Research Article

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Bioaccessibility and bioavailability of calcium in sprouted brown and golden flaxseed

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Abstract

BACKGROUND: Germination promotes changes in the composition of seeds by providing potential nutritional and health benefits compared with unsprouted seeds. This study investigated the influence of germination on the bioaccessibility and bioavailability of calcium in brown flaxseed (BF) and golden flaxseed (GF).

RESULTS: Germination did not influence the calcium levels of BF or GF, but the sprouted GF (SGF, 265.6 ± 12.9 mg) presented higher levels of calcium than the sprouted BF (SBF, 211.6 ± 3.20 mg). Tannin levels were similar among the groups (GF = 79.97 ± 3.49 mg; SGF = 78.81 ± 0.77 mg; BF = 81.82 ± 2.61 mg; SBF = 79.24 ± 4.58 mg), whereas phytate and oxalate levels decreased after germination. Germination reduced the phytate:calcium and oxalate:calcium molar ratios. In the *in vitro* study, germination increased calcium bioaccessibility (GF = 35.60 mg *versus* SGF = 41.45 mg; BF = 31.01 mg *versus* SBF = 38.84 mg). In the *in vivo* study, all groups present similar levels of urinary calcium (GF = 1.04 mg *versus* SGF = 2.06 mg; BF = 1.68 mg *versus* SBF = 1.35 mg) and fecal calcium (GF = 5.06 mg *versus* SGF = 6.14 mg; BF = 6.47 mg *versus* SBF = 8.40 mg). The calcium balance/day of the SBF group (37.97 mg) was smaller than the control group (47.22 mg). The germination maintained the plasma levels of calcium, phosphorus, creatinine, and alkaline phosphatase similar among the groups. No changes were observed in morphology and calcium levels of animal femurs.

CONCLUSION: The germination reduced the antinutritional factor in both flaxseed varieties. Although there was an improvement in the *in vitro* bioaccessibility of calcium, the germination did not increase calcium absorption and balance in the animals, which may be due to the interaction with other compounds in the organism.

Keywords: flaxseed; germination; calcium; bioaccessibility; bioavailability

INTRODUCTION

Germination occurs under suitable conditions, in which the embryonic axis returns to the development interrupted at the time of physiological maturation, with subsequent integument rupture by the root.[1] Germination involves an important increase of metabolic processes that, among other actions, influence nutrients' storage in seeds.[2] So, the germination process consists of three steps: soaking, biochemical processes (enzyme activity, cell respiration, and protein synthesis), and root emergence, with adequate temperature and water supply being the key elements for the seed to develop as desired.[3]

The germination process mobilizes nutritional reserves in the first stage of seed growth. Therefore, proteins are converted into amino acids, carbohydrates into simple sugars, lipids into fatty acids, vitamins and enzymes are synthesized, and minerals are mobilized. These modifications promote bioaccessibility, bioavailability, and digestibility of nutrients in the body. In addition, there is a reduction of antinutritional components; for example, protease and trypsin inhibitors, phytate, oxalate, and tannin.[4] However, the germination effects on chemical composition, nutritional aspects, and sensory characteristics vary with species, cultivars, and seed germination conditions.[5]

Flaxseed (*Linum usitatissimum* L.) is an oilseed, with the brown and golden types being the two most popular varieties.[6] Although they differ in a few nutritional aspects and flavor, their basic composition is composed of 35% lipids, 20% proteins, 40% carbohydrates (of which 30% are fiber), vitamins A, B, D, E, and K complex, and minerals such as calcium, phosphorus, and zinc. In this sense, flaxseed contains high concentration of calcium (25.5 g kg^{-1})[7] compared with other cereals, such as quinoa (0.63 mg kg^{-1}), amaranth (1.52 mg kg^{-1}),[8] and chia (6.31 g kg^{-1}).[9] These levels may vary according to variety, environment, seed processing, and analysis methods.[10] Also, flaxseed is considered a functional

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food,[11] so there is a trend to include it in the diet due to its potential health benefits, such as the anticarcinogenic and antiatherogenic properties provided by fibers, lignans, and α-linolenic acid ($\omega - 3$).[12]

Although flaxseed is a food rich in beneficial nutrients to the organism, the bioaccessibility and bioavailability of these nutrients are mainly affected by the presence of fibers and antinutritional factors, such as phytate and oxalate. However, the germination process is able to reduce these undesirable components.[13] Thus, the germination of flaxseed increases the content of bioactive compounds, such as flavonoids, polyphenols, tocopherols, and γ-aminobutyric acid, which can double the antioxidant value of the seed and increase the mineral content (including calcium) and vitamins (such as ascorbic acid), so allowing greater bioaccessibility and absorption of nutrients.[14]

Considering that flaxseed is a calcium-rich plant food, the germination process of golden flaxseed (GF) and brown flaxseed (BF) is potentially a simple and low-cost alternative to increase the bioaccessibility and bioavailability of calcium. So, it is a method that can encourage the inclusion of sprouted flaxseeds in diets in replacement of unsprouted seeds. Therefore, the objective of this study was to investigate the influence of germination on the bioaccessibility and bioavailability of calcium in GF and BF.

MATERIAL AND METHODS

Raw material

BF and GF (*Linum usitatissimum* L.; Nayna®) were purchased from a local market of Alegre, ES, Brazil.

Germination

The flaxseeds were placed in polyethylene trays previously demineralized with nitric acid and sterilized with peracetic acid. The trays with the flaxseeds were covered with a cloth moistened with ultrapure water and stored away from light at a room temperature of 26 ± 2 °C for 48 h. The seeds were moistened twice a day (morning and late afternoon) to ensure enough moisture for germination.[15,16]

Elaboration of flaxseed flour

The sprouted flaxseeds were oven dried at 50 ± 5 °C to constant weight. Next, the dried sprouted and unsprouted flaxseeds were ground in a blender and sifted to obtain a fine homogeneous meal (30 mesh). The flaxseed flour was stored in laminated packaging and refrigerated at −4 °C until use.

