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ORIGINAL CONTRIBUTION

Effect of a mixture of GOS/FOS® on calcium absorption and retention during recovery from protein malnutrition: experimental model in growing rats

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Abstract
Introduction During growth, protein deprivation impairs epiphyseal growth plate (EGP) height, bone volume (BV) and endochondral ossification. During catch-up growth, Ca availability becomes essential to ensure the extra amount needed to achieve optimal peak bone mass and strength. GOS and FOS improve mineral absorption in the colon.
Purpose The effect of a mixture of GOS/FOS® 9:1 added to a 0.5 %Ca (NCa) and a 0.3 %Ca (LCa) diets on Ca, P and Mg absorptions and bone mineralization, density and structure using an experimental model of growing rats recovering from early protein malnutrition was investigated.
Methods To induce protein malnutrition, rats were fed a low protein diet: 4 % (LPD) during 1 week and then were randomly assigned to recovery groups (R) until day 50 ($T = 50$) as follows: R0.5 %: NCa; RP0.5 %: NCa + 5.3 % GOS/FOS®; R0.3 %: LCa and RP0.3 %: LCa + 5.3 % GOS/FOS®. Control groups received the 0.5 %Ca or 0.3 %Ca diet from weaning until day 40 or 50.
Results Body weight and length increased in C groups throughout the study; both were arrested in all **R** during LPD consumption and increased immediately after re-feeding. Independently of dietary Ca content, LS counts, β-glucosidase and Ca, P and Mg absorption increased, whereas cecum pH, β-glucuronidase, urease and tryptophanase decreased in RP0.5 %: and RP0.3 %: as compared to the other studied groups ($p < 0.01$). Prebiotic consumption decreased CTX levels and increased femur Ca, Mg and P contents, total skeleton bone mineral content, proximal tibia and spine BMD, BV, EGP height and hypertrophic zone thickness, stiffness and elastic modulus as compared to recovery groups fed the prebiotic-free diets.
Conclusion Under the present experimental conditions, GOS/FOS® mixture induced colonic positive effects, which increased Ca, P and Mg absorption. Thus, consuming the prebiotic-containing diet resulted in an extra amount of minerals that improved bone development in growing rats recovering from protein malnutrition.

Keywords Calcium absorption · Calcium retention · Galacto-oligosaccharides · Fructo-oligosaccharides · Undernourished rats · Catch-up growth

Abbreviations

EGP	Epiphyseal growth plate
P	Phosphorus
CaI	Calcium intake
NDFO	Non-digestible fructo-oligosaccharides
GOS	Galacto-oligosaccharides
FOS	Long-chain fructo-oligosaccharides

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AIN	American Institute of Nutrition
LPD	Low protein diet
NCa	Normal Ca content diet
LCa	Low Ca content diet
U	Undernourished group
BW	Body weight
CO_2	Carbon dioxide
LS	*Lactobacillus*
CFU	Colony-forming units
I	Food consumption
F	Feces
Ab	Apparent mineral absorption
Mg	Magnesium
HCl	Hydrochloric acid
HNO_3	Nitric acid
BAP	Bone alkaline phosphatase
CTX	Type I collagen telopeptide
Alb	Albumin
tsBMC	Total skeleton bone mineral content
tsBMD	Bone mineral density
DXA	Dual-energy X-ray absorptiometry
CV	Coefficients of variation
ROI	Region of interest
EDTA	Ethylenediaminetetraacetic acid

BV/TV Bone volume fraction

GPC.Th	GPC thickness
HpZ.Th	Hypertrophic zone thickness
BL	Body length
TL	Tail length

Introduction

Nutrition influences linear growth and bone mass accumulation, modulating the genetic potential for bone development [1]. Although animal studies have clearly demonstrated the deleterious effects of protein malnutrition on linear growth, different parts of long bones respond differently to nutritional injury. In the growing rat, isocaloric protein deprivation affects epiphyseal growth plate (EGP) height rather than bone length; bone volume and endochondral ossification are also affected with negative consequences on bone formation [2–5]. Hence, abnormalities in bone length and composition will be observed unless the stopped growth is compensated for by catch-up growth, i.e., a growth velocity above the statistical limits of normality for age and/or maturity [6].

The main priority of recovery should be to regain linear growth and maturation without affecting body mass average to age ratio. For this reason, it is important to increase the quality rather than the quantity of the recovery diet. In this regard, re-feeding with a normal protein–calorie diet immediately initiates a catch-up response. In such a recovery process, calcium (Ca) and phosphorus (P) availability becomes essential to ensure the extra amount needed to achieve optimal peak bone mass and strength [7].

Whereas increasing Ca intake (CaI) would be the most effective strategy to supply the amount of Ca required for catch-up growth, improving Ca absorption is another important tool to optimize Ca bioavailability. Most Ca absorption occurs in the small intestine; however, if the insoluble, unabsorbed Ca coming from this part of the intestine is maintained in an ionic form, about 5–10 % could occur in the colon [8]. Non-digestible fructo-oligosaccharides (NDFO) are not hydrolyzed enzymatically in the small intestine but are fermented by the resident microbiota in the colon, inducing several changes that improve Ca and P absorption in the gut [9–12].

Besides inhibiting longitudinal bone growth, protein malnutrition has several devastating consequences such as malabsorption, increased intestinal permeability, gram-negative (enteric) bacteremia and suboptimal immune response [13–15]. NDFO modifies the colonic microbiota increasing the proliferation and activity of beneficial bacteria against pathogenic species [16–19]. This shift in the composition of the enteric microbiota dramatically changes the bacterial metabolic products released into the gut. Some of these products stimulate intestinal epithelium proliferation and increase its absorptive capacity, whereas others modulate the immune system.

We previously demonstrated that a combination of short- and long-chain inulin-type fructans increases the number of *Lactobacilli* in feces and enhances Ca and P absorption and bone mineralization during normal growth [20]. The hypothesis of the present report was that an increment in colonic mineral absorption as a result of feeding the prebiotic mixture would supply an extra amount of Ca and P to support the catch-up growth that would allow recovery from protein malnutrition. The present work was carried out to evaluate the effect of a 9:1 mixture of short-chain galacto-oligosaccharides (GOS) and long-chain fructo-oligosaccharides (FOS) (GOS/FOS®) during recovery from early protein undernutrition on Ca and P absorption, bone mineralization and bone density and structure. Several indicative colonic parameters (e.g., luminal pH, cecum weight, bacterial counts and intestinal enzymatic activity) were also evaluated to help support the findings of the present report. Because the effects of protein malnutrition on the skeleton might be most marked during early growth, we chose an experimental model in growing rats, in which the degree of protein restriction was high enough to induce mild growth retardation [21].

