and could increase bone resorption<sup>2</sup>. Current evidence on that issue is conflicting. Some studies reported a negative effect on bone health in both rats<sup>3</sup> and humans<sup>4</sup>. Conversely, other more recent studies in rodents<sup>5-7</sup> and humans<sup>8, 9</sup> have not observed any adverse osseous effects derived from HP diets consumption. Protein level seems to be crucial for the development of bone and muscle mass. In fact, several epidemiological and clinical studies point to a healthy effect of protein intakes above the current Recommended Dietary Allowance RDA (0.8 g/kg per day) for adults aged 19 and older<sup>10</sup>. On the other hand, a low protein intake could have a negative effect on bone mineral density (BMD), and epidemiological studies such as “The Framingham Osteoporosis Study”, have showed that elderly subjects in the lowest quartile of protein intake (<0.72 g/kg per day) showed the greatest bone loss<sup>11</sup>.

Whey protein (WP) and soy protein (SP) isolates have also become popular in the last years. Many studies have investigated the effects of soy foods, SP, or isoflavone extracts on bone markers and osteoporosis prevention, and have come to conflicting conclusions<sup>12-19</sup>. Soy foods are commonly associated to improved markers of bone status<sup>14</sup> and lower risk of bone fracture among postmenopausal women<sup>17</sup>. However, the optimal amounts and types of SP needed to support bone health are not yet clear, and some studies failed to find improvements<sup>12, 13, 15, 16</sup>. Studies analyzing the effects of WP on bone are scarce, but WP may stimulate bone formation by activating osteoblasts<sup>20</sup>.

Both protein sources appear to be important for bone health. Besides their protein content, both plant and animal foods provide other nutrients that can exert positive influences on bone status<sup>21</sup>. Until now, there is not enough evidence to support the notion that animal protein is better or worse than vegetable protein, or that WP or SP are more favourable than other protein sources on bone health<sup>22</sup>. Our group previously observed that the consumption of a high WP diet did not seem to affect bone status, as measured by femur ash content<sup>23</sup>, but bone properties were not extensively explored and we did not analyze the influence of the SP.

It is difficult to design and conduct well-controlled nutrition studies in humans to quantify the effect of nutrients on bone. The extrapolation of rodent studies to humans is widely found in the literature due to similar patterns of bone structure and metabolism<sup>7</sup>. Furthermore, the use of rodent experimental models is especially useful on bone metabolism, because years, not weeks, are required to assess BMD changes in humans. Moreover, most of available evidence comes from studies that examined the effect of specific interventions, i.e. focus on just the source of protein, or just the amount of protein in the diet. However, the combined effect and interactions taking place between the dietary amount and source of protein is unknown. Therefore, the present study aimed to examine the effects of the dietary amount (HP vs. normal-protein (NP) content) and source of protein (WP vs. SP) on bone status in rats, and the interactions taking place between these nutritional interventions.

## 2. MATERIALS AND METHODS

### 2.1 Animals and experimental design

A total of 140 albino male Wistar rats were allocated into four groups (n=35 each) derived of two main interventions: the concentration of protein in the diet (HP or NP),

and the source of protein (WP or SP) for 12 weeks. The animals, aged 8 weeks and with an initial body weight of  $172 \pm 15$  g, were located in a well-ventilated thermostatically controlled room ( $21 \pm 2^\circ\text{C}$ ), with relative humidity ranging from 40 to 60%. Throughout the experimental period all rats had free access to water and the animals consumed the diets *ad libitum*.

Body weight was measured weekly in all animals on the same day and hour and after a fasting period of 6 hours, and the amount of food consumed by each rat was registered daily. On day 74, a 12-hour urine sample from each animal was collected for biochemical analysis. Urine volumes were recorded and samples were transferred into graduated centrifuge tubes for pH, calcium, and citrate analysis.