Calcium composition

Calcium was determined by flame atomic absorption spectrometry with microwave-assisted digestion using nitric acid.[17] A Shimadzu AA-6200 (Barueri, SP, Brasil) instrument with a calcium hollow-cathode lamp and nitrous oxide–acetylene flame was used. Absorbance measurements were performed by using the calcium resonance line at $\lambda = 422.7$ nm, 0.7 nm slit width, and 10.0 mA lamp current. The microwave digestion (MARS™ 6 model; CEM Corporation) involved three steps: (i) 25 min temperature ramp up to 210 °C; (ii) maintain the temperature for 15 min at 210 °C; and (iii) decrease for 15 min to room temperature.

Antinutritional factors

Tannin levels were measured according to Price *et al.*[18] using a Multiskan GO® UV–visible spectrophotometer (Thermo Scientific, Porto Alegre, RS, Brazil).

Phytic acid levels were measured according to Latta and Eskin.[19] Briefly, previous extraction with hydrochloric acid was performed and then passed through an ion-exchange column with a stationary phase consisting of 1 × 4 100–200 mesh resin. The column was preconditioned with 2 mol L^{-1} sodium chloride (NaCl) and the extract obtained was carefully applied to it. The inorganic phosphors were eluted with 0.05 mol L^{-1} NaCl, followed by the elution of the retained phytate with 2 mol L^{-1} NaCl. The phytate was determined by colorimetric method in a spectrometer with the absorbance measured at 500 nm.

Oxalic acid levels were measured according to Iwuoha and Kalu,[20] with samples analyzed by permanganometry.

In vitro study: calcium bioaccessibility

The sprouted and unsprouted GF and BF meals were subjected to an *in vitro* enzyme-stimulated gastrointestinal digestion process.[21,22] Gastric digestion was performed, whereby the sample was mixed with ultrapure water, the pH adjusted to 2.0, and pepsin added. Then, the samples were incubated at 37 °C in a shaking water bath for 2 h. Afterwards, the pH was adjusted to 7.0 and pancreatin enzyme and bile salts were added. The samples were incubated again at 37 °C and shaken for 2 h. At the end of the digestive process, the enzymes were inactivated by heating at 75 °C for 20 min in a water bath. Next, the samples were cooled and centrifuged to separate the bioaccessible and residual fractions. Calcium analysis of the bioaccessible fraction was performed as previously described.[17]

In vivo study: calcium bioavailability

Ethical approval

The study was approved by the Animal Ethics Committee of the Federal University of Espírito Santo, Brazil, register no. 013/2018.

Experimental design

Forty weaned male Wistar rats were used, with an average body weight of 50 g, obtained from the Health Sciences Center, Universidade Federal do Espírito Santo. The animals were divided into five groups ($n = 8$): control (CT), GF, sprouted GF (SGF), BF, and sprouted BF (SBF). The total experimental period was 6 weeks. The animals were maintained in individual cages with controlled environment of 12 h photoperiod and average temperature of 23 ± 2 °C. The animals received ultrapure water and feeding *ad libitum*. Weight and food intake were monitored weekly. The feed efficiency ratio (FER) and caloric efficiency ratio (CER) were calculated. These are the ratios of total body weight gain (grams) to total dietary intake (grams) and total caloric intake (kilocalories) of the animals respectively.

At the end of the experimental period, the animals were anesthetized by using xylazine (15 mg kg^{-1}) and ketamine (60 mg kg^{-1}) administered intraperitoneally. Then, blood samples were collected by cardiac puncture, centrifuged to obtain the serum, and stored at −80 °C.

Experimental diets

The experimental diets (Table 1) were prepared based on the composition of the AIN-93G diet.[24] The flaxseed content in the diets was 100 g kg^{-1}, and the diets were prepared to provide 50% of the animals' daily calcium requirements. After preparation, the diets were wrapped in laminated packaging, labeled, and stored under refrigeration.

Calcium balance

The animals were placed in individual stainless-steel metabolic cages to collect feces and urine for 5 days at the sixth week of

Table 1. Composition of experimental calcium bioavailability diets (g kg^{-a} diet)

Ingredients	CT	GF	SGF	BF	SBF
Golden flaxseed	—	100	—	—	—
Sprouted golden flaxseed	—	—	100	—	—
Brown flaxseed	—	—	—	100	—
Sprouted brown flaxseed	—	—	—	—	100
Albumin[a]	257.4	236.8	235.4	237.5	235.6
Dextrinized starch	132	132	132	132	132
Sucrose	100	100	100	100	100
Soybean oil[b]	70	37.5	32.5	36.9	32.8
Fiber[c, d]	50	21.5	21.5	22.2	22.2
Mineral mix without Ca	35	35	35	35	35
Calcium carbonate ($CaCO_3$)[e]	6.2	5.7	5.6	5.7	5.8
Vitamin mix	10	10	10	10	10
L-Cystine	3.0	3.0	3.0	3.0	3.0
Choline bitartrate	2.5	2.5	2.5	2.5	2.5
Corn starch[f]	340	316	322.5	315.2	321.1
Total energy (kcal kg^{-1})	3923	3972	3947	3966	3945

CT, control; GF, golden flaxseed; SGF, sprouted golden flaxseed; BF, brown flaxseed; SBF, sprouted brown flaxseed; Ca, calcium.
[a] Amount adjusted according to flaxseed composition to provide 200 g kg^{-1} protein. Albumin: 777 g protein kg^{-1}.
[b] Amount adjusted according to flaxseed composition to provide 70 g kg^{-1} lipid.
[c] Amount adjusted according to flaxseed composition to provide 50 g kg^{-1} fiber.
[d] Fiber amounts based on the literature.[26]
[e] Amount adjusted according to flaxseed composition, to provide 50% of animal daily requirement. $CaCO_3$: 40 g Ca/100 g $CaCO_3$.
[f] Enough to complete 1 kg.

the experiment. During this period, the food intake was standardized at 20 g day^{-1} per animal for all groups in order to minimize possible errors.