Table 1 Centesimal composition of the experimental diets (g/100 g)

Diet	AIN93-G (containing 4 % of protein) (LP)	AIN93-G (containing 0.5 %Ca) (NCa)	AIN93-G (containing 0.5 %Ca) + 5.3 % GOS/FOS®	AIN93-G (containing 0.3 %Ca) (LCa)	AIN93-G (containing 0.3 %Ca) + 5.3 % GOS/FOS®
Energy (kcal)	395	395	395	395	395
Protein (g)*	4	17	17	17	17
Lipids (g)**	7	7	7	7	7
Ca-free salts mixture (g)#	3.5$	3.5$	3.5$	3.5$	3.5$
Vitamins (g)***	1$	1$	1$	1$	1$
Choline (g)	0.25	0.25	0.25	0.25	0.25
Cellulose (g)****	5	5	5	5	5
Dextrin&	To complete 100 g				
Calcium (Ca) (g)##	0.5	0.5	0.5	0.3	0.3
Phosphorus (P)¤	0.3	0.3	0.3	0.3	0.3
Magnesium (Mg)¤	0.0513	0.0513	0.0513	0.0513	0.0513
GOS/FOS® (9:1) (g)###	–	–	5.3	–	5.3

All diets were prepared according to AIN93-G, and they only varied in protein (4 or 17 %) or calcium (0.5 or 0.3 %) content

* *Sodium caseinate* (*Lactoprot GMBH*, *Germany*) containing 85.1 % of protein and 0.095 g % of Ca

** Commercial soy oil. Molinos Rio de la Plata. Argentina

*** *Vitamins* prepared according to AIN-93G that meet rat requirements during growth. Manufactured by the Department of Food Science School of Biochemistry, University of Buenos Aires (individual components from Sigma, Missouri, USA)

**** Choline citrate 0.71 % (*food grade*, *Anedra*, *Argentina)*

Ca-free salts mixture was prepared according to AIN93-G, except for Ca content

CaCO3 (*food grade individual components*, *Anedra*, *Argentina*) was added to obtain the required Ca concentration

¤ Potassium phosphate monobasic and magnesium oxide (food grade individual components, Anedra, Argentina) were added to obtain the required P and Mg concentration, respectively

& *Corn dextrin* from corn refinery, provided by Food SA Argentina, was added as carbohydrate (fiber) source to achieve 100 g of diet

GOS and FOS batch Nº 110710 and HPPGJ1AGJ, respectively

Materials and methods

Diets

All experimental diets were isocaloric for formulation and prepared according to the American Institute of Nutrition Rodent Diets Recommendations for growing animals settled in 1993 (AIN93-G) [22]. Low protein diet (LP) only contained 4 % of protein, while the other components were added according to AIN93-G. Diets containing prebiotics were prepared by adding 5.3 g % of a mixture of GOS/FOS® (9:1) (batch Nº 110710 and HPPGJ1AGJ, respectively). $CaCO_3$ (Analytical grade, Anedra, Argentina) was added to obtain the two dietary levels of Ca: 0.5 % (NCa) or 0.3 % (LCa; Table 1).

Animal

Male weanling Wistar rats (35–40 g) were obtained from the Biochemical and Oral Department, School of Dentistry, Buenos Aires University, Argentina. They were housed individually in stainless steel cages under 12-h light/dark cycles, at controlled room temperature (21 ± 2 °C) and humidity (50–55 %). Throughout the experimental period, rats were allowed access to deionized water and food "ad libitum."

Rats were maintained in keeping with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, NIH (Res 8023) and observed in the Ethics Code of the School of Dentistry and Pharmacy and Biochemistry of Buenos Aires University, Res. CD 325/2002 and Res CS 4081/04.

Experimental design

It has been shown previously in experimental models that early protein depletion by feeding an isocaloric low protein diet closely resembles what occurs in the human child suffering protein malnutrition shortly after weaning. Indeed, weaning rats fed this diet for 1 week show an arrest in body growth that leads to delayed growth for age [23, 24]. Given that one remodeling cycle in the rat lasts about 21 days and in order to evaluate changes in bone parameters, the experimental diets were supplied during a 28-day period.

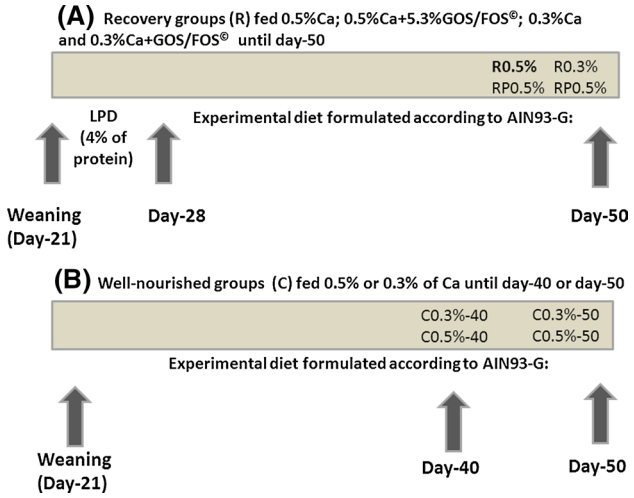

Fig. 1 Diagram of the experimental design

A diagram of the experimental groups is shown in Fig. 1a, b. To induce protein malnutrition, 32 weaning rats were fed the isocaloric low protein diet during 1 week. After that, rats were randomly assigned to recovery groups (R) receiving one of the four experimental diets until 50 days of age ($T = 50$; $n = 8$ per group; Fig. 1a).

Group R0.5 %: AIN93-G formulation containing 0.5 % of Ca (NCa)
Group RP0.5 %: AIN93-G formulation containing 0.5 % of Ca (NCa) + 5.3 % of a GOS/FOS® mixture 9:1
Group R0.3 %: AIN93-G formulation containing 0.3 % of Ca (LCa)
Group RP0.3 %: AIN93-G formulation containing 0.3 % of Ca + 5.3 % of a GOS/FOS® mixture 9:1

The results of recovery were compared to well-nourished control groups ($n = 8$ per group) receiving one of the following experimental diets from weaning ($T = 0$) until 50 days of age ($T = 50$; Fig. 1b).

Group C0.5 %-50: AIN93-G formulation containing 0.5 % of Ca (NCa)
Group C0.3 %-50: AIN93-G formulation containing 0.3 % of Ca (LCa)

To evaluate any possible stunting in height for age, the results of recovery were compared to two additional control groups fed one of the above diets until the age of 40 days ($T = 40$):

Group C0.5 %-40: AIN93-G formulation containing 0.5 % of Ca (NCa)
Group C0.3 %- 40: AIN93-G formulation containing 0.3 % of Ca (LCa; Fig. 1b)

The diets were offered as a powder. Food consumption and body weight (BW) were determined twice a week. Body and tail length (BL, TL, respectively) were measured once a week under moderate CO_2 sedation.

Fresh fecal samples were obtained weekly. Densitometry analysis was performed at the end of the study: $T = 50$ or $T = 40$. Fasting blood samples were collected under light anesthesia at the end of the study, and the serum samples were stored at −20 °C until analyses were performed. The animals were then killed by CO_2 inhalation, and cecum, femurs and tibiae were resected, in order to perform histological, biochemical and biomechanical studies.