At the end of the experimental period, the animals were anaesthetized with ketamine-xylazine and sacrificed by cannulation of the abdominal aorta. Blood was collected (with heparin as anticoagulant) and centrifuged at 3500 rpm for 25 min to separate plasma, which was frozen in liquid N and stored at  $-80^\circ\text{C}$  for subsequent biochemical analysis. Femurs were defleshed and weighed. The left femur was fixed in formalin and stored in 70% ethanol for BMD analysis, and the right femur was frozen in liquid N for femur ash analysis.

All experiments were undertaken according to Directional Guides Related to Animal Housing and Care<sup>24</sup>, and all procedures were approved by the Animal Experimentation Ethics Committee of the University of Granada.

## 2.2 Experimental diets

Experimental diets were formulated to meet the nutrient requirements of rats (NRC, 1995) based on the AIN-93M formulation described by Reeves et al, but included modifications in the protein source and content and in the oil source<sup>25</sup> (Table 1). We selected 45% protein content for the HP diet groups and 10% protein content for the NP

diet groups following previous studies in which HP diet had been compared with NP diets in rats<sup>7</sup>.

Commercial WP or SP isolates were used as the only source of protein since these proteins are widely available and frequently used by sportsmen and people interesting on losing weight, or improve health. The 45% protein content in the HP diet was achieved at the expense of complex carbohydrates (wheat starch). Prior to diet preparation, humidity and total protein concentration of the commercial WP and SP isolates was measured.

### 2.3 Chemical analyses

Total nitrogen (N) content of the protein isolates and diets was determined according to Kjeldahl's method. Crude protein was calculated as  $N \times 6.25$ . Bone ash was prepared by calcination at 500°C to a constant weight. Calcium content in bone, diets and urine and phosphorous and magnesium in the diets were determined by atomic absorption spectrophotometry (Perkin Elmer, Wellesley, MA, USA). Analytical results were validated by standard references certified reference material. Urinary pH was analysed using a bench pH-meter (Crison, Barcelona, Spain). Urinary Citrate was measured using a commercial kit (Spinreact, S.A. Gerona, Spain). Plasma urea, calcium and alkaline phosphatase, were measured using an autoanalyzer (Hitachi-Roche p800, F. Hoffmann-La Roche Ltd. Switzerland).

Plasma testosterone concentrations were measured in a subsample by radioimmunoassay using a commercially available TESTO-CTK I-125 Kit (Dia Sorin, Italy).

### 2.4 Bone turnover biomarkers

Osteocalcin was determined in the Rat-MID™ Osteocalcin enzymeimmunoassay (Immunodiagnosics System Ltd, Boldon, UK) from plasma samples. Degradation

products from C-terminal telopeptides of type I collagen were measured using a RatLaps™ enzymeimmunoassay (Immunodiagnosics System Ltd, Boldon, UK) from plasma samples. Tartrate-resistant acid phosphatase (TRACP 5b) was measured in rat serum using The RatTRAP™ assay (Immunodiagnosics System Ltd., Boldon, UK) which uses a highly characterized, specific monoclonal antibody prepared using baculovirus generated recombinant rat TRACP as antigen<sup>26</sup>.

### 2.5 Bone mineral density and structure

Volumetric BMD of the left femur was measured by peripheral quantitative computed tomography (pQCT) using a XCT Research M+pQCT machine (Stratec Medizintechnik, Pforzheim, Germany) as described<sup>27</sup>. One slice (0.2 mm thick) in the mid-diaphysis of the femur as a cortical bone site, and 3 slices in the distal femoral metaphysis located 1.5, 2, and 2.5 mm proximal to the articular surface of the knee joint as a site rich in trabecular bone were measured. Bone mineral density values of the distal femoral metaphysis were calculated as the mean over 3 slices. A voxel size of 0.070 mm and a threshold of 710 mg/cm<sup>3</sup> were used for calculation of cortical BMD. Trabecular BMD was calculated by using a threshold of 450 mg/cm<sup>3</sup>.