The total urine volume collected was centrifuged and the supernatant stored at −20 °C. The total fecal content collected was sieved, dried at 105 °C for 24 h, and then weighed and stored at −20 °C. The calcium content of the feces and urine supernatant was analyzed as previously described.[17] The calcium intake was calculated from the diet consumed over the experimental period multiplied by the calcium content of each diet. Also, we calculated the calcium balance:

Balance = Ingested calcium − Fecal calcium − Urinary calcium

and the percentage of calcium retention:

$$\text{Retention}\,(\%) = \frac{\text{Calcium intake} - \text{Urinary calcium} - \text{Fecal calcium}}{\text{Calcium intake}} \times 100$$

Biochemical analyses

The following plasma tests were performed: calcium (Calcium Arsenazo III; Bioclin®, Belo Horizonte, MG, Brazil), phosphorus (Phosphorus UV; Bioclin), creatinine (Creatinine Kinetic; Bioclin), and alkaline phosphatase (Kinetic Alkaline Phosphatase; Bioclin), by following the manufacturers' instructions and using a chemical analyzer (B5-200E model; Mindray, Shenzhen, China).

Femur analysis

After the euthanasia, the femurs of animals were removed, weighed, measured in length and diameter with a caliper, and stored at −20 °C. The femurs were dissolved in nitric acid for 16 h,[25] and calcium was analyzed as previously described.[17] The figures obtained were used to calculate the percentage of bone calcium:

$$\text{Bone calcium}\,(\%) = \frac{\text{Femur calcium (mg)}}{\text{Weight of femur}} \times 100$$

and bone retention:

$$\text{Bone retention}\,(\%) = \frac{\text{Femur calcium (mg)}}{\text{Total calcium ingested}} \times 100$$

Statistical analyses

The Kolmogorov–Smirnov test was performed to evaluate the normality of the data. Then, two-way analysis of variance (ANOVA) was used for physicochemical, antinutritional factors, and *in vitro* study data, with the Tukey test applied in cases of interaction. For the *in vivo* study, one-way ANOVA was applied, followed by the Tukey test. The results were expressed as mean ± standard deviation and considered significant at $P \leq 0.05$. Statistical analyses were performed using the GraphPad Prism® (version 5; GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Germination

The flaxseed germination process lasted 48 h (Fig. 1). Although germination time for both varieties was the same, BFs grew larger sprouts than the GFs did. This difference may be climate related, since BF is better adapted to the hot and humid climate in Brazil, whereas GF grows better in cold climates, or because the golden variety is able to absorb water faster than the brown variety due to the seed coat permeability.

Calcium composition

There was no influence on germination for calcium concentration ($P > 0.05$), although the levels were significantly higher in the SGF (265.60 mg) than in the SBF and not sprouted (211.60 mg and 212.80 mg respectively; $P \leq 0.05$) (Table 2).

Antinutritional factors

The tannin levels of the flaxseeds did not differ after germination or present any influence of the variety ($P > 0.05$). On the other

Figure 1. Digital records of (A) golden and (B) brown flaxseeds at the end of the 48 h germination process.

hand, phytate and oxalate levels decreased after germination in both varieties ($P \leq 0.05$) (Table 2).

Germination reduced the molar ratios of phytate:calcium and oxalate:calcium ($P \leq 0.05$) of the flaxseeds. Also, there was a significant difference between the varieties after the germination process, where the molar ratio of the GF was lower for phytate (4.09) and oxalate (0.91) than of the BF (5.11 and 1.16 respectively) ($P \leq 0.05$). Also, with regard to the oxalate:calcium ratio, the GF even without germinating was statistically different from the BF (1.09 *versus* 1.34; Table 2). Although the molar ratios of calcium to oxalate and to phytate decreased after germination, these values are still considered elevated.

In vitro study: calcium bioaccessibility

Calcium bioaccessibility increased in sprouted compared with unsprouted seeds ($P \leq 0.05$), and variety also significantly influenced the result ($P \leq 0.05$), with greater bioaccessibility in GF (not sprouted: 35.60%; sprouted: 41.45%) than in the BF (not sprouted: 31.01%; sprouted: 38.84%) (Fig. 2).

In vivo study: calcium bioavailability

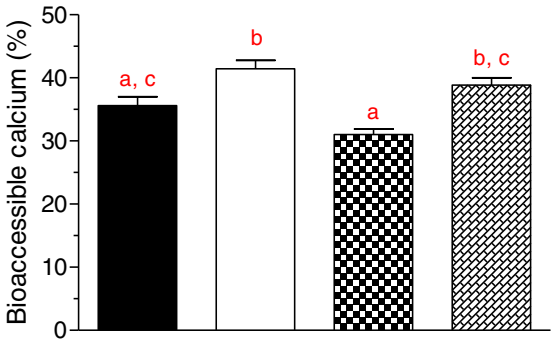
Food intake, animal weight, and feed and caloric efficiency ratios

Weekly food intake, daily food intake, daily caloric intake, daily calcium intake, weekly animal weight, total animal weight gain, FER, and CER showed no statistical difference between the groups

Table 2. Calcium content, antinutritional factors, and molar ratios of phytate and oxalate to calcium of dry sprouted and unsprouted golden and brown flaxseed meals (dry matter)

					P		
	GF	SGF	BF	SBF	Var	Ger	Int
Calcium (mg/100 g)	248.3 ± 5.67^{a,b}	265.6 ± 12.99^{a}	212.8 ± 26.29^{b}	211.6 ± 3.20^{b}	<0.001	0.378	0.317
Tannin (CE/100 g)	79.97 ± 3.49^{a}	78.81 ± 0.77^{a}	81.82 ± 2.61^{a}	79.24 ± 4.58^{a}	0.552	0.338	0.711
Phytate (mg/100 g)	1435.43 ± 84.70^{b}	1140.57 ± 75.09^{a}	1296.38 ± 57.91^{a,b}	1134.23 ± 21.43^{b}	0.086	<0.001	0.112
Oxalate (mg/100 g)	293.27 ± 6.51^{b}	252.85 ± 16.11^{a}	307.87 ± 8.04^{b}	258.20 ± 15.39^{a}	0.197	<0.001	0.532
Phytate:calcium	5.35 ± 0.28^{b}	4.09 ± 0.25^{a}	5.66 ± 0.26^{b}	5.11 ± 0.08^{b}	<0.001	<0.001	0.020
Oxalate:calcium	1.09 ± 0.03^{b}	0.91 ± 0.06^{a}	1.34 ± 0.03^{c}	1.16 ± 0.06^{b}	<0.001	<0.001	0.904

