Effects of dietary lipid composition and inulin-type fructans on mineral bioavailability in growing rats

Alexandre Rodrigues Lobo, M.Sc.[a], Jorge Mancini Filho, Ph.D.[a], Eliana Parisi Alvares, Ph.D.[b], Maria Lucia Cocato, M.Sc.[a], and Célia Colli, Ph.D.[a],*

[a] Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil
[b] Department of Cell and Developmental Biology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

Abstract

Objective: This study reports the effects of feeding with a combination of inulin-type fructans (ITF) and fish oil (FO) on mineral absorption and bioavailability as part of a semipurified diet offered to rats.

Methods: Male Wistar rats ($n = 24$) were fed a 15% lipid diet (soybean oil [SO] or a 1:0.3 fish:soybean oil mixture [FSO]) and diets containing the same sources of lipids supplemented with 10% ITF (Raftilose Synergy 1) *ad libitum* for 15 d. Feces and urine were collected for mineral analyses during the last 5 d of the test period. Fatty acid composition was determined in liver and cecal mucosa homogenates. Liver and bone mineral analyses were performed by atomic absorption spectrophotometry. Bone biomechanical analyses were evaluated by a 3-point bending test.

Results: Compared with the controls, ITF-fed rats had enlarged ceca and a significant decrease in cecal content pH ($P < 0.001$). The apparent mineral absorption was improved in these rats, and this effect was enhanced by dietary combination with FO for all minerals except for magnesium. Addition of ITF to the diet resulted in higher bone mineral content (calcium and zinc) and bone strength, but increased bone mineral content was only statistically significant in FO-fed animals. A decrease in liver iron stores ($P = 0.015$) was observed in rats fed FO, considering that ITF consumption returned to levels comparable to the SO control group.

Conclusion: These findings confirm the positive influence of ITF on mineral bioavailability, which was potentiated by addition of FO to the diet.   © 2008 Published by Elsevier Inc.

Keywords: Inulin-type fructans; Fish oil; Polyunsaturated fatty acids; Mineral absorption; Bioavailability

Introduction

Inulin-type fructans (ITF; fructo-oligosaccharides [FOSs] and inulin) have been studied as prebiotic non-digestible oligosaccharides because they modulate the composition and metabolic activity of the intestinal microbiota, favoring the growth of bifidogenic bacteria rather than other species considered to be pathogenic to the host [1]. Bacterial fermentation of ITF in the large intestine has been implicated in increased intestinal absorption and bioavailability of calcium (Ca) and magnesium (Mg) in animal (rat, pig) [2–8] and human [9–11] studies. In humans, the effects on intestinal Ca absorption seem to occur under conditions of greater mineral demands, such as during adolescence and after menopause [9–11]. In contrast, studies examining the effects of ITF on micromineral absorption (mainly copper [Cu], iron [Fe], and zinc [Zn]) are relatively scarce and present contradictory results [2].

The fermentative process favors the production of short-chain fatty acids (FAs), which reduce the luminal pH and enhance mineral solubility [2,5,12]. These effects are accompanied by modifications in the architecture of the intestinal mucosa, which affect the cellularity and number of crypts, mechanisms that could contribute to an increase in the absorptive surface for mineral uptake [2,3,5,12,13]. Furthermore, dietary ITF seems to affect intestinal mineral absorption in rats by altering intestinal tight junction permeability and the gene expression of mineral transporters such as calbindin-D_{9k} (for Ca), DMT-1, ferroportin (for Fe),

and ZnT1 (for Zn), supporting an additional effect on para-cellular and transcellular absorption [5,6,14,15]. All of these effects are reflected by increases in mineral bioavailability [2–12]. Nevertheless, one should keep in mind that these physiologic effects depend on some experimental conditions, such as short-term or long-term consumption of these carbohydrates, the food matrix, and the age and/or nutritional/physiologic status of the animals [2,3,6–8,12].

Other dietary components also have the potential to influence mineral absorption and bioavailability. It has been well documented by *in vivo* and *in vitro* studies that dietary lipid composition plays an important role in skeletal biology and bone health [16]. It has been demonstrated that dietary supplementation with polyunsaturated FAs (PUFAs) improves Ca balance and bone Ca content in animal and human studies [16]. Specifically, long-chain (LC) ω-3 PUFAs (eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]) appear to modulate bone remodeling by altering the synthesis of eicosanoids in bone tissues, which improves bone formation and inhibits bone resorption [16–19]. In addition, the dietary ω-6/ω-3 FA ratio may be important [17,19], because bone prostaglandin E_2 was lower and serum alkaline phosphatase activity was higher in rats fed diets containing a low ω-6/ω-3 ratio, supporting a positive effect of ω-3 PUFAs on bone formation [19].

In addition, changes in the level of unsaturation and acyl chain length in intestinal cell membranes might result in increased intestinal mineral absorption by altering membrane fluidity and function [20]. In this aspect, it has been observed that the consumption of fish oil (FO) rich in ω-3 PUFAs could affect Fe homeostasis by enhancing membrane lipid peroxidation [21]. This in turn indirectly influences intestinal Fe absorption and liver Fe stores (i.e., Fe bioavailability) [21].

This considered, a combination of functional components in the food with the potential to modulate intestinal mineral absorption might represent an interesting approach to improving mineral bioavailability. Moreover, the body's store of minerals should be considered to influence their absorption capacity and, most likely, the levels of these elements in their target tissues. Hence, the aim of the present study was to determine the effect of feeding healthy growing rats a semipurified diet containing a combination of ITF and ω-3 PUFA-rich FO on mineral absorption and bioavailability. The apparent intestinal absorption and organ (bone, liver) retention of minerals were evaluated as a measurement of mineral bioavailability. Bone biomechanical testing, which provides important information about bone mechanical integrity [22,23], was also investigated.