### 2.6 Statistical analysis

Results are presented as mean and standard deviation, unless otherwise indicated. The effects of the dietary amount of protein and the dietary source of protein, including their two-way interactions in the model (i.e. protein amount\*protein source), were analysed by two-way factorial ANOVA, with protein content and protein source as fixed factors. The overall P value is that reported for the main effects of the fixed factor (e.g. source of protein) as provided by the ANOVA. A significant P value indicates that there are differences at least between two of the groups. Additionally, multiple comparisons between groups were made considering Bonferroni's adjustment in order to control type



I error propagation and to identify between which groups the differences were significant (e.g. normal-WP vs. high-SP groups). All analyses were performed using the Statistical Package for Social Sciences (IBM-SPSS, version 20.0 for Windows), and the level of significance was set at 0.05.

### 3. RESULTS

#### *Final body weight, food intake and muscle N retention*

Food intake was lower in the HP compared to the NP diet ( $p < 0.001$ ), without differences regarding the protein source. No differences between groups were observed in gastrocnemius N content and final body weight in any of the interventions (all,  $p > 0.05$ ). Protein intake more than a 300% higher in the HP compared to the NP diet ( $p < 0.001$ ), without differences regarding the source of protein. Calcium intake was 28% lower for the WP compared to the SP diet ( $p < 0.001$ ), (Table 2).

#### *Plasma parameters*

Plasma urea was 46% higher for the HP compared to the NP diet ( $p < 0.001$ ). Pairwise comparisons showed higher differences mainly between the high-WP diet group and the rest of interventions. An interaction was found in plasma urea concentration, which was the highest in the high-WP diet group ( $p < 0.001$ ).

Regarding plasma bone remodelling biomarkers, no differences between groups were observed in plasma alkaline phosphatase and TRACP 5b, whereas plasma osteocalcin concentration was 19% higher for the NP compared to the HP groups ( $p < 0.05$ ) and 25% for the SP compared to the WP diet ( $p < 0.01$ ). Finally, C-terminal telopeptides of type I collagen were the highest in the HP from SP source ( $p < 0.01$ ), (Table 2).

In order to analyse a possible hormonal effect, plasma testosterone was measured in a

subsample of sixty animals (data not shown) and we observed that animals fed the HP diet had higher levels of plasma testosterone than those fed the NP diet ( $2.83 \pm 2.0$  vs.  $1.73 \pm 1.2$  ng/ml,  $p=0.017$ ).

#### *Urinary parameters*

Urinary calcium was 65% higher for the HP diet compared to the NP diet and 60% higher for the WP compared to the SP diet (all,  $p<0.01$ ). Urinary pH was 8% lower (more acidic) in the HP compared to the NP diet and 4% for the WP compared to the SP diet (all,  $p<0.001$ ). Pairwise comparisons showed differences mainly between the normal-SP diet group and the rest of interventions. Urinary citrate was 50% lower for the HP diet compared to the NP diet ( $p<0.001$ ) and 40% lower for the WP compared to the SP diet ( $p<0.001$ ). Urinary volume was 36% higher in the HP compared to the NP diet ( $p<0.01$ ) without differences attending to the protein source. An interaction was found in urinary citrate, which was the highest in the normal-SP group ( $p<0.001$ ), (Table 2).

#### *Bone mineral content, density and structure*

Femur ash percentage was 3% higher in the SP compared to the WP diet ( $p<0.001$ ). Femur calcium content, expressed as mg/g ash as well as expressed in mg/g dry matter, was 7% higher for the SP compared to the WP diet (both,  $p<0.001$ ), (Table 3).

Metaphyseal and diaphyseal total cross-sectional areas were 3% higher for the HP compared to the NP diet ( $p<0.05$  for metaphyseal and  $p<0.01$  for diaphyseal, respectively). Metaphyseal trabecular areas and diaphyseal cortical areas were also 3% higher for the HP compared to the NP diet (all,  $p<0.05$ ). Diaphyseal periosteal and endocortical perimeters were 3% higher for the HP compared to the NP diet groups (both,  $p<0.01$ ). Finally, diaphyseal cortical areas and cortical thickness were 3% higher

for the SP compared to the WP diet ( $p < 0.05$ ). No differences between groups were observed in femur length, metaphyseal or diaphyseal BMD, (Table 3).