Fecal and cecum determinations

Fresh feces were directly obtained by rectal stimulation and immediately transferred into sterile tubes and stored at 4 °C until analyses were performed. Fecal samples were homogenized and diluted with 0.1 M phosphate buffer (containing 0.5 % cysteine). An aliquot was poured onto selective Lactobacilli MRS agar (Britania, Argentina) and incubated at 37 °C for 48 h under an anaerobic atmosphere (5–10 % CO_2). Lactobacillus (LS) colonies were counted, and the number of colony-forming units (CFU) was expressed as log CFU per gram of feces.

A second aliquot was used to assess fecal enzyme activities. β-glucosidase activity was evaluated using p-nitrophenyl β-D-glucopyranoside as substrate (Sigma, USA), and β-glucuronidase activity was assessed using *p*-nitrophenyl-β-D-glucuronide as substrate (Sigma, USA). Tryptophanase activity was assayed using tryptophan as substrate, and urease activity was determined using urea as substrate. The reactions were measured by spectrophotometry at 405, 550 or 610 nm, respectively (Metrolab 2100, Argentina) [16, 25, 26].

At the end of the study, ceca were excised, weighed and split open; pH was directly recorded by inserting a glass electrode into the cecum content (Adwa AD110, Hungary).

Ca, P and Mg absorption

At the beginning and during the last 3 days of the study, the animals were individually lodged in plastic metabolic cages. Food consumption (I) and feces (F) were collected to calculate apparent mineral absorption (Ab) (mg/day). Apparent absorption, expressed as a percentage of intake (Ab %), was calculated according to the following equation: $\text{Ab } \% = (I - F/I) \times 100$. Feces were dried under infrared light and pounded. Diets and feces were digested

with nitric acid using Parr bombs to evaluate Ca, P and magnesium (Mg) content [20].

Femur analysis

The left femurs were cleaned of soft tissue, dried in an oven at 100 °C for 72 hs and defatted using a chloroform–methanol (3:1) solution for 2 weeks. Defatted samples were dried at 100 °C and weighed (Denver instrument, USA). The longitudinal size was assessed using a vernier caliper (VIS, Poland). The defatted femurs were digested in a glass tube containing a mixture of HCl–HNO_3 (1:1) to evaluate Ca, Mg and P.

Biochemical determination

Ca concentration in serum, feces, diet and femur was determined by atomic absorption spectrophotometry [20]. Lanthanum chloride (6500 mg/L in the final solution) was added as interference suppressor. P and Mg concentration in serum, feces, diet and femur was evaluated following habitual methods using an automated analyzer (Abbott Laboratories, Abbott Park, IL, USA). Serum bone alkaline phosphatase (BAP) (IU/L) was measured using a colorimetric method after bone isoenzyme precipitation with wheat germ lectin [20]. Serum type I collagen telopeptide (CTX) (ng/mL) was assessed employing immunoassay (ELISA) (Rat Laps. Osteometer. BioTech, Herlev, Denmark). Total protein and albumin (Alb) in serum were analyzed following habitual methods using an automated analyzer (Abbott Laboratories, Abbott Park, IL, USA).

Densitometry

At the end of the study, and before killing the rats, total skeleton bone mineral content (tsBMC) and bone mineral density (tsBMD) were determined "in vivo" under light anesthesia (0.1 mg ketamine hydrochloride/100 g BW and 0.1 mg acetopromazine maleate/100 g BW), by dual-energy X-ray absorptiometry (DXA). A whole body scanner and specifically designed software for small animals (DPX Alpha, Small Animal Software, Lunar Radiation Corp. Madison WI) were used as described in a previous report [27]. In brief, all rats were scanned using an identical scan procedure. Precision was assessed by measuring one rat five times with repositioning between scans, on the same day and on different days. The coefficients of variation (CV) for BMC and BMD were 3.0 and 0.9 %, respectively.

The different subareas were analyzed on the image of the animal on the screen using a region of interest (ROI) for each segment. CVs were 1.8 % for lumbar spine, 0.8 % for femur and 3.5 % for proximal tibia [27, 28].

Histology

Immediately after euthanasia, the right tibia was resected and cleaned of soft tissue, and weight and length were recorded. The tibiae were fixed in 10 % buffered formaldehyde solution for 48 h, decalcified in ethylenediaminetetraacetic acid (EDTA, Sigma), pH 7.4, for 30 days and embedded in paraffin. One 8- to 10-µm-thick longitudinally oriented section of subchondral bone was obtained and stained with hematoxylin–eosin. The section was microphotographed (AXIOSKOP, Carl ZEISS) to perform histomorphometric measurements on the central area of the metaphyseal bone displayed on the digitalized image (Image pro plus 4.5). Bone volume fraction (BV/TV) (%) as the percentage of cancellous bone within the total measured area was evaluated [29].

Growth plate cartilage (GPC) height, defined as the full thickness of the GPC (GPC.Th), and hypertrophic zone thickness (HpZ.Th) were determined on EGP sections at 12 random sites along an axis oriented 90° to the transverse plane of the EGP and parallel to the longitudinal axis of the tibia. The 12 measures were averaged to obtain the mean GPC.Th and HpZ.Th value per section.

Biomechanical analysis

The right femurs were excised, cleaned of soft tissues, weighed and frozen (-20 °C) until analyses were performed. Bone breaking strength, elastic modulus and stiffness were measured using a three-point bending test (Instron, 4411). The load was applied perpendicularly to the long axis, at the mid-length region of the femur (displacement rate of 0.01 mm/s, sampling rate of 100 Hz). The distance between the supporting points was 10 mm.

Statistical methods

Results were expressed as mean ± standard deviation (SD). Normality of variables was evaluated by the Shapiro–Wilk test and homogeneity of variances by Levene's test. Data with a normal distribution were analyzed using 3-factors analysis of variance (ANOVA). Nonparametric data (count of LS) were analyzed using the Kruskal–Wallis test. Bonferroni multiple comparisons test was performed when significant differences were observed. Histological data were analyzed by a single-point factorial analysis. Statistical analyses were carried out using SPSS for Windows 19.0 (SPSS, Inc. Chicago, IL). A value of $p < 0.05$ was considered statistically significant.

Results

Zoometric parameters

All animals remained in good health and showed no signs of diet-related side effects, such as diarrhea, throughout the study. Regarding the internal organs, no significant differences were found among groups, except for an increase in the size of the colon (data not shown) and in cecum weight of the prebiotic groups, independently of dietary Ca content ($p < 0.01$; Table 2).

No significant differences in BW (g) or BL (cm) were observed among the studied groups at weaning. As expected, both parameters increased in the nourished groups throughout the study. Conversely, BW and BL were arrested during the period of LPD consumption in all R groups and then increased immediately after refeeding independently of the prebiotic mixture content. No differences in BW gain were observed among the 4 R groups during the re-feed period, i.e., when comparing dietary calcium levels and prebiotic content (Table 2).