Materials and methods

Animals and experimental diets

Twenty-four male Wistar rats (6 wk old, average weight 116 ± 2 g) were obtained from a colony at the Faculty of Pharmaceutical Sciences, University of São Paulo. The experimental protocol was approved by the ethics commission on animal experiments of the Faculty of Pharmaceutical Sciences of the University of São Paulo (CEEA 45/2004 FCF-USP), according to the guidelines of the Brazilian College on Animal Experimentation. The rats were maintained in stainless-steel wire-mesh cages (to limit coprophagy) under controlled temperature (22 ± 2°C) and relative humidity (55 ± 10%), with a 12-h dark/light cycle (lights off from 8 pm to 8 am). The ITF tested (Raftilose Synergy 1; Orafti-Active Food International, Tienen, Belgium) was donated by Clariant S/A (São Paulo, Brazil). Raftilose Synergy 1 is a 1:1 mixture of FOS (degree of polymerization [DP] ranging between 2 and 8, with a DP average of 4) and high-performance inulin (DP ranges between 10 and 65, with a DP average of 25), which is obtained by physically removing the lower-DP fraction from the native inulin. Soybean oil (SO) was provided by Cargill Agrícola S/A (Mairinque, São Paulo, Brazil). FO (rich in EPA [20:5ω-3] and DHA [22:6ω-3]) made from fish, derived from the *Sardina pilchardus* and *Sardinella anchovia* families, was provided by Cardinal Health Brasil (Sorocaba, São Paulo, Brazil). Oils assessed in the present study did not contain antioxidants (<100 IU/g of vitamin A for FO). Therefore, the antioxidant *tert*-butyl hydroquinone (0.03%) was added to the oils to prevent their oxidation [24].

During the acclimatization period (7 d), all animals were fed a standard rat chow (Nuvilab CR1, irradiated [10 kGy]; Nuvital Nutrientes S/A, Paraná, Brazil) consisting of 22% protein, 4% lipids, 9% ash, 55% nitrogen-free extract, 11% moisture, 19 mg of Ca/g, 3615 μg of Mg/g, 19 μg of Cu/g, 928 μg of Fe/g, and 103 μg of Zn/g. During this period, every cage contained two rats. After the acclimatization period, the animals were stratified based on their body weights and transferred to individual cages where they received semipurified AIN-93G [25] based diets modified with 15% (wt/wt) lipids, as SO, or a 1:0.3 (wt/wt) FO and SO mixture (FSO) and diets containing the same lipid sources supplemented with 10% ITF (SO + ITF and FSO + ITF, respectively; Table 1) for 15 d. Dietary lipid was provided at 15% (30% energy from fat) to simulate the current recommendations for humans [26]. The FO diets contained 3.5 g of SO/100 g to ensure that essential FA requirements were met. The following factors were used for energy calculations: 4 for carbohydrates and proteins, 9 for lipids, and 1 for ITF [27].

Food consumption was measured daily, and body weight was recorded every 48 h. Rats were provided with a fresh diet every day, and the feeders were removed and washed daily. Food and demineralized water were offered *ad libitum*. The feeding efficiency was determined as the weight gain per gram of food intake at the end of the experiment. Feces and urine were quantitatively collected during the last 5 d of the test period, pooled, and stored at −20°C.

Table 1
Formulation of experimental diets

Ingredient (%)	SO	SO + ITF	FSO	FSO + ITF
Casein*	20.0	20.0	20.0	20.0
Fiber	5.0	5.0	5.0	5.0
SO[†]	15.0	15.0	3.5	3.5
Fish oil[‡]	—	—	11.5	11.5
L-cystine	0.30	0.30	0.30	0.30
Choline bitartrate (41.1% choline)	0.25	0.25	0.25	0.25
Vitamin mix[§]	1.00	1.00	1.00	1.00
Mineral mix[‖]	3.50	3.50	3.50	3.50
Sucrose	10.00	—	10.0	—
Corn starch	44.95	44.08	44.95	44.08
Raftilose Synergy 1[¶]	—	10.87	—	10.87

FSO + ITF, fish and soybean oil mixture + inulin-type fructans; FSO, fish and soybean oil mixture (1:0.3, wt/wt); SO + ITF, soybean oil + inulin-type fructans; SO, soybean oil

* Eighty-five percent protein.

[†] Cargill Agrícola S.A. (Mairinque, São Paulo, Brazil).

[‡] Cardinal Health Brasil (Sorocaba, São Paulo, Brazil).

[§] AIN-93-VX vitamin mixture [25].

[‖] AIN-93G-MX mineral mixture [25].

[¶] Orafti Active Food International (Clariant, São Paulo, Brazil), 92% ITF.

Sampling procedures

At the end of the experiment, the rats were anesthetized with a 1:2 (v/v) mixture of ketamine (10 mg/kg) and xylazine (25 mg/kg) [3,4] and sacrificed. The liver and cecum with its contents were then removed and weighed. The hind limbs were removed, and the bones (femurs and tibias) were cleaned of soft tissue, wrapped in a bandage previously soaked in saline solution, hermetically sealed in plastic bags, and stored at −20°C until mineral analyses and biomechanical testing, considering that storage at −20°C does not alter the mechanical properties of the bones [28]. Total liver Fe was determined from the left lateral lobe of the liver by atomic absorption spectrophotometry (AAnalyst 100, Perkin Elmer, Norwalk, CT, USA). Liver Fe was not corrected for Fe content in the residual blood. The pH of the cecal contents was determined *in situ* by inserting an electrode (UP-25; Denver Instrument, Denver, CO, USA) through the ileocecal junction. The cecal wall was flushed clean with ice-cold saline, blotted dry, and weighed. The cecal mucosa was scraped off with a glass slide and stored at −20°C until analysis. In addition, tissue samples of the cecum were fixed in formaldehyde solution and stored in 70% ethanol. For histologic examination, the fixed tissue fragments were embedded in paraffin and approximately 5-μm-thick sections were obtained and stained with hematoxylin and eosin. In each crypt, the cells in the left-hand column were counted (cells/hemicrypt) from the bottom to the top of the cecal crypt using an optical microscope. At least 30 hemicrypts per animal were assessed, and only crypts cut lengthwise were considered.

Chemical composition of experimental diets

The following components were determined: moisture, ash, and total lipids [29]; protein by the micro-Kjeldahl method [30] (conversion factor of 6.25); and total dietary fiber by the enzymatic-gravimetric method [31]. Dietary Ca, Mg, Cu, Fe, and Zn concentrations were determined by atomic absorption spectrophotometry employing a hollow cathode lamp at 422.7, 202.6, 324.8 283.4, and 213.9 nm, respectively, and slits of 0.7, 1.3, 1.3, 0.2, and 1.3 nm, respectively, after wet digestion (HNO_3:H_2O_2, 5:1; v/v) and addition of 0.1% (wt/v) lanthanum as La_2O_3 (for Ca and Mg analyses). The working standard solutions were prepared with $CaCl_2$, $MgCl_2$, $CuCl_2$, $FeCl_3$, and $ZnCl_2$ (Titrisol, Merck, Darmstadt, Germany). There were no significant differences in the mineral concentrations of the diets (4 mg of Ca/g, 430 μg of Mg/g, 27 μg of Cu/g, 66 μg of Fe/g, and 44 μg of Zn/g in the SO control group). FA composition was measured by gas chromatography, as described below, and expressed as the percentage of total FAs (Table 2).