#### 4. DISCUSSION

The main findings of this study were: i) HP diet consumption led to higher acidity markers (i.e. higher plasma urea and lower urinary pH). ii) Despite this, bone remodelling biomarkers, mineral content and structure were not negatively affected, but rather HP diet had a moderately positive effect on bone. iii) SP showed a positive role on such urinary and plasma acidity markers and on bone ash and calcium content compared to the WP, especially when it was included in the HP diet, where the SP decreased plasma urea and calciuria at the same time that increased urinary pH.

##### *4.1 High versus normal-protein diets and bone*

The role of HP diets on bone health has been a controversial topic in the last decade<sup>3-9, 11, 28</sup>. On the one hand, dietary amino acids have been considered important to support bone remodelling while on the other hand there have been concerns that the dietary acid load associated with protein consumption promotes hypercalciuria and loss of bone calcium stores<sup>2, 29, 30</sup>.

Dietary calcium content of the present study design was at the recommended levels for all the experimental diets. Urinary calcium excretion is strongly related to net renal acid excretion<sup>30</sup>. We have observed higher calciuria in the groups fed the HP diet, especially in the rats fed the WP (animal) source. Theoretically, the acid load might decrease osteoblastic activity and increase osteoclastic activity, resulting in net bone resorption with mobilization of calcium<sup>31</sup>. Nevertheless, nowadays it is assumed that whole body

calcium retention is not changed by the amount or type of protein because it is offset by changes in calcium absorption or endogenous secretion<sup>28</sup>, and recent studies suggest that dietary protein works synergistically with calcium to improve calcium retention and bone metabolism<sup>32, 33</sup>. Under our experimental conditions, even with this higher calciuria, the HP diet did not negatively affect BMD or bone geometry; rather, some bone properties were improved. In this regard, and similarly, Heaney *et al.*<sup>34</sup> demonstrated that HP diets have adverse effects on bone health only if dietary calcium and potassium intakes were not at the recommended levels. Therefore, our data also support substantial literature showing beneficial effects of HP consumption on skeletal metabolism when such level of protein is consumed together with adequate calcium, potassium, and other minerals, regardless of the amount or source of protein<sup>30</sup>. Accordingly to our results, Pye *et al.*<sup>35</sup> observed that a mixed HP diet containing adequate calcium levels (such as all our formulated diets) was safe for long-term bone health in rats.

High-protein intakes may positively impact bone health by several mechanisms, such as the stimulation of the secretion of insulin-like growth factor-1, or the enhancement of lean body mass<sup>32, 33</sup>. Besides, bone is approximately 50% protein matrix, so dietary protein is an essential nutrient for the development of maximum peak bone mass, although recent evidence also suggests that dietary protein could have an important role in skeletal health throughout adulthood and elderly<sup>36, 37</sup>. Another hypothesis to explain the better bone structure found in the HP diet groups could be mediated by the androgens effects, since the groups fed the HP diet presented higher levels of testosterone. Loss of androgens increases the rate of bone remodelling and also causes a focal imbalance between resorption and formation by prolonging the lifespan of osteoclasts and shortening the lifespan of osteoblasts<sup>38</sup>. Conversely, androgens maintain

bone mass and integrity, regardless of age or sex<sup>38</sup>. Under a rodent model, Reim *et al.*<sup>39</sup> observed tibia cortical bone loss in androgen deficiency-induced rats. The authors reported that this lower bone mass was mainly at expenses of the increased endocortical bone remodelling, which is consistent with our results since the HP diet group showed higher endocortical femur perimeters.