At the end of the study, all LCa groups had significantly lower BW and BL than their NCa counterparts ($p < 0.01$). All the groups studied until $T = 50$ showed significantly higher BW than those studied until $T = 40$ ($p < 0.01$), although remaining significantly lower than control group values at $T = 50$, BL and BW were positively affected by the prebiotic mixture. No differences in BW were observed between R0.5 % and RP0.5 %; however, the difference between R0.3 % and RP0.3 % almost reached statistical significance ($p = 0.055$). BW remained significantly lower in RP0.3 % and RP0.5 % compared to C0.3 %-50 and C0.5 %-50, respectively ($p < 0.01$). BL values observed in U groups were similar to control values at $T = 40$. Regardless of dietary Ca content, BL increased in RP groups as compared to the control group at $T = 40$ and to the R group ($p < 0.01$). These changes were evidenced in BL gain from weaning to the end of the study. Tibia length followed the same pattern as BL. No differences in TL gain were observed among the eight studied groups throughout the study (Table 2).

Bacteriological analysis, cecum weight, pH and fecal enzymes

At $T = 0$, no differences in LS counts in fresh feces were observed among the eight studied groups. This parameter was significantly increased in RP0.5 % and RP0.3 % as compared to the other studied groups ($p < 0.01$), from the first week of prebiotic consumption to the end of the study. No differences were observed between RP0.5 % and RP0.3 % (Table 2).

Cecum pH was significantly lower in RP0.5 % and RP0.3 % as compared to the other studied groups ($p < 0.01$), with no differences between these two groups (Table 2).

At $T = 0$, no differences in the activity of the studied enzymes were observed among the eight studied groups (data not shown). At the end of the study, β-glucosidase activity was significantly higher, whereas β-glucuronidase, urease and tryptophanase activities were significantly lower in RP0.5 % and RP0.3 % than in the remaining groups ($p < 0.01$), showing no differences between these groups (Table 2).

Biochemical determinations

At $T = 50$, no differences in serum Ca, P, Mg, total protein and albumin levels were observed among the eight studied groups. BAP levels were lowest in C0.5 %-50 and C0.3 %-50 ($p < 0.01$), showing no differences between the two groups. Consumption of the prebiotic mixture had no significant effects on BAP levels. CTX levels were significantly higher in the groups fed the LCa diet than in their NCa diet counterparts ($p < 0.01$). Independently of dietary Ca content, prebiotic consumption decreased CTX in the R groups; the observed decrease only reached statistical significance when comparing RP0.3 % and R0.3 % ($p < 0.01$). CTX levels were significantly lower in RP0.3 % and RP0.5 % than in C0.3 %-50 and C0.5 %-50, respectively ($p < 0.01$). R0.5 % and R0.3 % presented similar CTX levels to those of C0.5 %-40 and C0.3 %-40, respectively, and significantly lower CTX values than C0.5 %-50 and C0.3 %-50, respectively ($p < 0.01$; Table 3).

Mineral absorption

No differences in daily food consumption were observed among the eight studied groups either during the two balance periods or throughout the study (data not shown). CaI was directly related to dietary Ca content, whereas P and Mg intakes were similar in the eight studied groups.

At $T = 0$, CaAb expressed as mg/day was higher in the NCa group as compared to their respective LCa group (C groups: 53.8 ± 4.3 vs. 32.1 ± 3.0; R groups: 58.2.5 ± 4.6 vs. 36.6 ± 2.5, respectively; $p < 0.01$); no differences in MgAb or PAb were observed among groups (data not shown).

At the end of the study, fecal Ca, Mg and P excretions were significantly lower and their corresponding Abs (mg/day) were significantly higher in RP0.5 % and RP0.3 % as compared to the remaining studied groups ($p < 0.01$). The lowest P and Mg Abs (mg/day) were observed in R0.5 % and R0.3 %, showing no differences between the two groups (Table 3).

Table 2 Effect of GOS/FOS® in food consumption, body weight (BW) and body (BL), tibia and tail (TL) length, lactobacillus colonies (LS) at weaning ($T = 0$) and at the end ($T = F$) and cecum weight and pH and enzymatic activity at $T = 50$

	C0.5 %-40	C0.5 %-50	R0.5 %	RP0.5 %	C0.3 %-40	C0.3 %-50	R0.3 %	RP0.3 %
Zoometric determinations								
Food consumption (g/d)	11.5 ± 1.0	13.6 ± 1.7	12.9 ± 1.8	13.2 ± 2.1	11.7 ± 1.4	12.1 ± 0.9	11.1 ± 1.6	11.2 ± 1.2
BW (g) ($T = 0$)	42.3 ± 2.7	41.8 ± 1.9	41.6 ± 3.2	41.1 ± 2.4	41.6 ± 3.1	42.6 ± 3.5	41.3 ± 3.7	40.7 ± 3.0
BW (g) (after 1 week)[a,b]	67.1 ± 2.21	81.7 ± 5.3	40.8 ± 2.8*,**	42.8 ± 3.5*,**	70.5 ± 5.3	87.1 ± 7.5	41.5 ± 4.2*,**	41.4 ± 4.0*,**
BW (g)($T = F$)[a,c]	133.4 ± 9.6	175.6 ± 3.1	164.7 ± 5.1*,**	167.7 ± 4.8*,**	116.6 ± 5.5¥	166.9 ± 6.6¥,*	147.3 ± 7.2¥,*,**	156.0 ± 5.9¥,*,**
BW gain (g/day)[c]	4.31 ± 0.52	4.61 ± 0.31	4.21 ± 0.27	4.41 ± 0.51	3.79 ± 0.27	4.21 ± 0.22	3.62 ± 0.31¥	3.88 ± 0.34¥
BL ($T = 0$) (cm)	11.9 ± 0.2	12.0 ± 0.2	11.6 ± 0.4	11.7 ± 0.3	11.8 ± 0,3	11.8 ± 0.2	11.8 ± 0.5	11.7 ± 0.2
BL (after 1 week) (cm)[a,b]	14.5 ± 0.6	14.5 ± 0.42	12.1 ± 0.3*,**	12.3 ± 0.4*,**	14.3 ± 0.6	14.4 ± 0.5	12.1 ± 0.6*,**	12.3 ± 0.2*,**
BL ($T = F$)(cm)[a,b,c]	17.1 ± 0.3	20.3 ± 0.8*	16.8 ± 0.6**	18.1 ± 0.3*,**,#	16.2 ± 0.4¥	18.1 ± 0.5¥,*	15.8 ± 0.6¥,**	17.2 ± 0.3¥,*,**,#
TL gain (cm)	5.7 ± 0.1	5.9 ± 0.5	6.1 ± 0.3	6.0 ± 0.3	5.0 ± 0.5	5.7 ± 0.2	5.0 ± 0.4	5.1 ± 0.3
Tibia length (cm)[a,b,c]	3.21 ± 0.03	3.41 ± 0.03*	3.19 ± 0.05**	3.29 ± 0.04*,**,#	2.93 ± 0.05¥	3.18 ± 0.03¥,*	2.87 ± 0.04¥,*,**	3.07 ± 0.06¥,*,**,#
Bacteriological analysis, cecum weight, pH and fecal enzymes								
LS (log UFC/g feces) ($T = 0$)[&]	5.5 ± 0.1	5.4 ± 0.1	5.4 ± 0.1	5.5 ± 0.2	5.5 ± 0.1	5.6 ± 0.1	5.6 ± 0.1	5.5 ± 0.1
LS (log UFC/g feces) ($T = F$)[&]	5.7 ± 0.2	5.6 ± 0.2	5.2 ± 0.2	7.3 ± 0.3*,**,#	5.6 ± 0.1	5.5 ± 0.2	5.6 ± 0.2	7.2 ± 0.2*,**,#
Cecum pH ($T = F$)[a,b]	7.1 ± 0.2	7.1 ± 0.2	7.1 ± 0.1	6.4 ± 0.2*,**,#	7.1 ± 0.1	7.2 ± 0.2	7.1 ± 0.2	6.4 ± 0.2*,**,#
Cecum weight (g)[a,b,c]	1.3 ± 0.2	1.5 ± 0.2	1.2 ± 0.3	2.4 ± 0.3*,**,#	1.3 ± 0.1	1.5 ± 0.2	1.0 ± 0.1	2.1 ± 0.2*,**,#
β-glucosidase ($T = F$)[a,b,c]	1.60 ± 0.10	1.64 ± 0.14	1.61 ± 0.10	1.73 ± 0.33*,**,#	1.64 ± 0.07	1.74 ± 0.12	1.87 ± 0.08	2.22 ± 0.31*,**,#
β-glucuronidase ($T = F$)[a,b,c]	2.04 ± 0.10	1.91 ± 0.14	1.89 ± 0.16	1.16 ± 0.13*,**,#	2.10 ± 0.08	1.95 ± 0.14	1.90 ± 0.09	1.42 ± 0.08*,**,#
Urease ($T = F$)[a,b,c]	1.73 ± 0.16	1.70 ± 0.15	1.83 ± 0.16	1.28 ± 0.14*,**,#	1.82 ± 0.08	1.79 ± 0.15	1.85 ± 0.07	1.52 ± 0.09*,**,#
Tryptophanase ($T = F$)[b,c]	0.120 ± 0.015	0.19 ± 0.00	0.131 ± 0.013	0.078 ± 0.008*,**,#	0.123 ± 0.012	0.115 ± 0.08	0.138 ± 0.005	0.093 ± 0.07*,**,#