Apparent mineral absorption and balance

Dry feces (105°C, 15 h) were milled, and the powdered samples and the urine samples were utilized for mineral analyses, as described previously for the mineral analyses of

Table 2
Fatty acid composition of the experimental diets*

Fatty acids (%)	Dietary treatment			
	SO	SO + ITF	FSO	FSO + ITF
14:0	0.20	0.19	6.03	5.94
16:0	11.64	11.63	16.59	16.89
18:0	3.45	3.40	3.47	3.56
22:0	0.43	0.42	0.74	0.74
24:0	0.13	0.13	ND	ND
16:1	0.12	0.14	6.82	6.73
18:1(ω-9)	24.79	24.96	15.50	16.03
20:1(ω-9)	0.17	0.18	1.21	1.18
18:2(ω-6)	54.07	54.22	14.24	14.58
20:4(ω-6)	ND	ND	0.72	0.72
18:3(ω-3)	4.58	4.38	1.54	1.55
18:4(ω-3)	ND	ND	2.74	2.68
20:5(ω-3)	ND	ND	14.17	14.06
22:5	ND	ND	1.48	1.41
22:6(ω-3)	ND	ND	8.62	8.62
SATs	15.85	15.78	26.83	27.13
Total MUFAs	25.07	25.27	23.52	23.95
Total PUFAs	58.72	58.60	43.51	43.63
ω-6 PUFAs	54.07	54.22	14.96	15.31
ω-3 PUFAs	4.65	4.38	28.55	28.32
ω-6:ω-3 PUFAs	11.69	13.20	0.52	0.54

FSO + ITF, fish and soybean oil mixture + inulin-type fructans; FSO, fish and soybean oil mixture (1:0.3, wt/wt); MUFAs, monounsaturated fatty acids; ND, not detected; PUFAs, polyunsaturated fatty acids; SATs, total saturated fatty acids; SO + ITF, soybean oil + inulin-type fructans; SO, soybean oil

* Expressed as area percentage of total fatty acids.

the diets. Apparent absorption and balance were calculated by the following equations:

$$\text{Apparent absorption (\%)} = 100 \times (\text{ingestion} - \text{fecal excretion})/\text{ingestion}$$

$$\text{Mineral balance (mg/d)} = (\text{ingestion} - [\text{fecal excretion} + \text{urinary excretion}])$$

Lipid analyses

Lipids were extracted from the diets and liver and cecal mucosa homogenates by acidic hydrolysis using pyrogallic acid to minimize oxidative degradation of FAs during analysis [30]. The samples were extracted with ether and then methylated to FA methyl esters using boron trifluoride (BF_3) in methanol (7%; wt/wt). After removing the solvent under a stream of N_2, FA methyl esters were resuspended in chloroform and analyzed with a CG17A Gas Chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector linked to a C-30021 integrator-processor (CG Instrumentos Científicos Ltda, São Paulo, Brazil). An SP-2560 (100 m × 0.25 mm inner diameter, 20-μm film thickness; Supelco Park, Bellefonte, Palo Alto, CA, USA) gas-liquid chromatographic column was used. The column temperature was programmed from 125°C to 175°C at a rate of 1°C/min and held at 175°C for 25 min. The detector and injector temperatures were 285°C and 225°C, respectively. The percentage of individual FAs was calculated according to the peak areas relative to the total area (total FAs were set at 100%). The unsaturation index (UI) in each tissue was calculated by multiplying the amount of a certain unsaturated FA by the number of double bounds in this FA. The sum of the values was then calculated to obtain a number representing the UI of the FAs [32].

Bone mineral and biomechanical assessment

Bone biomechanical properties (peak load, yield load, stiffness, resilience, and absorbed energy) were evaluated in the mid-diaphyseal region of the left tibia using a 3-point bending test with a TA-TX2 texture analyzer coupled to Texture Expert 1.2 (Stable Micro Systems, Haslemere, United Kingdom), as previously described [3,4]. The bones were thawed at room temperature, and saline solution was regularly applied to the specimens to prevent drying. Tibial length and diameter (measured in the mid-diaphyseal region) were measured using a digital caliper (Mitutoyo Sul America Ltda., Suzano, São Paulo, Brazil). The center of the bones was fractured under the following conditions: 16-mm sample space, 0.2-mm/s plunger speed, and 25-kg load range. The fractured tibiae and the left femurs were used for Ca and Zn analyses, as previously described for mineral analyses of the diets.

Statistical analysis

Results are expressed as mean ± standard deviation of six animals per group. Statistical analysis was performed using Minitab Statistical Software 14 (available at *http://www.minitab.com*). Data were analyzed by analysis of variance, followed by Tukey's multiple comparisons test after checking the homogeneity of variances (Bartlett's test). $P < 0.05$ was considered statistically significant.

Results

Food intake, body weight, and intestinal parameters

A significant decrease ($P < 0.05$) in total food intake was observed in ITF-fed rats in comparison with control rats

Table 3
Body weight gain, food intake, fecal output, and cecal parameters of rats fed 15% lipid diets (SO or FSO) and diets containing the same lipid sources supplemented with 10% ITF (SO + ITF and FSO + ITF, respectively) for 15 d*

Variable	Dietary treatment				P		
	SO	SO + ITF	FSO	FSO + ITF	Fructan	Lipid	Fructan × lipid
Body weight gain (g)	85.2 ± 8.7[a]	72.9 ± 10.3[b]	88.2 ± 6.9[a]	80.6 ± 10.7[b]	0.015	NS	NS
Food intake (g/15 d)	224.9 ± 11.9[a]	204.4 ± 11.5[b]	235.9 ± 10.8[a]	202.7 ± 21.4[b]	<0.05	NS	NS
Food efficiency	0.38 ± 0.03	0.36 ± 0.04	0.37 ± 0.02	0.40 ± 0.04	NS	NS	NS
Fecal output (dry g/5d)	7.6 ± 0.7	7.1 ± 1.0	7.7 ± 1.0	6.4 ± 0.9	NS	NS	NS
Fecal water content (%)	22.8 ± 2.5[a]	43.4 ± 5.9[b]	22.7 ± 2.8[a]	51.5 ± 13.4[b]	<0.001	NS	NS
Cecal wall weight (wet g/100 g rat)	0.25 ± 0.08[a]	0.45 ± 0.07[b]	0.25 ± 0.06[a]	0.51 ± 0.06[b]	<0.001	NS	NS
Cecal content weight (wet g/100 g rat)	0.98 ± 0.41[a]	2.21 ± 0.34[b]	0.98 ± 0.36[a]	2.54 ± 0.55[b]	<0.001	NS	NS
Cecal content pH	6.8 ± 0.1[a]	5.9 ± 0.2[b]	7.0 ± 0.3[a]	5.8 ± 0.1[b]	<0.001	NS	NS
No. of cells/crypt	21.9 ± 1.2[a]	35.7 ± 5.4[b]	22.1 ± 2.3[a]	34.7 ± 5.6[b]	<0.001	NS	NS
Liver weight (wet g/100 g rat)	5.1 ± 0.4[a]	4.9 ± 1.1[a]	5.8 ± 0.7[b]	5.7 ± 0.4[b]	NS	0.012	NS

FSO + ITF, fish and soybean oil mixture + inulin-type fructans; FSO, fish and soybean oil mixture (1:0.3, wt/wt); SO + ITF, soybean oil + inulin-type fructans; SO, soybean oil.