Thus, HP diet does not seem to lead to bone loss, and the role of protein appears to be complex and probably dependent on other dietary factors and the presence of other nutrients in the diet<sup>28</sup>. Notably is that in the general human population, HP diets usually contain low amounts of fruits and vegetables, which yet appear to be beneficial to bone health<sup>21</sup>. Therefore, it appears reasonable to avoid HP diets when they are associated with low calcium, fruits and vegetables intake<sup>21, 40</sup>.

#### 4.2 *Whey versus soy protein diets and bone*

Urine pH is an indicator of dietary acid-base balance. Welch *et al.*<sup>41</sup> investigated the relationship between urine pH and dietary acid-base load in 22,034 men and women aged 39-78 years. A more alkaline diet based on high fruit and vegetable intake and lower consumption of meat was associated with a more alkaline urine pH<sup>41</sup>. Decreased urinary pH, hypocitraturia and hypercalciuria, are risk factors for kidney stone formation, specifically by increased urinary saturation of calcium salts<sup>7, 42</sup>. In our study, the WP diet increased calciuria excretion and decreased urine pH and citrate levels, which could be also explained by the higher content in sulphur aminoacids in the WP supplement. Therefore, animals on a high WP diet could be at an increased risk of nephrolithiasis than those that consume the SP source<sup>7, 42</sup>.

A number of studies have been conducted in female experimental animals and post-menopausal women using soy products, SP or soy-associated isoflavones as an alternative to classic estrogens to restore bone loss<sup>43</sup>. Soy protein is notable for its low

fat content and for containing phytochemicals such as isoflavones but also saponins, and phytates. Despite concern that they may reduce calcium absorption, there is some evidence that they are beneficial for a better bone health<sup>44</sup>. In the study by Zhang *et al.*<sup>15</sup> SP isolate and 17beta-estradiol had different effects on bone turnover prior to puberty in rats. Approximately half of the genes were regulated in the same direction by 17beta-estradiol or SP isolate, but in combination, SP isolate blocked the estrogenic effects and returned the profile towards control levels<sup>15</sup>. In the cross-sectional studies performed in postmenopausal women by Horiuchi *et al.*<sup>18</sup> and Ho *et al.*<sup>19</sup>, SP was associated with increased BMD at hip, total body, and spine, and lower levels of bone resorption, especially in high versus low isoflavone concentrations. On the other hand, the study performed by Zhou *et al.*<sup>45</sup> in premenopausal women, incorporating approximately 19 grams of SP from soy foods daily for 10 weeks did not cause significant changes in bone resorption. Dietary pattern evidence suggests that regular consumption of soy foods may be useful for optimal bone health as an integral part of a dietary pattern that is built largely on whole plant foods<sup>16</sup>.

Only the study by Chen *et al.*<sup>14</sup> performed a direct comparison between the osseous effects of SP and WP diets. The authors also examined bone quality with pQCT in ovariectomized rapidly growing female rats, showing that both protein sources had positive effects on either BMD or bone mineral content (BMC) compared to the casein protein. The authors observed a positive effect of SP over WP on BMD and BMC. Moreover, SP increased serum bone formation markers and osteoblastogenesis *in vivo*. In agreement with our results, plasma osteocalcin levels were higher in the SP compared to the WP diet. Serum osteocalcin is a sensitive and specific marker of osteoblastic activity and its serum level thus reflects the rate of bone formation<sup>46, 47</sup>. In the above mentioned study<sup>14</sup>, SP also suppressed the bone resorption marker *C-terminal*

*telopeptides of type I collagen*, whereas we have not found differences. Consequently, the authors suggested a beneficial bone effect of a SP diet in rapidly growing animals and the potential for early soy consumption to increase peak bone mass. Finally, despite we have found better bone quality and calcium content, we cannot confirm a higher BMD in the groups fed the SP diet. Nevertheless, some of the lack of differences could be due to the fact that we have employed adult male Wistar rats whereas Chen *et al.*<sup>14</sup> and others studies used ovariectomized rapidly growing female rats.