Groups: R0.5 %: AIN93-G containing 0.5 % of Ca (NCa); RP0.5 %: NCa + 5.3 % of a GOS/FOS® mixture 9:1 (P); R0.3 %: AIN93-G containing 0.3 % of Ca (LCa); RP0.3 %: LCa + P; C0.5 %-50: NCa until day 50; C0.3 %-50: LCa until day 50; C0.5 %-40: NCa until day 40; C0.3 %-40: LCa until day 40

Results were expressed as mean ± SD ($n = 8$); * $p < 0.01$ R and P groups compared to C40 groups; ** $p < 0.01$ R and P groups compared to C50 groups

Notice that the groups compared here were RP0.5 % and R0.5 %versus C0.5 %-50 and C0.5 %-40 or RP0.3 % and R0.3 % versus C0.3 %-50 and C0.3 %-40

[#] $p < 0.01$ R compared to RP groups. Notice that the groups compared here were RP0.5 % versus R0.5 % and RP0.3 % versus R0.3 %

[¥] $p < 0.01$ LCa diet compared to NCa diet. Notice that the groups compared here were C0.3 %-40 versus C0.5 %-40; C0.3 %-50 versus C0.5 %-50; R0.3 % versus R0.5 %; and RP0.3 % versus RP0.5 %. Data were analyzed by ANOVA three factors ([a,b,c] $p < 0.05$: main effects related to age, prebiotic and Ca content, respectively). Bonferroni was used as a post hoc test

[&] *Lactobacillus* was analyzed by Kruskal–Wallis followed by a multiple comparisons test

Table 3 Effect of the GOS/FOS® mixture in calcium (Ca), magnesium (Mg) and phosphorus (P) absorptions during the last 3 days of the study (from $T = 47$ to $T = 50$) and serum biochemical parameters at the end of the experimental period

	C0.5 %-40	C0.5 %-50	R0.5 %	RP0.5 %	C0.3 %-40	C0.3 %-50	R0.3 %	RP0.3 %
Mineral absorption								
Ca intake (mg/d)[c]	85.5 ± 2.8	84.1 ± 3.6	82.9 ± 2.1	81.7 ± 2.9	47.2 ± 2.1	47.8 ± 2.8	47.3 ± 1.9	48.3 ± 1.3
Fecal Ca (mg/d)[a,b,c]	18.8 ± 2.1	21.5 ± 2.1	21.0 ± 0.4	12.5 ± 0.3*,**,#	6.3 ± 0.6	7.2 ± 0.3	6.5 ± 0.4	4.3 ± 0.3*,**,#
Ca absorption (mg/d)[a,b,c]	66.7 ± 2.2	62.2 ± 2.9	61.9 ± 1.9	71.2 ± 2.8*,**,#	40.9 ± 2.1	40.7 ± 1.9	40.7 ± 1.6	45.2 ± 1.2*,**,#
Ca absorption (%)[a,b,c]	78.3 ± 0.9	75.1 ± 0.9	74.6 ± 0.6	84.7 ± 0.7*,**,#	87.3 ± 0.8	85.1 ± 0.6	86.3 ± 0.6	91.8 ± 0.8*,**,#
P intake (mg/d)	51.3 ± 3.7	50.7 ± 2.2	49.8 ± 1.6	49.0 ± 1.6	47.2 ± 2.3	47.5 ± 0.9	47.3 ± 1.9	47.2 ± 1.8
Fecal P (mg/d)[a,b]	10.6 ± 0.7	10.8 ± 0.6	11.8 ± 0.6	6.9 ± 0.4*,**,#	9.4 ± 1.1	10.8 ± 0.7	11.6 ± 0.6	6.0 ± 0.3*,**,#
P absorption (mg/d)[b]	40.6 ± 0.8	40.0 ± 1.0	37.9 ± 0.7	42.7 ± 0.9**,#	37.8 ± 2.0	36.7 ± 1.5	35.6 ± 1.2	41.9 ± 1.1**,#
P absorption (%)[a,b,c]	79.8 ± 0.8	79.5 ± 1.0	76.2 ± 0.8*,**	86.5 ± 1.0*,**,#	80.8 ± 2.0	77.2 ± 1.5	75.2 ± 1.2*,**	87.9 ± 0.8*,**,#
Mg intake (mg/d)	8.7 ± 0.5	8.6 ± 0.3	8.5 ± 0.2	8.3 ± 0.3	8.0 ± 0.3	8.2 ± 0.2	8.0 ± 0.3	8.0 ± 0.2
Fecal Mg (mg/d)[a,b,c]	3.6 ± 0.2	3.8 ± 0.1	3.6 ± 0.2	2.1 ± 0.2*,**,#	3.1 ± 0.3	3.5 ± 0.1	3.6 ± 0.2	2.0 ± 0.1*,**,#
Mg absorption (mg/day)[a,b,c]	5.2 ± 0.3	4.9 ± 0.1	4.8 ± 0.2	6.2 ± 0.2*,**,#	4.9 ± 0.4	4.7 ± 0.2	4.5 ± 0.1	5.9 ± 0.3*,**,#
Mg absorption (%)[a,b,c]	59.2 ± 0.4	58.3 ± 1.3	56.5 ± 1.1*	74.6 ± 0.8*,**,#	61.3 ± 1.7	57.3 ± 1.2*	56.4 ± 0.8*	73.8 ± 1.7*,**,#
Biochemical determinations								
BAP (UI/L)[b,c]	85 ± 4	74 ± 4*	87 ± 6**	81 ± 6**	85 ± 3	78 ± 5*	87 ± 5**	94 ± 7**
sCTX (ng/mL)[a,b,c]	107 ± 6	123 ± 6*	98 ± 6**	96 ± 5*,**	133 ± 7¥	170 ± 6¥,*	138 ± 3¥,**	102 ± 2b¥,*,**,#
Ca (mg/dL)	10.2 ± 0.3	10.3 ± 0.3	10.2 ± 0.6	10.2 ± 1.0	10.1 ± 0.3	10.2 ± 0.3	10.2 ± 0.6	10.1 ± 0.7
P (mg/dL)	10.6 ± 0.4	10.6 ± 0.6	10.2 ± 0.9	10.1 ± 1.0	10.5 ± 0.3	10.7 ± 0.7	10.5 ± 1.0	10.3 ± 1.4
Mg (mg/dL)	2.6 ± 0.2	2.5 ± 0.3	2.4 ± 0.1	2.5 ± 0.3	2.6 ± 0.3	2.6 ± 0,2	2.4 ± 0.1	2.3 ± 0.2
Total Protein (mg/dL)	5.3 ± 0.5	5.4 ± 0.4	5.6 ± 0.7	5.4 ± 0.4	5.7 ± 0.5	5.4 ± 0.5	5.6 ± 0.5	5.9 ± 0.6
Albumin (mg/dL)	3.5 ± 0.3	3.4 ± 0.3	3.7 ± 0.3	3.6 ± 0.42	3.5 ± 0.3	3.3 ± 0.3	3.6 ± 0.2	3.6 ± 0.4