Data expressed as mean plus/minus standard deviation. *P*: two-way ANOVA values. Different letters on the same line differ from each other ($P \leq 0.05$) by the Tukey test.
GF, golden flaxseed; SGF, sprouted golden flaxseed; BF, brown flaxseed; SBF, sprouted brown flaxseed. CE; catechin equivalent. Var, flaxseed variety; Ger, germination; Int, interaction between variety × germination.



	p		
Var	Ger	Int	
0.018	< 0.001	0.438	

Figure 2. Percentage of bioaccessible calcium in sprouted and unsprouted golden and brown flaxseed meal following *in vitro* digestion. GF, golden flaxseed; SGF, sprouted golden flaxseed; BF, brown flaxseed; SBF, sprouted brown flaxseed; Var; flaxseed variety; Ger, germination; Int, interaction between variety × germination. Data expressed as mean and standard deviation. *P*: two-way ANOVA values. Different letters mean statistical difference ($P \leq 0.05$) by one-way ANOVA test followed by Tukey test.

($P > 0.05$; Fig. 3). The diets provided were isocaloric, which contributed to adequate intake and similar weight gain among the groups.

Calcium balance

Food and calcium intake during the balance was similar among the groups ($P > 0.05$), showing that all animals consumed similar amounts of food and this factor had a positive correlation with the study, with no interference with the final balance results. No statistical difference was observed in urinary and fecal calcium either ($P > 0.05$), with a similar behavior in all groups and compared wth the control group (Fig. 4).

Regarding calcium balance per day, sprouted brown flaxseed was smaller than the control group ($P \leq 0.05$), but the other groups did not differ from the control group ($P > 0.05$). The percentage of calcium retention, in turn, showed no difference between any of the groups ($P > 0.05$), all being above 90% retention.

Biochemical analyses

Regarding biochemical analyses (Fig. 5), no statistical differences were observed between the sprouted and unsprouted varieties in relation to the control group over the 6 week intervention period ($P > 0.05$), with all being within normal levels.

Femur analyses

Regarding the morphological evaluation of femurs, no statistical differences were observed between the flaxseed groups or in relation to the control group over the 6 week intervention period ($P > 0.05$). In the same way, calcium (mg)/total femur weight ratio, femur calcium concentration, and bone retention rate did not vary between the treatments ($P > 0.05$; Fig. 6).

DISCUSSION

There are a few studies comparing the functional properties of BF and GF. Most studies performed with this seed have used the GF variety, whereas BF, which grows in Brazil and is more affordable in the domestic market, has been poorly investigated.[26] The golden variety is mostly cultivated in cold regions, such as in Canada and the USA,[11] whereas the brown variety has been cultivated in regions with a hot and humid climate. The brown variety is the most cultivated worldwide, serving as a raw material for industries and as animal and human food. GF is a variety that grows better in cold weather, owing to its greater sensitivity to attack by pests and fungi, and its production is lower.[6] They differ from each other in some aspects, mainly nutrition and flavor, but in their basic composition they provide 35% lipids, 20% proteins, 40% carbohydrates (of which about 30% is in the form of fibers), vitamins A, B, D, E, and K complex, and minerals such as calcium, phosphorus, and zinc. These levels can vary according to the variety, the environment, seed processing, and analysis methods.[10] Currently, there is a growing incentive to include flaxseed in the diet owing to its potential health benefits, being considered a functional food,[11] due to the presence of a large amount of fibers, lignans, and α-linolenic fatty acid (ω−3).[11,12,26]

The alterations in the mineral content of seeds after germination may be related to differences in the seed composition, germination time, content of antinutritional factors, activation of endogenous enzymes, and extent of mineral binding in the matrix or interaction of these factors.[27] The results for calcium content after germination are still controversial: whereas some have verified a reduction in the content of calcium,[15] others have observed a 47% increase in flaxseed calcium levels after germination.[13]

In this sense, the increase in mineral levels of sprouted seeds may be related to the loss of dry matter caused by changes in the seeds' metabolism, which can improve the mineral extraction. The improvement in mineral levels is related to phytate hydrolysis, since phytase enzymatic activity increases during germination. Moreover, the absorption of some minerals from the growth medium could interfere in their increase during germination.[28] On the other hand, mineral loss can be attributed to the leaching caused by soaking before germination.[29]

In our study, germination was able to decrease the levels of phytate and oxalate. The antinutritional action of tannin and phytate is related to their ability to form insoluble complexes with minerals, proteins, and starches that are biologically unavailable for humans under normal physiological conditions, whereas the oxalate content is nutritionally important due to interference in calcium bioavailability.[30] In contrast to our results, Oloyo[31] observed an 82% reduction of tannin levels in legumes after 48 h germination. On the other hand, Ahmed *et al.*[30] observed a 51% increase in tannin levels after 36 h of germination, which was associated with the solubilization of insoluble tannin that caused the tannin to migrate from the integument to the seed nucleus. Other studies reported that the activity of chelating enzymes of phytate, oxalate, and tannin increased during seed germination, contributing to reduction of anti-nutritional