* Results expressed as mean ± SD ($n = 6$); mean values followed by the same superscript letter are not significantly different ($P < 0.05$).

(Table 3). Furthermore, body weight was negatively affected by ITF consumption: the average body weight gain was significantly lower ($P = 0.015$) in ITF-fed rats compared with controls, regardless of the presence of FO in the diet (Table 3). In contrast, no statistically significant differences in food efficiency were observed among the groups.

Loose stools were observed for some of the rats in the FSO + ITF group within the first 5 d of the study period. However, the consistency of feces returned to normal (compared with the SO group) during subsequent days. Fecal output during the last 5 d, cecal parameters, and liver weights are presented in Table 3. Fecal dry weight did not differ significantly among groups, whereas fecal water content was positively influenced by ITF consumption ($P < 0.001$). ITF supplementation induced an enlargement of the cecum (wall and contents) and a decrease in cecal content pH ($P < 0.001$) compared with the controls. The number of cells per hemicrypt was significantly larger ($P < 0.001$) in ITF-fed animals than in controls. FO feeding induced an increase ($P = 0.012$) in the relative mean wet weight (expressed as g/100 g of body weight) of the liver compared with SO-fed groups (Table 3).

FA composition

Rat tissue FA incorporation was significantly influenced by dietary lipid composition. Higher linoleic acid (18:2ω-6), α-linolenic acid (18:3ω-3), arachidonic acid (20:4ω-6), total ω-6 PUFA, and ω-6/ω-3 PUFA ratios, but lower myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1), 20:5ω-3, docosapentaenoic acid (22:5ω-3), 22:6ω-3, and total ω-3 PUFA values were observed in the livers of rats given SO-based diets (SO and SO + ITF) compared with those fed FO diets (FSO and FSO + ITF). Incorporation of FAs into the cecal mucosa and liver were similarly affected by dietary lipid composition, excluding higher amounts of oleic acid (18:1ω-9) and lower amounts of 20:4(ω-6) in the SO-compared to the FO-fed groups. The UI was higher ($P < 0.05$) in liver and cecal mucosa homogenates in the FO-fed groups when compared with the SO-fed groups.

Mineral absorption and balance

In general, the apparent mineral absorption was increased by ITF consumption, whereas this effect was improved by dietary combination with FO (Fig. 1). ITF-fed rats presented a higher Ca balance than those fed control diets ($P < 0.001$; Fig. 1B). In contrast, although Mg absorption increased ($P < 0.05$) as a consequence of ITF consumption, it was negatively influenced by dietary FO (Fig. 1C). Cu absorption was positively affected by ITF ($P < 0.05$) and potentiated when in combination with FO ($P < 0.001$). In addition, apparent Fe absorption was significant and positively affected only when ITF was accom-
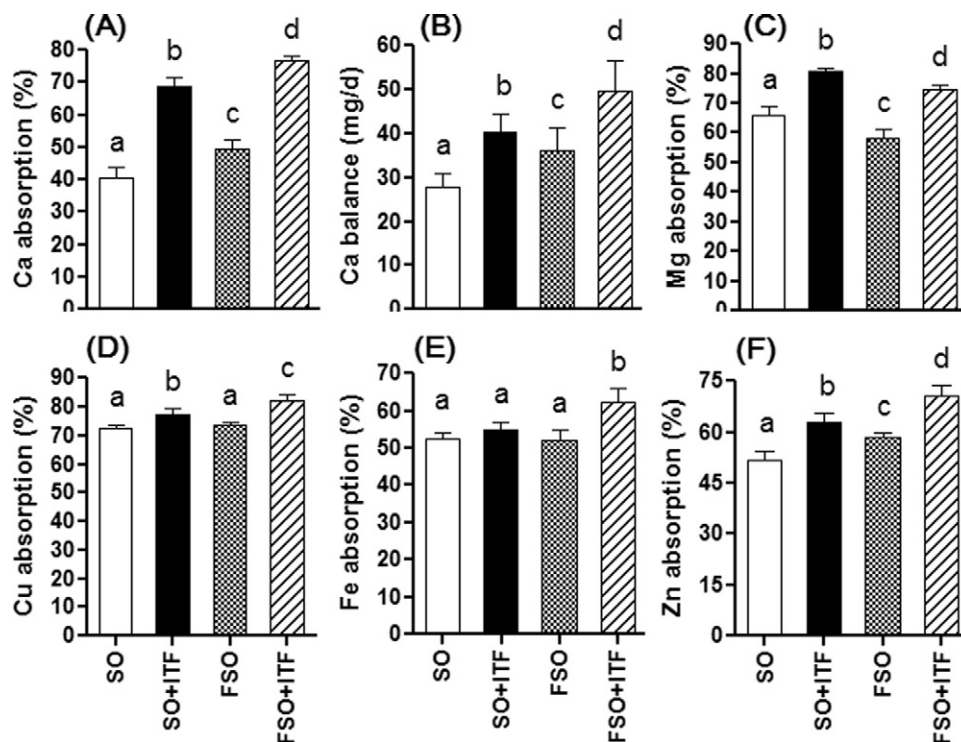