Groups: R0.5 %: AIN93-G containing 0.5 % of Ca (NCa); RP0.5 %: NCa + 5.3 % of a GOS/FOS® mixture 9:1 (P); R0.3 %: AIN93-G containing 0.3 % of Ca (LCa); RP0.3 %: LCa + P; C0.5 %-50: NCa until day 50; C0.3 %-50: LCa until day 50; C0.5 %-40: NCa until day 40; C0.3 %-40: LCa until day 40

Results were expressed as mean ± SD ($n = 8$); * $p < 0.01$ R and RP groups compared to C40 groups; ** $p < 0.01$ R and P groups compared to C50 groups

Comparisons were established among RP0.5 %, R0.5 %, C0.5 %-50, C0.5 %-40 or RP0.3 %, R0.3 %, C0.3 %-50, C0.3 %-40

# $p < 0.01$ R compared to RP groups. Comparisons were established between RP0.5 % versus R0.5 % and between RP0.3 % versus R0.3 %

¥ $p < 0.01$ LCa diet compared to NCa diet. Comparisons were established between C0.3 %-40 versus C0.5 %-40; C0.3 %-50 versus C0.5 %-50; R0.3 % versus R0.5 %; and RP0.3 % versus RP0.5 %. Data were analyzed by ANOVA three factors ([a,b,c] $p < 0.05$: main effects related to age, prebiotic and Ca content, respectively). Bonferroni was used as a post hoc test

At $T = 0$, the percentage of Ca, Mg and P Abs (Abs %) showed no significant differences among the eight studied groups (data not known). At the end of the study, mineral Abs % was significantly higher in RP0.5 % and RP0.3 % as compared to the remaining groups ($p < 0.01$).

Bone analysis

As expected, all the studied bone parameters were significantly lower in groups fed the 0.3 % Ca diets as compared to their respective 0.5 % Ca counterparts at $T = 50$ ($p < 0.01$).

Femur Ca and P contents were increased by prebiotic consumption; however, only RP0.5 % reached C0.5 %-50 values, whereas RP0.3 % remained significantly lower than C0.3 %-50 ($p < 0.01$). The significantly lowest femur Ca and P contents were observed in R0.5 % and R0.3 % as compared to the other studied groups ($p < 0.01$). Prebiotic consumption increased femoral Ca/P ratio to control levels ($p < 0.01$), while it remained lower in R groups versus controls ($p < 0.01$).

Prebiotic consumption increased femur Mg content; however, only the difference between RP0.5 % and R0.5 % reached statistical significance ($p < 0.05$). Femur Mg content in R0.5 % and R0.3 % remained as low as in C0.3 %-40 and C0.5 %-40 (Table 4).

Independently of dietary Ca content, prebiotic mixture consumption increased tsBMC/BW. RP0.3 % and RP0.5 % reached the values observed in C0.3 %-50 and C0.5 %-50. R0.3 % showed the lowest value ($p < 0.01$), which was

Table 4 Effect of GOS/FOS® on femur calcium (Ca), phosphorus (P) and magnesium (Mg) content and Ca/P ratio, total skeleton (ts) bone mineral content (BMC), and total skeleton (ts), lumbar spine and proximal tibia bone mineral densities (BMDs); bone volume (BV/TV), total and hypertrophic growth plate cartilage thickness (GPC.Th and HpZ.Th, respectively) and on biomechanical parameters related to bone structure at the end of the experimental period