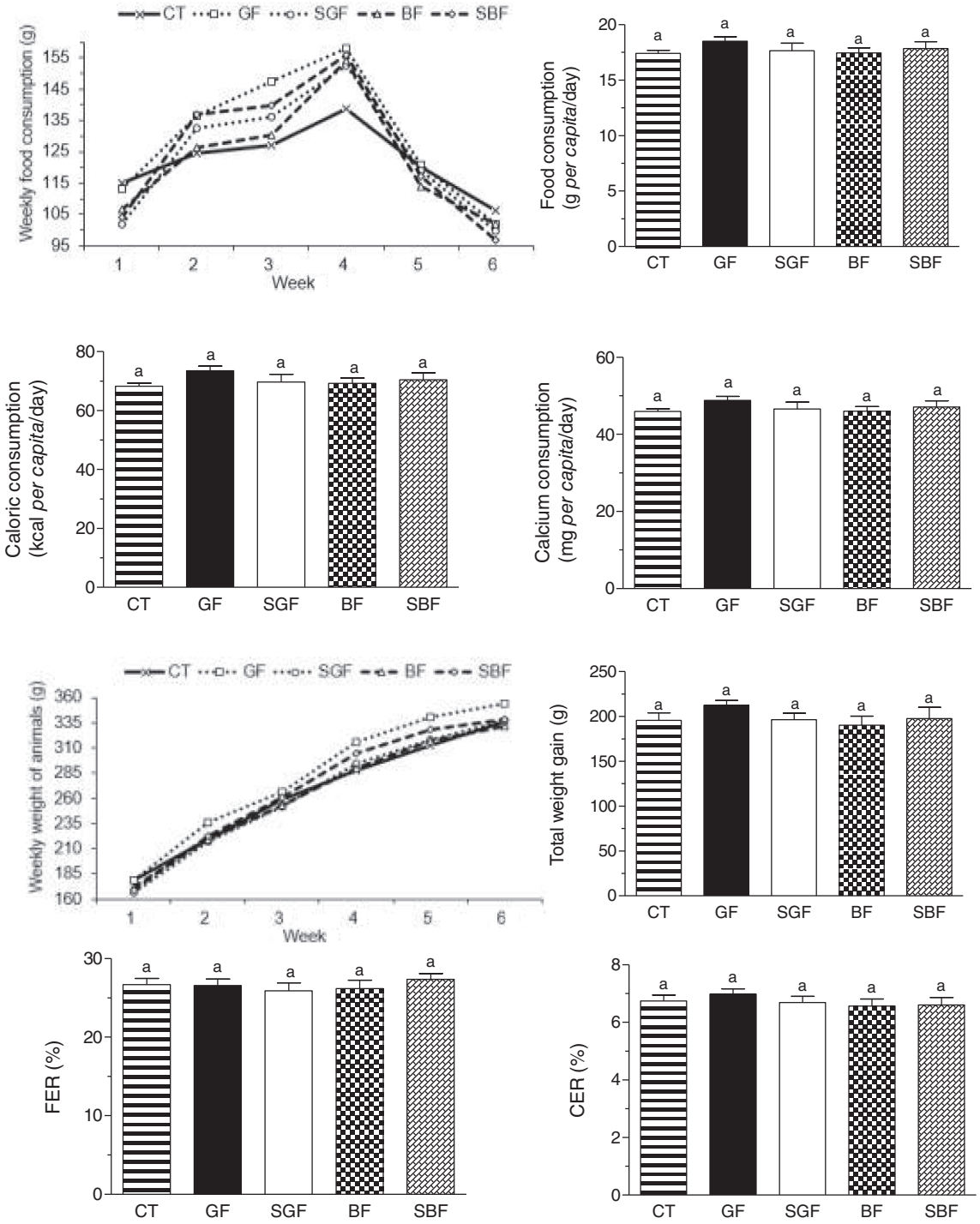


Figure 3. Weekly food intake, daily *per capita* food intake, daily *per capita* caloric intake, daily *per capita* calcium intake, weekly animal weight, total animal weight gain, feed efficiency ratio (FER), and caloric efficiency ratio (CER) of the *in vivo* experiment. CT, control; GF, golden flaxseed; SGF, sprouted golden flaxseed; BF, brown flaxseed; SBF, sprouted brown flaxseed. Data expressed as mean and standard deviation. Different letters mean statistical difference ($P \leq 0.05$) by one-way ANOVA test followed by Tukey test.

factors.[15,29,32] In turn, Hemalatha *et al.*[33] observed no significant reduction in phytate levels after germination.

Although phytic acid levels decrease during germination, the increase of this enzyme's activity will rely on the germination conditions investigated, as well as the type and time of soaking before germination. Therefore, this wide variation in phytic acid levels suggests that germination conditions have a key role in the final product composition.[32] Williams and Taylor[34] observed that phytate hydrolysis by bacterial phytase in the large intestine is mainly responsible for phytate breakdown when rats are fed diets without phytase. Thus, intestinal phytase may increase calcium bioavailability and the differences in phytate levels between diets and the phytate:calcium ratio, as observed in our study, may not be sufficient to affect calcium absorption in rats.