Fig. 1. Intestinal Ca absorption (A) and balance (B) and intestinal Mg (C), Cu (D), Fe (E), and Zn (F) absorption in rats fed 15% lipid diets (SO or FSO) and diets containing the same lipid sources supplemented with 10% ITF (SO + ITF and FSO + ITF, respectively) for 15 d. Results are expressed as mean ± SD ($n = 6$). Mean values followed by the same superscript letter are not significantly different ($P < 0.05$). Ca, calcium; Cu, copper; Fe, iron; FSO + ITF, fish and soybean oil mixture + inulin-type fructans; FSO, fish and soybean oil mixture (1:0.3, wt/wt); Mg, magnesium; SO + ITF, soybean oil + inulin-type fructans; SO, soybean oil; Zn, zinc.
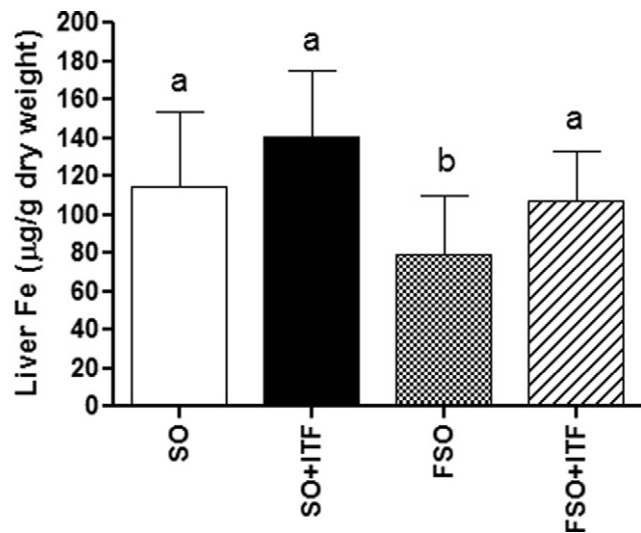
Fig. 2. Liver iron concentration of rats fed 15% lipid diets (SO or FSO) and diets containing the same lipid sources supplemented with 10% ITF (SO + ITF and FSO + ITF, respectively) for 15 d. Results are expressed as mean ± SD (*n* = 6). Mean values followed by the same superscript letter are not significantly different (*P* < 0.05). Ca, calcium; FSO + ITF, fish and soybean oil mixture + inulin-type fructans; FSO, fish and soybean oil mixture (1:0.3, wt/wt); SO + ITF, soybean oil + inulin-type fructans; SO, soybean oil; Zn, zinc.

panied by FO in the diet (*P* = 0.004; Fig. 1E). Compared with SO, FO significantly affected Ca, Mg, and Zn absorption and Ca balance (Fig. 1A–C,F).

Mineral retention in the organs

Liver Fe stores were decreased (−45%, *P* = 0.015) in rats fed the FSO diet, considering that ITF consumption returned to levels comparable to the SO control group (Fig. 2). Bone Ca and Zn retention results are illustrated in Figure 3. Bone mineral content was enhanced as a conse-

quence of ITF consumption, but the increase was statistically significant only for FO-fed animals (FSO + ITF group).

Bone biomechanical assessment

Table 4 presents extrinsic biomechanical properties at the mid-diaphyseal region of the left tibia. No significant differences were observed in bone wet weight and length among the experimental groups (0.4 g and 34.9 mm, respectively, for the SO control group). However, the 3-point bending test revealed a significant and positive effect of ITF and FO feedings on peak and yield load variables (Table 4). ITF supplementation also positively influenced stiffness (*P* = 0.04) and resilience (*P* = 0.02) compared with the control groups. In addition, dietary FO affected absorbed energy (*P* = 0.032) when compared with SO-based diets.

Discussion

Mineral bioavailability could be influenced by several factors in the diet, the magnitude of which depends on inhibitors and promoters contained in a meal and, hence, the diet composition [33]. ITF (FOS and inulin) are the most investigated dietary non-digestible oligosaccharides with respect to their positive effects on mineral bioavailability [2–12]. In the present study, dietary supplementation with 10% ITF as a combination of short-chain and LC ITF (Raftilose Synergy 1) for 15 d resulted in higher intestinal absorption of some macrominerals (Ca and Mg) and microminerals (Cu and Zn) in growing rats (Fig. 1). These effects were reflected in (non-statistically significant) higher bone mineral content (Fig. 3) and greater bone breaking strength at the tibia mid-diaphysis (Table 4). Furthermore, it was hypothesized that changes in mineral bioavailability as a result of ITF feeding might be influenced by dietary lipid
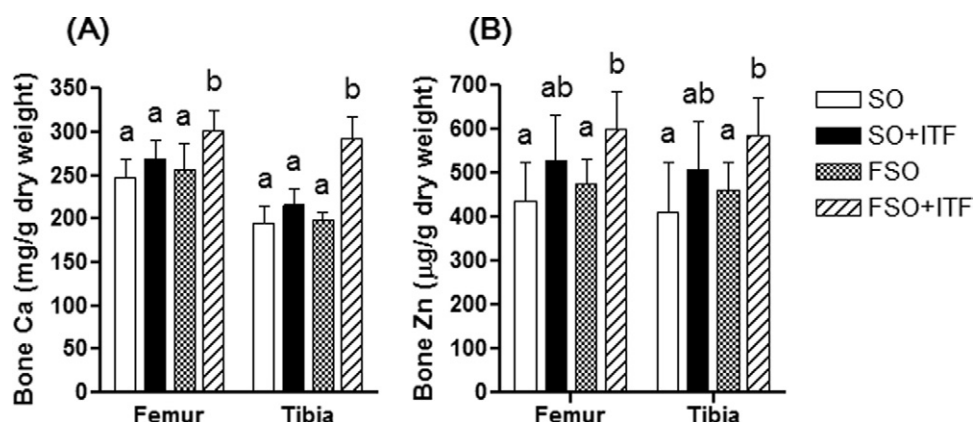

Fig. 3. Calcium (A) and zinc (B) concentrations in femurs and tibias of rats fed 15% lipid diets (SO or FSO) and diets containing the same lipid sources supplemented with 10% ITF (SO + ITF and FSO + ITF, respectively) for 15 d. Results are expressed as mean ± SD (*n* = 6). Mean values followed by the same superscript letter are not significantly different (*P* < 0.05). Fe, iron; FSO + ITF, fish and soybean oil mixture + inulin-type fructans; FSO, fish and soybean oil mixture (1:0.3, wt/wt); SO + ITF, soybean oil + inulin-type fructans; SO, soybean oil.