Finally, to note is that most of the urinary and plasma acidity markers analyzed in the present study were in the normal range for rats. However, as much acidity, higher is the risk of complications in the long time (especially of renal origin)<sup>7, 42,48</sup>. The high WP diet was close to the limits in Wistar rats for most of the urinary and plasma markers related to the acid-base balance measured (e.g. urea levels).

#### 4.3 Limitation and strengths

The present study has some limitations that need to be mentioned. First, the current physiological results obtained in rodents must be confirmed in human subjects. Second, we used single sources of purified protein (whey or soy), which do not exactly reflects the human diet, despite they are widely used by different population groups. Third, the bone markers employed reflect bone turnover on a systemic but not on a site specific level. An alternative approach to assess bone remodelling might have been the use of dynamic histomorphometry. On the other hand, the present study involved a large number of rats allocated in different groups, so that the main effects of the HP diet, the protein source, and the interactions taking place between them, provided a good opportunity to comprehensively investigate how these dietetic behaviours can influence bone health. Moreover, bone parameters were analysed with pQCT, which is one of the most advanced and innovative techniques nowadays, and we have described a large

number of structural parameters, and analysed some plasma bone resorption markers.

## 5. CONCLUSION

Overall, a HP diet appears to better maintain bone properties than a NP diet does, even with affecting some acidity markers such as plasma urea and urinary pH, which could be neutralized by the intake of protein from soy sources (vegetable protein). Soy and WP are the two more common commercial protein sources widely used by sportsmen and people interested in loss weight. This study also aimed to try to clarify which of them is the most adequate in order to preserve health in the long term. Our findings lead us to recommend the use of SP instead of WP. Indeed, the use of high SP diets, or SP supplements, may be useful for a better bone health in weak populations (e.g. elderly, or perimenopausal women).

Future studies, developed in humans and under long-term interventions are needed to confirm or contrast the present findings.

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## Conflict of interest

The authors declare that no conflict of interest exists in this study.

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**Table 1.** Composition of the experimental diets

<i>Nutritional Composition (g/100g DM)</i>	<b>Normal protein diet</b>		<b>High-protein diet</b>	
	<b>Soy</b>	<b>Whey</b>	<b>Soy</b>	<b>Whey</b>
Whey protein supplement	-	13.8	-	63.6
Soy protein supplement	13.1	-	57.4	-
Mineral mix (AIN-93M-MX)	3.5	3.5	3.5	3.5
Vitamin mix (AIN-93-VX)	1	1	1	1
Fat (olive oil)	4	4	4	4
Choline chloride	0.50	0.50	0.50	0.50
Cellulose	5	5	5	5
Starch	62.4	61.7	28.6	22.4
Methionine	0.5	0.5	-	-
Sucrose	10	10	-	-
<b><i>Mineral composition of the experimental diets (mg/g DM)</i></b>				
Ca (mg/g DM)	6.08 (0.76)	5.39 (0.23)	7.52 (0.08)	5.48 (0.24)
P (mg/g DM)	3.63 (0.13)	2.50 (0.10)	6.25 (0.13)	3.18 (0.15)
Mg (mg/g DM)	0.49 (0.02)	0.52 (0.02)	0.83 (0.01)	0.56 (0.01)
<b><i>Sulphur aminoacids of the protein supplement (mg/100g DM)*</i></b>				
<i>L-methionine</i>	123	304	539	1399
<i>L-cysteine</i>	123	304	539	1399

DM, dry matter; \* Reported by the producer.