	C0.5 %-40	C0.5 %-50	R0.5 %	RP0.5 %	C0.3 %-40	C0.3 %-50	R0.3 %	RP0.3 %
Femur mineral content								
Ca Content (mg/g)^{a,b,c}	117.0 ± 1.1	126.7 ± 0.9	98.7 ± 2.3*,**	120.0 ± 2.1$^{*,\#}$	102.4 ± 0.3$^{¥}$	113.5 ± 0.7$^{¥,*}$	78.0 ± 0.8$^{¥,*,**}$	102.6 ± 1.0$^{¥,**,\#}$
P content (mg/g)^{a,b,c}	70.0 ± 1.1	75.7 ± 0.7*	61.7 ± 1.4*,**	72.9 ± 1.3$^{\#}$	60.9 ± 0.9$^{¥}$	67.6 ± 0.6$^{¥,*}$	49.4 ± 1.7$^{¥,*,**}$	58.8 ± 1.3$^{¥,**,\#}$
Mg content (mg/g)^{b,c}	1.34 ± 0.03	1.41 ± 0.03*	1.21 ± 0.02*,**	1.30 ± 0.03**	1.18 ± 0.03	1.38 ± 0.07*	1.17 ± 0.05**	1.21 ± 0.04$^{**,\#}$
Femoral Ca/P ratio^{b,c}	1.67 ± 0.01	1.67 ± 0.02	1.61 ± 0.01*,**	1.67 ± 0.01$^{\#}$	1.68 ± 0.02	1.68 ± 0.01	1.59 ± 0.01*,**	1.65 ± 0.01$^{\#}$
Densitometric parameters								
tsBMC/BW (g/100 g BW)^{a,b,c}	1.77 ± 0.06	2.22 ± 0.07*	2.02 ± 0.06*,**	2.18 ± 0.09$^{*,\#}$	1.43 ± 0.06$^{¥}$	1.56 ± 0.08$^{¥,*}$	1.36 ± 0.10$^{¥,*,**}$	1.58 ± 0.09$^{¥,*,\#}$
tsBMD (mg/cm^2)^{a,c}	227 ± 6	258 ± 5*	250 ± 5*	256 ± 7*	222 ± 5$^{¥}$	247 ± 10$^{¥,*}$	231 ± 10$^{¥,*}$	240 ± 12$^{¥,*}$
Lumbar spine DMO^{a,b,c} (mg/cm^2)	175 ± 2	185 ± 6*	171 ± 6**	180 ± 5*	156 ± 2$^{¥}$	177 ± 6$^{¥,*}$	157 ± 6$^{¥,**}$	173 ± 3$^{¥,*,\#}$
Proximal tibia DMO^{a,b,c} (mg/cm^2)	169 ± 4	179 ± 4*	165 ± 4**	181 ± 7$^{*,\#}$	155 ± 6$^{¥}$	168 ± 7$^{¥}$	156 ± 7$^{¥,**}$	171 ± 6$^{¥,*,\#}$
Histomorphometry determinations$^{\&}$								
BV/TV (%)^{a,b,c}	27.5 ± 2.5	33.0 ± 1.7*	27.1 ± 1.9**	32.7 ± 1.9$^{*,\#}$	18.6 ± 0.8$^{¥}$	21.3 ± 1.0$^{¥,*}$	16.4 ± 1.3$^{¥,*,**}$	22.9 ± 1.0$^{¥,*,\#}$
GPC.Th (μm)^{a,b,c}	525 ± 6	512 ± 7*	490 ± 6*,**	517 ± 8$^{*,\#}$	463 ± 7$^{¥}$	424 ± 6$^{¥,*}$	376 ± 8$^{¥,*,**}$	440 ± 6$^{¥,*,\#}$
HpZ.Th (μm)^{a,b,c}	267 ± 4	297 ± 4*	234 ± 7*,**	281 ± 9$^{*,\#}$	195 ± 7$^{¥}$	216 ± 3$^{¥,*}$	156 ± 3$^{¥,*,**}$	205 ± 5$^{¥,*,\#}$
Biomechanical parameters in femur								
Bone breaking strength (N)^{a,b,c}	46.4 ± 3.6	54.6 ± 2.8*	32.8 ± 2.1*,**	39.3 ± 1.1$^{*,**,\#}$	35.6 ± 2.9$^{¥}$	41.7 ± 3.0$^{¥,*}$	20.3 ± 1.8$^{¥,*,**}$	26.1 ± 2.8$^{¥,*,**,\#}$
Stiffness (N/mm)^{a,b,c}	103 ± 10	119 ± 14*	74 ± 6*,**	98 ± 5$^{\#}$	89 ± 4$^{¥}$	94 ± 6$^{¥,*}$	50 ± 7$^{¥,*,**}$	69 ± 6$^{¥,*,**,\#}$
Elastic modulus (Mpa)^{a,b,c}	710 ± 17	760 ± 34*	516 ± 45*,**	722 ± 36$^{\#}$	416 ± 24$^{¥}$	447 ± 30$^{¥,*}$	363 ± 28$^{¥,*,**}$	456 ± 20$^{¥,*,\#}$

Groups: R0.5 %: AIN93-G containing 0.5 % of Ca (NCa); RP0.5 %: NCa + 5.3 % of a GOS/FOS® mixture 9:1 (P); R0.3 %: AIN93-G containing 0.3 % of Ca (LCa); RP0.3 %: LCa + P; C0.5 %-50: NCa until day 50; C0.3 %-50: LCa until day 50; C0.5 %-40: NCa until day 40; C0.3 %-40: LCa until day 40

Results were expressed as mean ± SD ($n = 8$); * $p < 0.01$ R and P groups compared to C40 groups; ** $p < 0.01$ R and P groups compared to C50 groups

Notice that the groups compared here were RP0.5 % and R0.5 %versus C0.5 %-50 and C0.5 %-40 or RP0.3 % and R0.3 % versus C0.3 %-50 and C0.3 %-40

$^{\#}$ $p < 0.01$ R compared to RP groups. Notice that the groups compared here were RP0.5 % versus R0..5 % and RP0.3 % versus R0.3 %

$^{¥}$ $p < 0.01$ LCa diet compared to NCa diet. Notice that the groups compared here were C0.3 %-40 versus C0.5 %-40; C0.3 %-50 versus C0.5 %-50; R0.3 % versus R0.5 %; and RP0.3 % versus RP0.5 %. Data were analyzed by ANOVA three factors (^{a,b,c} $p < 0.05$: main effects related to age, prebiotic and Ca content, respectively). Bonferroni was used as a post hoc test