Table 4

Biomechanical properties of left tibia of rats fed a 15% lipid diet (SO or FSO) and diets containing the same lipid sources supplemented with 10% ITF (SO + ITF and FSO + ITF, respectively) for 15 d*

Variable	Dietary treatment				P		
	SO	SO + ITF	FSO	FSO + ITF	Fructan	Lipid	Fructan × lipid
Peak load (N)	35.2 ± 2.5[a]	43.0 ± 5.6[b]	43.4 ± 5.0[b]	46.3 ± 2.1[b]	0.005	0.003	NS
Yield load (N)	29.5 ± 2.2[a]	34.7 ± 3.2[b]	34.3 ± 1.9[b]	34.5 ± 2.9[b]	<0.05	<0.05	NS
Stiffness (N/mm)	47.5 ± 6.3[a]	53.0 ± 2.2[b]	48.4 ± 5.7[a]	53.8 ± 3.2[b]	0.04	NS	NS
Resilience (Nmm)	9.6 ± 2.6[a]	11.7 ± 1.9[b]	10.2 ± 3.0[b]	12.7 ± 1.9[b]	0.02	NS	NS
Absorbed energy (Nmm)	21.5 ± 5.7[a]	27.2 ± 2.2[ab]	28.6 ± 9.2[ab]	32.0 ± 6.8[b]	NS	0.032	NS

FSO + ITF, fish and soybean oil mixture + inulin-type fructans; FSO, fish and soybean oil mixture (1:0.3, wt/wt); SO + ITF, soybean oil + inulin-type fructans; SO, soybean oil

* Results expressed as mean ± SD ($n = 6$); mean values followed by the same superscript letter are not significantly different ($P < 0.05$).

composition. To our knowledge, this is the first report demonstrating improved mineral bioavailability in rats fed a combination of dietary ITF and FOs rich in LC ω-3 PUFAs (especially EPA and DHA).

Although the small intestine is the major site of mineral absorption, the large intestine also plays an important role, because some dietary fibers (including ITF) are fermented by bacteria in the colon. ITF fermentation gives rise to short-chain FAs (particularly butyrate), lactate, and gases, which are accompanied by enlargement of the cecal tissue and decreased luminal pH [2,5,12,34,35]. Accordingly, in the present study, ITF-fed rats presented greater cecal mass (wall and contents) and lower luminal pH than control rats (Table 3) as an indirect result of enhanced biomass, which may have been a response to greater bacterial metabolic activity. Furthermore, these effects were concomitant with greater crypt cellularity (Table 3), which is probably attributable to an increase in crypt cell proliferation [36]. These trophic effects are believed to be mediated by butyrate, a potent stimulator of cell division and the acknowledged major energy source of colonocytes [34–36], although this assumption is still questionable and some investigators have argued about the beneficial effects of butyrate [37,38]. In this context, a previous study showed an increase in the depth and number of total and bifurcated crypts in the cecum of rats fed 7.5% low-DP ITF from yacon (*Smallanthus sonchifolius*) tuberous roots [3].

The present results demonstrate that tissue FA composition reflected the dietary lipid treatment: rats fed FO-based diets (FSO and FSO + ITF groups) showed significantly larger amounts of total ω-3 PUFAs (particularly EPA and DHA) and a reduction in total ω-6 PUFAs compared with those fed SO-based diets. The changes induced by FO feeding led to a significant increase in the UI of FAs incorporated into the liver and cecal mucosa homogenates. These findings are in agreement with previous reports demonstrating that FA incorporation in tissues and cells is markedly modified by dietary lipid composition [32,39]. It is well known that membrane lipid peroxide levels increase as membrane PUFA content and UI increase [39]. In this respect, changes in dietary lipid composition have also been related to alterations in Fe homeostasis. Miret et al. [21]

demonstrated that feeding rats an FO-rich diet increased lipid peroxidation and affected Fe metabolism. After 16 wk, total liver and spleen Fe stores were depleted in rats fed diets containing 5% lipid as FO compared with those fed diets containing the same amount of lipid as maize or olive oil [21]. Such effects were accompanied by an increase in the percentage of reticulocytes, indicating accelerated erythrocyte turnover. It was suggested that membrane PUFA oxidation could be associated with cell aging and that higher Fe incorporation into the erythrocytes of rats fed a ω-3 PUFA-rich diet is due to their shorter life span [40]. Accordingly, the present results showed that rats fed an FO-based diet (FSO group) had lower liver Fe stores compared with SO control rats, whereas the association of FO and ITF in the diets re-established liver Fe stores compared with those of the SO control group (Fig. 2). Therefore, although hematologic parameters were not evaluated in the present study, it is possible that greater LC ω-3 PUFA membrane incorporation (mainly EPA and DHA) occurred in the FO-fed groups (FSO and FSO + ITF), which may have increased erythrocyte turnover and liver Fe demand.

Feeding rats diets supplemented with 10% ITF induced a significant increase in apparent mineral absorption and balance when compared with control rats, and such effects were enhanced when rats were fed ITF diets containing FO (Fig. 1). In the present study, a mixture of ITF with different chain lengths (Raftilose Synergy 1) was used to improve mineral absorption efficiency along the large intestine. Short-chain ITFs (FOS) are more active in the proximal part, whereas LC ITFs (high-performance inulin) are more effective in the distal part of the large intestine [2]. These findings confirm those of Raschka and Daniel [41] who demonstrated a significant increase in intestinal absorption and retention of Ca, Mg, and Zn after feeding rats with Raftilose Synergy 1 for 15 d. Moreover, our previous studies demonstrated greater Ca and Mg absorption in rats fed short-chain ITF compared with controls, an increase observed as soon as 8 d after initiation of dietary treatment [3,4].

In contrast, the effect of FAs on mineral absorption may be exerted directly on mineral uptake or indirectly by altering the FA composition of the intestinal brush border membrane, which in turn modifies membrane properties and

function [20]. Incorporation of unsaturated FAs in membranes leads to greater fluidity and permeability. Furthermore, some membrane enzymes, such as adenosine triphosphatase, are activated by the unsaturated environment [16]. In the present study, rats fed FO presented a higher UI as a consequence of greater LC ω-3 PUFA incorporation into cecal mucosa homogenates. Therefore, we deduced that this might have contributed to the greater mineral absorption observed in FO-fed rats (Fig. 1).

Even though the majority of studies about Ca and Mg absorption using rats have been performed in the short term, the improvement in bone mineral and structure by ITF feeding has been reported more frequently in long-term studies [2]. In the present short-term study (15 d), bone mineral content was improved by ITF consumption, although this effect was statistically significant only when FO was the lipid source (Fig. 3). Bone remodeling is a dynamic, lifelong process that involves organic matrix synthesis by osteoblasts and bone resorption by osteoclasts. In healthy growing rats fed 5% ITF over a 4-wk period, Kruger et al. [8] observed lower urinary excretion of type 1 collagen fragments, a biochemical marker of bone resorption. The antiresorptive effect of ITF was also confirmed by Nzeusseu et al. [6] who demonstrated a significant decrease in serum levels of C-telopeptide in growing rats after 3 mo of dietary treatment. In agreement with these studies, recent data from our group showed 62% and 63% decreases in the number of osteoclasts (N.Oc/T.Ar) and the osteoclast surface (Oc.S/BS), respectively, in rat femurs, as measured by bone histomorphometry after 33 d of feeding 10% low-DP ITF (Lobo AR, Cocato ML, Jorgetti V, Vaz RTC, Colli C, unpublished results).