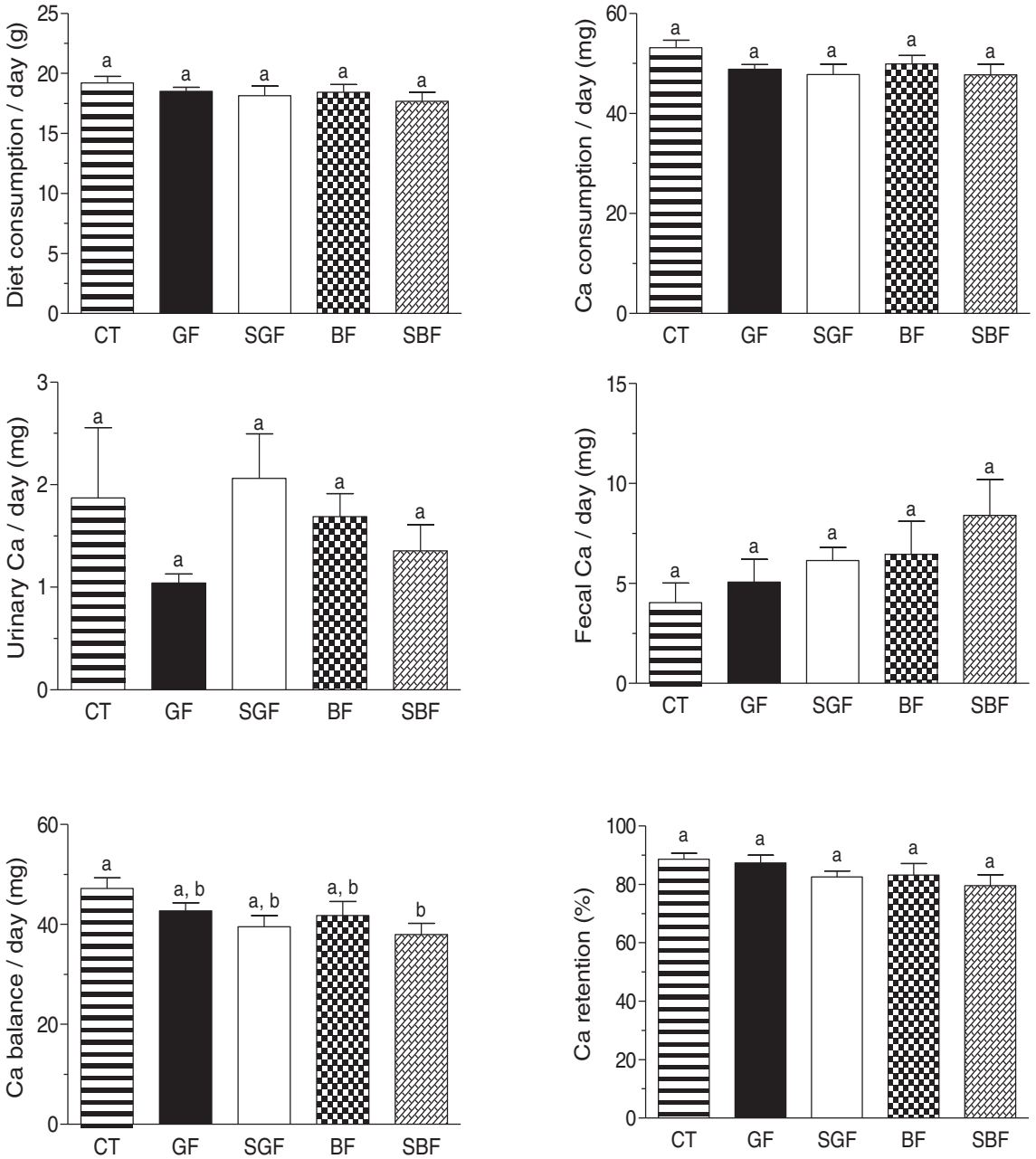


Figure 4. Dietary intake/day, calcium intake/day, urinary calcium/day, fecal calcium/day, calcium balance/day and percentage of calcium retention of the *in vivo* experiment. Ca, Calcium; CT, control; GF, golden flaxseed; SGF, sprouted golden flaxseed; BF, brown flaxseed; SBF, sprouted brown flaxseed. Data expressed as mean and standard deviation. Different letters mean statistical difference ($P \leq 0.05$) by one-way ANOVA test followed by Tukey test.

The simulated *in vitro* digestion has been used to obtain preliminary information on the estimated bioavailability of certain nutrients due to its positive correlation with *in vivo* experiments models. Other studies have also shown an increase in calcium bioaccessibility after germination in several types of seeds.[35,36] In this sense, the beneficial effect of germination on calcium bioaccessibility can probably be attributed to the decreased phytate and oxalate levels because of germination. Among the mineral bioaccessibility inhibitors inherent in seeds, phytate and oxalate have been observed to be dominant compared with fibers or tannin.[35] Although phytate in plant foods forms complexes with essential minerals and makes them biologically unavailable for absorption, the phytase activity increases with germination by resulting in the catabolism of phytic acid and polyphenols, so improving mineral bioaccessibility.[36] Ghavidel and Prakash[36] verified a negative correlation between calcium bioaccessibility and phytate levels in different types of legumes germinated for 24 h. Therefore, there are a number of factors that may interfere with calcium bioavailability.[37] In general, the amount of calcium absorbed in humans depends on a variety of factors, including the length of the intestinal segment, the time that the chyme goes by a specific segment of the intestine, the calcium concentration in the intestinal lumen, and the mineral bioavailability in the food.[38]

In this study, calcium absorption was similar between BF and GF and their respective germinations. Albeit significant, the