**Table 2. Effect of normal vs. high-protein diet and whey vs. soy protein diet on final body weight, plasma and urinary markers**

	Normal protein diet			High-protein diet			P		
	Soy	Whey	% *	Soy	Whey	% *	Amount effect	Source Effect	Source × Amount interaction
Final body weight (g)	331.0(32.3)	323.2(36.4)	-2.4	318.1(35.1)	332.6(46.5)	4.6	0.782	0.587	0.072
Gastrocnemius N content (g/100g DM)	13.6(0.4)	13.9(0.8)	2.2	13.7(0.7)	13.5(1.2)	-1.5	0.541	0.784	0.411
Food intake in DM (g/day)	16.0(2.0) <sup>ab</sup>	14.6(1.4)	-8.8	13.9(1.2) <sup>b</sup>	13.5(2.3) <sup>a</sup>	-2.9	<0.001	0.068	0.291
Protein intake (g/day)	1.65(0.2)	1.62(0.2)	-1.8	6.89(0.6)	6.74(0.5)	-12.7	<0.001	0.056	<0.001
Calcium intake (g/day)	102.5(11.2)	83.8(9.9)	-18.2	116.6(12.8)	73.5(9.1)	-37.2	0.428	<0.001	<0.001
<b>Plasma parameters</b>									
Urea (mg/dl)	27.7(5.4) <sup>ad</sup>	22.9(6.0) <sup>be</sup>	-17.3	33.3(8.9) <sup>cde</sup>	41.3(13.5) <sup>abc</sup>	24.0	<0.001	0.250	<0.001
Calcium (mg/dl)	19.0(10.1)	14.5(10.8)	-23.7	23.7(13.6)	17.6(15.7)	-25.7	0.141	0.046	0.757
Alkaline phosphatase (UI/L)	88.2(46.5)	102.9(19.4)	16.7	108.6(45.5)	99.2(44.6)	-8.7	0.480	0.823	0.307
Osteocalcin (ng/mL)	241.6(100) <sup>a</sup>	203.9(58.0)	-15.6	216.9(81.8) <sup>b</sup>	142.2(48.3) <sup>ab</sup>	-34.4	0.013	0.001	0.364
C-terminal telopeptides of type I collagen (ng/mL)	12.7(3.6)	14.0(5.1)	10.2	16.2(7.3)	11.5(5.1)	-29.0	0.712	0.146	0.007
TRACP 5b (UI/L)	2.98(0.07)	2.98(0.09)	0.0	2.97(0.08)	3.00(0.07)	1.0	0.766	0.344	0.395
<b>Urinary parameters</b>									
Calcium (mg/L)	1.68(0.8) <sup>a</sup>	2.17(0.9) <sup>b</sup>	29.2	2.31(0.7) <sup>c</sup>	3.78(3.4) <sup>abc</sup>	63.6	0.002	0.006	0.164
Citrate (g/L)	2.82 (1.5) <sup>abc</sup>	0.99(1.0) <sup>a</sup>	-66.9	0.75(0.5) <sup>b</sup>	0.63(1.2) <sup>c</sup>	-16.0	<0.001	<0.001	<0.001
pH	7.00(0.6) <sup>abc</sup>	6.58(0.3) <sup>ad</sup>	-6.0	6.42(0.3) <sup>b</sup>	6.23(0.2) <sup>cd</sup>	-3.0	<0.001	<0.001	0.120
Urinary volume (mL)	2.75(1.1) <sup>ab</sup>	3.92(1.6)	42.5	4.60(1.6) <sup>a</sup>	4.43(0.6) <sup>b</sup>	-3.7	0.004	0.360	0.751

Values expressed as mean(SD). DM, dry matter; TRACP 5b, tartrate-resistant acid phosphatase; \* Percentage of difference between whey and soy groups was computed as ((whey-soy)/whey) x 100. <sup>a,b,c,d</sup> Common superscripts in a same row indicate a significant difference (P<0.05) between the groups with the same letter. Pairwise comparisons were performed with Bonferroni's adjustment.