Today, accumulating evidence indicates that dietary lipids, particularly LC ω-3 PUFAs, have strong effects on mineral absorption and bioavailability. The dietary ω-6/ω-3 ratio affects bone metabolism because bone formation and bone resorption are influenced by prostaglandins and cytokines [16,18,19], which in turn are regulated by these FAs. A high dietary ω-6/ω-3 ratio has been observed to reduce bone formation capacity and stimulate bone resorption activity through increased endogenous production of prostaglandin E$_2$ [18,19]. Furthermore, ω-3 PUFA treatment has been related to decreased osteoclastogenesis in vivo and in vitro, because DHA (more efficiently than EPA) downregulates several osteoclast-specific genes, such as tartrate-resistant acid phosphatase and osteoclast-specific transcription factor (c-Fos), and the osteotropic proinflammatory cytokine tumor necrosis factor-α [16,42].

Bone tissue is a combination of mineral and non-mineral matrix constituents. The major minerals in bone are Ca and phosphorus, which are present as constituents of hydroxyapatite and play a role in the maintenance of skeletal mechanical strength. However, other minerals and vitamins are crucial for carrying out reactions and metabolic processes in bone [43]. For instance, Zn is a cofactor for alkaline phosphatase that has been implicated in bone formation and mineralization [44]. A reduction in bone integrity in moderately Zn-deficient versus Zn-adequate rats has been observed, as demonstrated by diminished tibia biomechanical properties [45].

The present results indicate that rats fed a dietary combination of ITF and FO presented a greater tibial peak and yield load compared with SO control rats (Table 4). Furthermore, there was a significant effect of ITF on stiffness and resilience and a significant effect of FO on absorbed energy (Table 4). Therefore, the positive effects on intestinal absorption of bone-relevant minerals observed in the present study could have contributed to greater bone mineral retention and quality in rats fed ITF. Nevertheless, it should be taken into account that bone strength also depends on the architectural disposition of bone material (bone geometry parameters, e.g., cross-sectional area, moment of inertia, cortical thickness) and other factors unrelated to mineralization, such as crystal arrangement and size, the degree of collagen cross-linking, and the amount and distribution of tissue microdamage [46]. For instance, if collagen fibers provide resistance to the propagation of a crack through bone, loss of their cross-linking would lead to reduced resistance to the crack, and the bone would be more readily fractured [46]. In this regard, our previous results showed an increase in peak and yield load in the femurs of rats fed a 5% short-chain ITF diet, suggesting an influence of ITF on bone organic constituents [4]. In contrast, Nzeusseu et al. [6] observed a greater polar stress/strain index in rats fed LC ITF (inulin; 13.5% increase) than those fed short-chain ITF (oligofructose; 6.7% increase) compared with control rats, as assessed by peripheral quantitative computerized tomographic measurements, suggesting an increase in cortical bone resistance to bending.

In summary, this study examined the effects of a dietary combination of ITF and FO, which are hypothesized to enhance mineral absorption and bone parameters in healthy growing rats. The present results revealed an improvement in apparent mineral absorption, bone (femur and tibia) mineral retention, and bone strength variables in the tibial midshafts of rats fed ITF and FO rich in LC ω-3 PUFAs. These findings indicate that dietary lipid composition influenced the effects of ITF on mineral bioavailability. More accurate studies expanding these results on bone biochemical and architectural parameters could elucidate the likely mechanisms involved in these findings. If these observations are applicable to humans, then a dietary combination of ITF (or other types of fermentable carbohydrates) and FO might be useful in maintaining skeletal integrity and improving bone health. Furthermore, such a combination of ITF and FO may be interesting because FO consumption is currently recommended, although the occurrence of peroxidation in an individual consuming a diet rich in these lipids might result in Fe deficiency as a consequence of lower body stores.

Acknowledgments

References

[1] Roberfroid M. Prebiotics: the concept revisited. J Nutr 2007;137:830S–7.

[2] Scholz-Ahrens K, Schrezenmeir J. Inulin and oligofructose and mineral metabolism: the evidence from animal trials. J Nutr 2007;137:2513S–23.

[3] Lobo AR, Colli C, Alvares EP, Filisetti TMCC. Effects of fructans-containing yacon (*Smallanthus sonchifolius* Poepp & Endl.) flour on the morphometry of cecal mucosa, calcium and magnesium balance and bone calcium retention in growing rats. Br J Nutr 2007;97:776–85.

[4] Lobo AR, Colli C, Filisetti TMCC. Fructooligosaccharides improve bone mass and biomechanical properties in rats. Nutr Res 2006;26:413–20.

[5] Raschka L, Daniel H. Mechanisms underlying the effects of inulin-type fructans on calcium absorption in the large intestine of rats. Bone 2005;37:728–35.

[6] Nzeusseu A, Dienst D, Haufroid V, Depresseux G, Devogelaer JP, Manicourt DH. Inulin and fructo-oligosaccharides differ in their ability to enhance the density of cancellous and cortical bone in the axial and peripheral skeleton in growing rats. Bone 2006;37:394–9.

[7] Zafar TA, Weaver CM, Zhao Y, Martin BR, Wastney ME. Non-digestible oligosaccharides increase calcium absorption and suppress bone resorption in ovariectomized rats. J Nutr 2004;134:399–402.

[8] Kruger MC, Brown KE, Collett G, Layton L, Schollum LM. The effect of fructooligosaccharides with various degrees of polymerization on calcium bioavailability in the growing rat. Exp Biol Med 2003;228:683–8.

[9] Van Der Heuvel EGHM, Muys T, Van Dokkum W, Schaafsma G. Oligofructose stimulates calcium absorption in adolescents. Am J Clin Nutr 1999;69:544–8.

[10] Abrams SA, Griffin IJ, Hawthorne KM. Young adolescents who respond to an inulin-type fructan substantially increase total absorbed calcium and daily calcium accretion to the skeleton. J Nutr 2007;137:2524S–6.

[11] Holloway L, Moynihan S, Abrams SA, Kent K, Hsu AR, Friedlander AL. Effects of oligofructose-enriched inulin on intestinal absorption of calcium and magnesium and bone turnover markers in postmenopausal women. Br J Nutr 2007;97:365–72.

[12] Rémésy C, Levrat MA, Gamet L, Demigné C. Cecal fermentations in rats fed oligosaccharides (inulin) are modulated by dietary calcium level. Am J Physiol Gastrointest Liver Physiol 1993;264:G855–62.