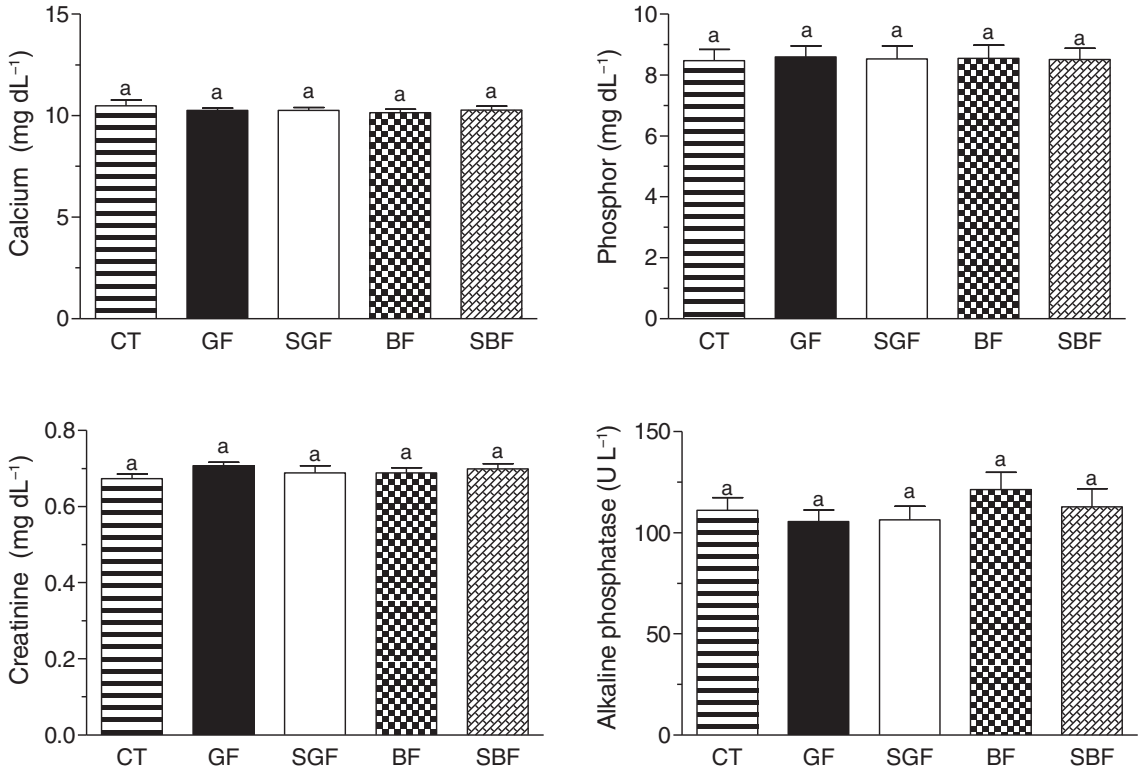


Figure 5. Plasma calcium, plasma phosphorus, and plasma creatinine and alkaline phosphatase in animals. CT, control; GF, golden flaxseed; SGF, sprouted golden flaxseed; BF, brown flaxseed; SBF, sprouted brown flaxseed. Data expressed as mean and standard deviation. Different letters mean statistical difference ($P \leq 0.05$) by one-way ANOVA test followed by Tukey test.

reduction in phytate and oxalate levels was probably insufficient to affect calcium absorption. The results obtained in this work are similar to those of Mason *et al.*,[39] in which different phytate levels (0.74 and 1.7%) in soy protein isolate did not influence calcium absorption in rats. During digestion, calcium is uncoupled and released in a soluble and ionized form for absorption. For this, it requires the activity of digestive enzymes and a relatively acidic pH. However, low molecular weight complexes, such as calcium oxalate and calcium carbonate, can be absorbed intact.[40] Furthermore, the molar ratios reduction of phytate and oxalate to calcium between unsprouted and sprouted flaxseeds may have been sufficient to increase calcium bioaccessibility. However, despite this reduction, the phytate and oxalate molar ratios are still considered elevated, so preventing the calcium absorption improvement.[23] Weaver *et al.*[23] used different bean varieties and observed that common beans with higher oxalate:calcium and phytate:calcium ratios did not reduce the calcium absorption in humans compared with white and red beans with lower molar ratios.

Despite being performed with animals, this study attempted to use reference values for humans. Thus, as the average calcium intake of the population is around 50% of the recommended daily intake,[41] this percentage was chosen instead of the 100% of daily requirement. Moreover, the use of a diet providing 50% of the animals' daily requirement is supported, since dietary calcium deficiency can reduce the calcium absorbed in the intestine, which leads to lower calcium levels in the blood. In this sense, these metabolic changes stimulate the release of parathyroid hormone to restore the blood levels, which in turn decreases urinary calcium excretion and increases its intestinal absorption and bone mobilization.[23] A study that evaluated 50% and 100% of the daily calcium intake requirement of animals verified that the levels of urinary and fecal calcium excretion of animals that consumed low calcium levels were lower than those with 100% intake.[42] This lower calcium loss can be a protective mechanism of the organism to save serum calcium levels for its physiological functions. Germinated fava beans increased the calcium bioavailability in rats to a value statistically similar to that of some animal products, so showing that germination is associated with an improvement of mineral bioavailability.[43] Therefore, the mineral bioavailability in foods relies on conditions such as the type of food matrix and method used, the activation of endogenous enzymes, the extent of mineral bonding within the matrix, the presence of antinutritional factors in the food, and an interaction of these factors.[27]

The normal serum calcium level remains tightly controlled, varying no more than 5% over a 24 h period.[42] It is regulated by a system of controlling factors and feedback mechanisms, which involve interaction of calciotropic hormones (parathyroid hormone, vitamin D, i.e. $1,25(OH)_2D_3$, and calcitonin) with organs such as the intestine, kidneys, and bones.[37] Among the risk factors that interfere with bone metabolism, those related to nutrition have a predominant role.[44] It has been suggested that part of the variation in bone mass is predetermined by diet, such as low calcium intake or conditions that alter its intestinal absorption, such as the presence of antinutritional factors.[37] Therefore, processes such as germination, which alter food composition and may improve the bioaccessibility and bioavailability of nutrients such as calcium, can have beneficial effects on the bone matrix. Nonetheless, there are factors that stimulate calcium absorption and contribute positively to bone metabolism.