Table 3. Effect of normal vs. high-protein diet and whey vs. soy protein diet on bone mineral content, density and structure

	Normal protein diet			High-protein diet			Amount effect	P	Source × Amount interaction
	Soy	Whey	% *	Soy	Whey	% *			
Femur dry weight (g)	0.565(0.05)	0.553(0.05)	-2.1	0.568(0.05)	0.581(0.07)	2.3	0.094	0.935	0.182
Femur ash percentage (%)	66.7(1.7) <sup>ac</sup>	64.7(1.7) <sup>a</sup>	-3.0	66.0(2.1) <sup>b</sup>	64.9(1.7) <sup>bc</sup>	-1.7	0.348	<0.001	0.590
Femur humidity percentage (%)	30.2(2.3)	30.2(5.5)	0.0	29.9(3.6)	30.7(3.0)	2.7	0.898	0.520	0.112
Femur calcium content (mg/g DM)	239.3(20.2) <sup>ac</sup>	221.7(22.9) <sup>ab</sup>	-7.4	240.5(16.1) <sup>bd</sup>	225.0(23.3) <sup>bcd</sup>	-6.4	0.517	<0.001	0.760
Femur calcium content (mg/g ashes)	367.0(30.6) <sup>ac</sup>	338.6(28.4) <sup>ab</sup>	-7.7	364.0(24.5) <sup>bd</sup>	343.8(32.7) <sup>bcd</sup>	-5.5	0.815	<0.001	0.405
Femur length (cm)	3.46(0.1)	3.51(0.1)	1.4	3.48(0.1)	3.47(0.1)	-0.3	0.606	0.209	0.067
Metaphyseal BMD (mg/cm <sup>3</sup> )	605.5(48.7)	598.7(51.6)	-1.1	604.4(44.1)	586.1(38.9)	-3.0	0.380	0.107	0.462
Diaphyseal BMD (mg/cm <sup>3</sup> )	896.6(55.0)	909.3(50.3)	1.4	906.8(50.2)	889.1(48.9)	-2.0	0.563	0.774	0.080
Metaphyseal total cross-sectional area (mm <sup>2</sup> )	15.7(1.5)	15.4(1.4)	-1.9	16.1(1.8)	16.5(2.1)	2.5	0.011	0.787	0.261
Diaphyseal total cross-sectional area (mm <sup>2</sup> )	9.65(0.8)	9.33(0.7) <sup>ab</sup>	-3.3	9.98(0.8) <sup>a</sup>	9.91(1.0) <sup>b</sup>	-0.7	0.002	0.182	0.376
Metaphyseal trabecular area (mm <sup>2</sup> )	9.12(1.3)	9.06(1.3)	-0.7	9.48(1.5)	9.93(1.7)	4.7	0.011	0.415	0.290
Metaphyseal cortical area (mm <sup>2</sup> )	6.78(0.5)	6.68(0.4)	-1.5	6.92(0.4)	7.00(0.5)	1.2	0.202	0.253	0.417
Diaphyseal cortical area (mm <sup>2</sup> )	5.98(0.5)	5.77(0.4) <sup>a</sup>	-3.5	6.15(0.5) <sup>a</sup>	6.00(0.6)	-2.4	0.016	0.031	0.764
Diaphyseal cortical thickness (mm)	0.67(0.04)	0.66(0.04)	-1.5	0.68(0.04)	0.66(0.03)	-2.9	0.535	0.014	0.627
Diaphyseal periosteal perimeter (mm)	11.0(0.5)	10.8(0.4) <sup>ab</sup>	-1.8	11.2(0.5) <sup>a</sup>	11.1(0.6) <sup>b</sup>	-0.9	0.002	0.177	0.393
Endocortical perimeter (mm)	6.78(0.5)	6.68(0.4) <sup>a</sup>	-1.5	6.91(0.4)	7.00(0.5) <sup>a</sup>	1.3	0.003	0.886	0.243

Values expressed as mean (SD); DM, dry matter; BMD, bone mineral density; \* Percentage of difference between whey and soy groups was computed as ((whey-soy)/whey) x 100. <sup>a,b,c,d</sup> Common superscripts in a same row indicate a significant difference (P<0.05) between the groups with the same letter. Pairwise comparisons were performed with Bonferroni's adjustment.