[13] Kleessen B, Hartmann L, Blaut M. Fructans in the diet cause alterations of intestinal mucosal architecture, released mucins and mucosa-associated bifidobacteria in gnotobiotic rats. Br J Nutr 2003;89:597–606.

[14] Tako E, Glahn RP, Welch RM, Lei X, Yasuda K, Miller DD. Dietary inulin affects the expression of intestinal enterocyte iron transporters, receptors and storage protein and alters the microbiota in the pig intestine. Br J Nutr 2008;99:472–80.

[15] Tako E, Yasuda K, Glahn RP, Welch RM, Lei X, Miller DD. Dietary inulin upregulates the expression of iron and zinc membrane transporters in intestinal enterocytes in the pig. FASEB J 2006;20:Ib439.

[16] Poulsen RC, Moughan PJ, Kruger MC. Long-chain polyunsaturated fatty acids and the regulation of bone metabolism. Exp Biol Med 2007;232:1275–88.

[17] Watkins BA, Li Y, Seifert MF. Dietary ratio of n-6/n-3 PUFAs and docosahexaenoic acid: actions on bone mineral and serum biomarkers in ovariectomized rats. J Nutr Biochem 2006;17:282–9.

[18] Watkins BA, Li Y, Lippman HE, Feng S. Modulatory effect of omega-3 polyunsaturated fatty acids on osteoblast function and bone metabolism. Prostaglandins Leukot Essent Fatty Acids 2003;68:387–98.

[19] Watkins BA, Li Y, Allen KGD, Hoffmann WE, Seifert MF. Dietary ratio of (n-6)/(n-3) polyunsaturated fatty acids alters the fatty acid composition of bone compartments and biomarkers of bone 7 ormation in rats. J Nutr 2000;130:2274–84.

[20] Stubbs CD, Smith AD. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. Biochim Biophys Acta 1984;779:89–137.

[21] Miret S, Sáiz MP, Mitjavila MT. Effects of fish oil– and olive oil–rich diets on iron metabolism and oxidative stress in the rat. Br J Nutr 2003;89:11–8.

[22] Shimano MM, Shimano AC, Volpon JB. Histerese de fêmures de ratas submetidos a ensaio de flexão, em diferentes faixas etárias. Rev Bras Eng Biomed 2002;18:88–97.

[23] Turner CH, Burr DB. Basic biomechanical measurements of bone: a tutorial. Bone 1993;14:595–608.

[24] Fritsche KL, Johnston PV. Rapid autoxidation of fish oil in diets without added antioxidants. J Nutr 1988;118:425–6.

[25] Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 Purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on reformulation of the AIN-76A rodent diet. J Nutr 1993;123:1939–51.

[26] National Academy of Sciences (USA). Dietary reference intakes for energy, carbohydrates, fat, fatty acids, cholesterol, protein and amino acids. Washington, DC: National Academy Press; 2005.

[27] Roberfroid M, Gibson GR, Delzenne N. The biochemistry of oligofructose, a nondigestible fiber: an approach to calculate its caloric value. Nutr Rev 1993;51:137–46.

[28] Sedlin ED, Hirsch C. Factors affecting the determination of the physical properties of femoral cortical bone. Acta Orthop Scand 1966;37:29–48.

[29] Instituto Adolfo Lutz. Normas analíticas do Instituto Adolfo Lutz: Métodos químicos e físicos para análise de alimentos. 4th ed. São Paulo: Instituto Adolfo Lutz; 2005, p. 98–117.

[30] Association of Official Analytical Chemists. Official methods of analysis. 18th ed. Washington, DC: Association of Official Analytical Chemists; 2005.

[31] Prosky L, Asp NG, Schweizer TF, DeVries JW, Furda I. Determination of insoluble, soluble, and total dietary fiber in foods and food products: interlaboratory study. J AOAC 1988;71:1017–23.

[32] Guimarães ARP, Kuga E, Torres RP, Colquhoun A, Curi R, Mancini Filho J. Composition of fatty acids in the liver and lymphoid organs of rats fed fatty acids–rich diets. Biochem Mol Biol Int 1995;36:451–61.

[33] Gibson RS. The role of diet- and host-related factors in nutrient bioavailability and thus in nutrient-based dietary requirement estimates. Food Nutr Bull 2007;28:S77–100.

[34] Le Blay GM, Michel CD, Blottière HM, Cherbut CJ. Prolonged intake of fructo-oligosaccharides induces a short-term elevation of lactic acid-producing bacteria and a persistent increase in cecal butyrate in rats. J Nutr 1999;129:2231–5.

[35] Le Blay GM, Michel CD, Blottière HM, Cherbut CJ. Raw potato starch and short-chain fructooligosaccharides affect the composition

and metabolic activity of rat intestinal microbiota differently depending on the caecocolonic segment involved. J Appl Microbiol 2003;94:312–20.

[36] Topping DL, Clifton PM. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol Rev 2001;81:1031–64.

[37] Goodlad RA, Englyst HN. Redefining dietary fibre: potentially a recipe for disaster. Lancet 2001;358:1833–4.

[38] Goodlad RA. Editorial: Fiber can make your gut grow. Nutrition 2007;23:434–5.

[39] Turini ME, Thomson ABR, Clandinin MT. Lipid composition and peroxide levels of mucosal cells in the rat large intestine in relation to dietary fat. Lipids 1991;26:431–40.

[40] Rodríguez MC, Sáiz MP, Muntané J, Mitjavila MT. Fatty acid composition of erythrocyte membranes affects iron absorption in rats. J Nutr 1996;126:3109–17.

[41] Raschka L, Daniel H. Diet composition and age determine the effects of inulin-type fructans on intestinal calcium absorption in rat. Eur J Nutr 2005;44:360–4.

[42] Rahman MM, Bhattacharya A, Fernandes G. Docosahexaenoic acid is more potent inhibitor of osteoclast differentiation in RAW 264.7 cells than eicosapentaenoic acid. J Cell Physiol 2008;214:210–9.

[43] Nieves JW. Osteoporosis: the role of micronutrients. Am J Clin Nutr 2005;81:1232S–9.

[44] Yamaguchi M, Yamaguchi R. Action of zinc on bone metabolism in rats. Increases in alkaline phosphatase activity and DNA content. Biochem Pharmacol 1986;35:773–7.

[45] Scrimgeour AG, Stahl CH, McClung JP, Marchitelli LJ, Young AJ. Moderate zinc deficiency affects biomechanical properties of rat tibiae independently of body composition. J Nutr Biochem 2007;18:813–9.

[46] Burr DB. The contribution of the organic matrix to bone's material properties. Bone 2002;31:8–11.