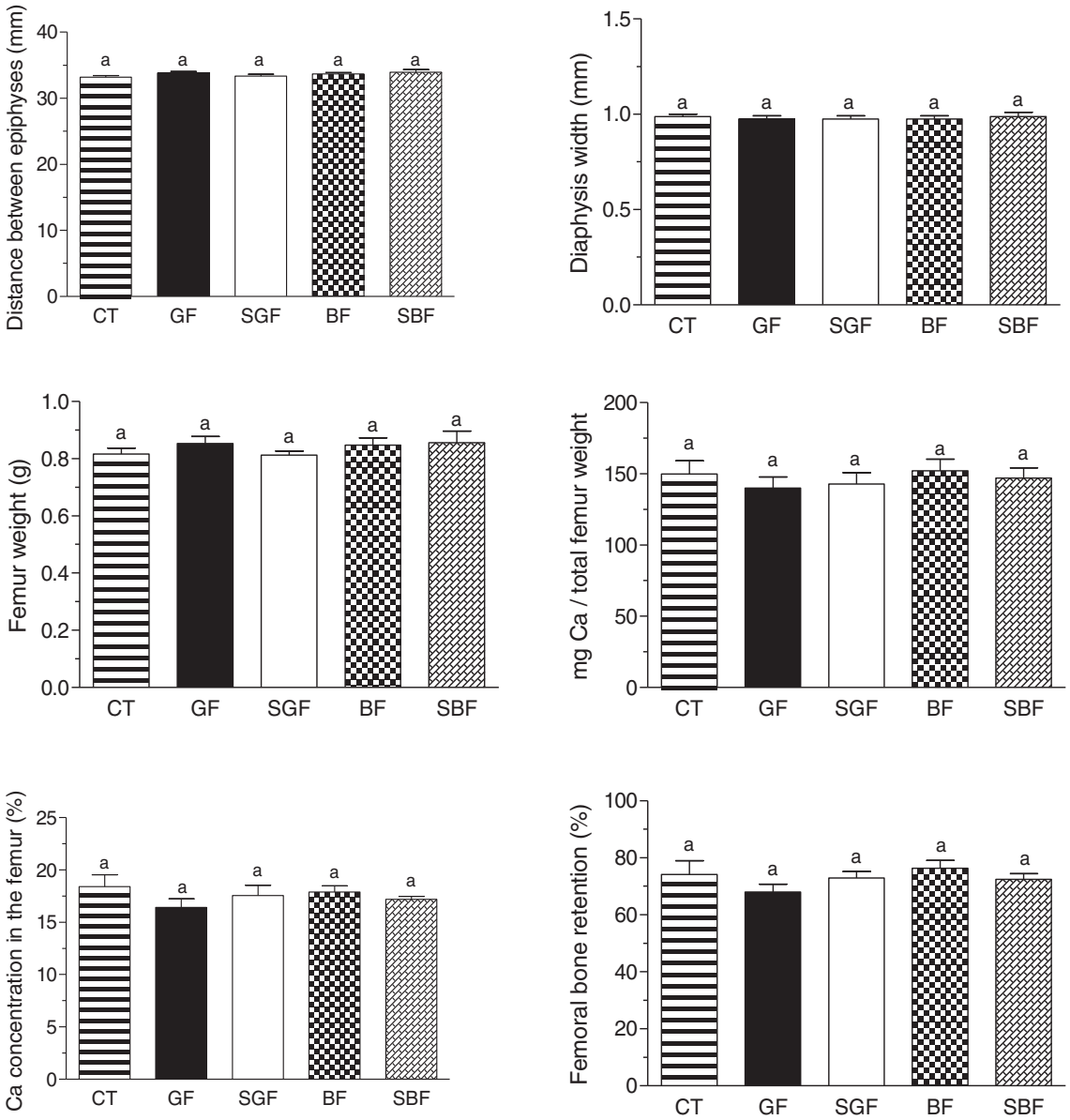


Figure 6. Distance between epiphyses, diaphysis width, femur weight, calcium mg/total femur weight ratio, femur calcium level and percentage of bone retention. Ca, calcium; CT, control; GF, golden flaxseed; SGF, sprouted golden flaxseed; BF, brown flaxseed; SBF, sprouted brown flaxseed. Data expressed as mean and standard deviation. Different letters mean statistical difference ($P \leq 0.05$) by one-way ANOVA test followed by Tukey test.

Among the nutrients involved in bone biosynthesis and maintenance, calcium is one of the most discussed and studied due to the high incidence of its deficiency in our usual diet.[41] Adequate calcium intake and absorption ensure the mineralization during periods of rapid growth and help to preserve bone mass and density during critical development periods.[44] In this context, flaxseed has been shown to be a good source of calcium.[44] In addition, flaxseed contains high levels of α-linolenic acid.[26] Omega-3 fatty acids reduce osteoclastogenesis and osteoclast maturation, contributing to a decrease in bone resorption by downregulation of the β-nuclear receptor activator. For osteoblasts, α-linolenic acid preserves bone mass by increasing the expression of major transcription factors, such as osteocalcin, which provide maturation of mature osteoblasts.[45] Besides α-linolenic acid, flaxseed has high levels of lignans, mainly as a secoisolariciresinol diglucoside (SDG), which are the precursors of the active forms of enterodiol and enterolactone. The structural similarity of enterodiol and enterolactone with the most predominant and active form of estrogen in the body, estradiol, allows these lignans to bind to estrogen receptors and exert weak estrogen or antiestrogen effects.[46] Therefore, SDG supplementation stimulated osteogenesis in both sexes in adulthood and may be associated with most of the flaxseed effects on bone metabolism, especially those related to the action of phytoestrogens, by mimicking the effects of estrogen on bone parameters.[47] Sacco *et al.*[48] demonstrated that supplementation with low-dose estrogen flaxseed in ovariectomized rats improved the bone microarchitecture and protected bone tissue due to reduced bone turnover. In addition, Figueiredo *et al.*[47] observed that the ingestion of flaxseed lignan by female rats during lactation improved bone parameters in

adulthood in male and female offspring, so suggesting that dietary SDG may benefit bone development. In the same way, weaned male pups with mothers treated with a flaxseed-enriched diet during lactation showed improvement in bone mineral content and bone area and higher serum levels of osteoprotegerin and osteocalcin.[49]

It is noteworthy that there are few studies evaluating the effect of flaxseed on bone metabolism, and these studies are still controversial. Aguilar *et al*.[26] verified that flaxseed supplementation, regardless of variety, had no significant effect on bone formation and resorption biomarkers in perimenopause overweight women. Conversely, Ribeiro *et al*.[50] showed that flaxseed meal contributed to bone mineralization in rats, by improving the biomechanical properties of the femur and contributing to the improvement of bone health.

CONCLUSIONS

This study showed that 48 h germination reduced the phytate and oxalate levels of flaxseed. The golden variety had a higher calcium content and lower phytate:calcium and oxalate:calcium molar ratios.

In vitro calcium bioaccessibility was higher in sprouted flaxseeds compared than in unsprouted seeds, and the golden variety showed higher bioaccessibility of this mineral than the brown variety did. However, the *in vivo* calcium bioavailability did not cause significant changes in metabolic markers, morphology, and calcium retention of femurs, but they did not differ from the control group either.

The nutritional benefits provided by germination may lead to an increase in the consumption and versatility of flaxseed. It is noteworthy that BF has a lower cost in the Brazilian market and in most analyses behaved similarly to the golden variety, being a good alternative for human nutrition.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

AVP, TAV, NMBC, and AGVC were responsible for the conception and study design. AVP and TAV performed the data collection. AVP and JCCS were responsible for the statistical analysis of the data. All authors contributed to interpretation of data. AVP, NMBC, and AGVC prepared the draft of the manuscript. JCSC, NMBC, and AGVC supervised the study. All authors contributed to manuscript revision, read, and approved the submitted version.

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