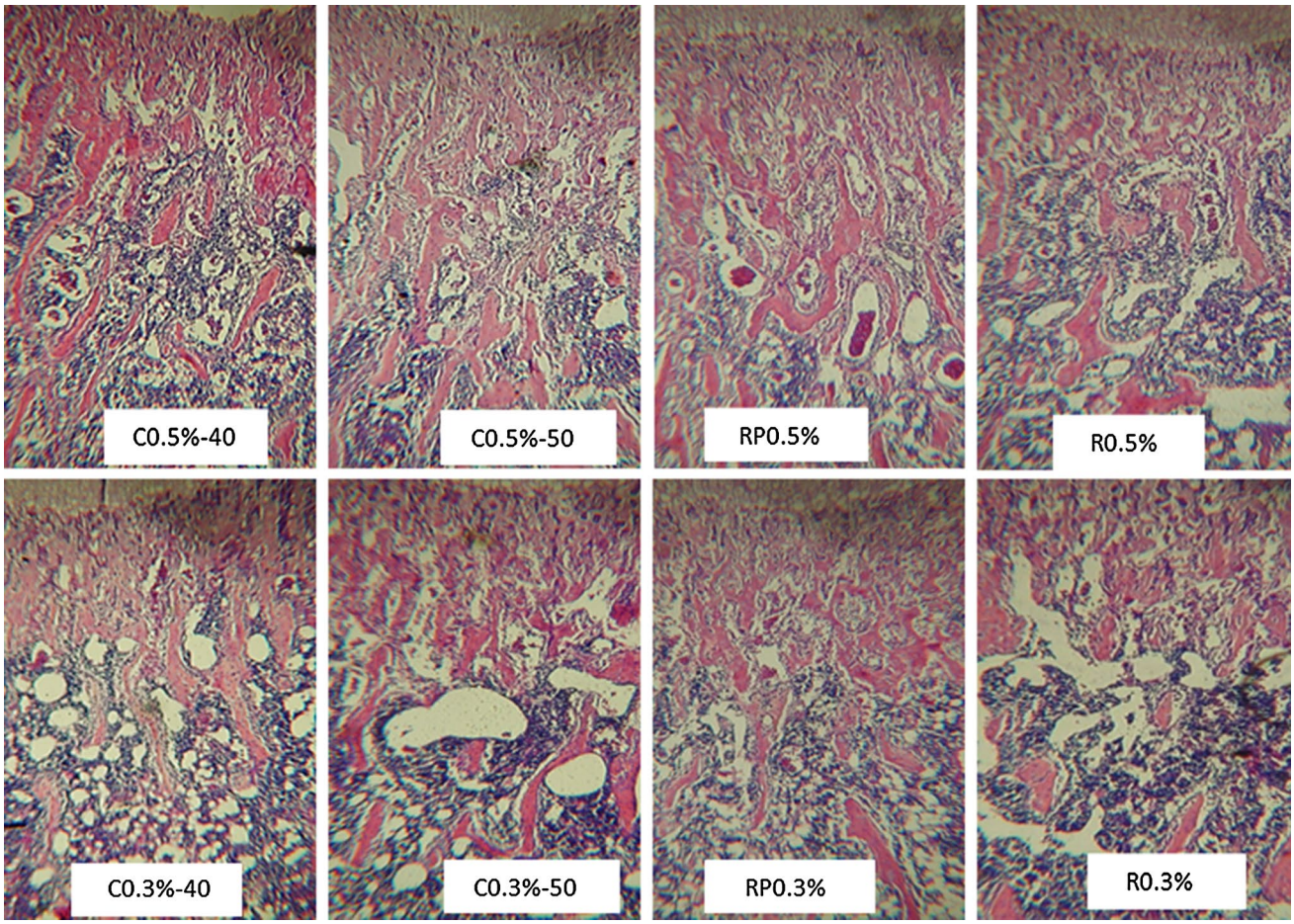

Fig. 2 Bone volume of the proximal third of the right tibia at end of the study. Groups: R0.5 %: AIN93-G containing 0.5 % of Ca (NCa); RP0.5 %: NCa + 5.3 % of a GOS/FOS® mixture 9:1 (P); R0.3 %: AIN93-G containing 0.3 % of Ca (LCa); RP0.3 %: LCa + P; C0.5 %-50: NCa until day 50; C0.3 %-50: LCa until day 50; C0.5 %-40: NCa until day 40; C0.3 %- 40: LCa until day 40. Section of subchondral bone at the level of the middle third, including primary and secondary spongiosa; Hematoxylin–eosin staining technique shows trabeculae stained in red (X50)

even lower than C0.3 %-40 ($p < 0.01$); R0.5 % had significantly lower tsBMC/BW than C0.5 %-50 ($p < 0.01$). Total skeleton BMD was not affected by protein undernutrition or prebiotic consumption; however, it was affected by dietary Ca consumption and time of experience. In this regard, the rats studied at $T = 40$ had significantly lower total skeleton BMD than those studied at $T = 50$ ($p < 0.01$), and rats fed the LCa reached significantly lower values than their NCa counterparts ($p < 0.01$). Lumbar spine and proximal tibia BMDs were increased by prebiotic consumption. RP0.3 % reached values similar to C0.3 %-50 in both parameters. Whereas only tibia BMD reached C0.5 %-50 values in RP0.5 %, the increase observed in lumbar spine BMD almost reached significance ($p = 0.058$). R0.5 % and R0.3 % showed values similar to C0.3 %-40 and C0.5 %-40 in both parameters, respectively (Table 4).

BV/TV, GPC.Th and HpZ.Th were significantly lower in LCa groups than in their NCa counterpart. Regardless of dietary Ca content, prebiotic mixture consumption increased tibia BV/TV, GPC.Th and HpZ.Th; the observed values were not significantly different as compared to C0.3 %-50 and C0.5 %-50, respectively (Table 4). BV/TV was significantly lower in R0.5 % versus C0.5 %-50 and in R0.3 % versus C0.3 %-40 and C0.3 %-50 ($p < 0.01$). GPC.Th and HpZ were significantly lower in R0.5 % and R0.3 % even compared to C0.5 %-40 and C0.3 %-40, respectively ($p < 0.01$). Figure 2 shows the higher trabecular number in the groups fed the prebiotic mixture as compared to R0.3 % and R0.5 %, and the similar values observed in RP0.5 % and C0.5 %-50.

Independently of dietary Ca content, the prebiotic mixture did not significantly increase bone strength, which was similar in RP and R groups. In addition, bone strength was found to remain significantly lower in RP than in the nourished groups, both at $T = 50$ and $T = 40$ ($p < 0.01$). Conversely, irrespective of dietary Ca content, the prebiotic mixture increased

stiffness and elastic modulus in the RP groups as compared to their respective U counterparts ($p < 0.01$). RP0.5 % showed values similar to C0.5 %-50 in both the latter parameters. The only parameter similar to C0.3 %-50 observed in RP0.3 %, however, was elastic modulus, with stiffness remaining significantly lower than C0.3 %-40 and C0.3 %-50 ($p < 0.01$). Neither parameter increased in the R groups (Table 4).

Discussion

To our knowledge, this is the first experimental study evidencing the beneficial effects of diets containing the prebiotic mixture GOS/FOS (9:1) on mineral absorption, gut microbiota, bone resorption and bone retention, during the catch-up growth period.

The "ad libitum" isocaloric low protein-fed rat model rapidly induces protein depletion. It is known that isocaloric protein depletion during early growth preserves vital functions while arresting linear growth. In addition, such a depletion also arrests body weight gain, since muscle and fat mass are metabolized to preserve body functions [1, 5]. The present report confirmed the stop in body weight and body length gain during the week of feeding the 4 % protein diet. Catch-up response after re-feeding with a normal caloric protein diet is immediate and profound and could be estimated by determining changes in body weight as an end point [30]. In the present report, BW gain was similar among the 4 recovery groups after the re-feeding period with the normal protein diet. This result was not unexpected, since food consumption was similar in all groups and the studied diets were isocaloric. Independently of the prebiotic and Ca consumption, the delay in BW gain observed in the undernourished groups was not recovered during the catch-up growth period studied here. In this regard, it is important to take into account that the transformation of consumed energy into BW (food efficiency) is similar in rats fed isocaloric diets, which supply the same percentage of protein, lipids and carbohydrate [31].

Prebiotics act primarily by modulating the composition of the gut microflora and the fermentation process, which releases metabolic products that improve the absorption of micronutrients that participate in bone growth, such as Ca, P and Mg. This effect is important during recovery from early protein malnutrition, in order to optimize the peak bone mass achieved at the end of the growth period [32].

In a previous study, we found that the prebiotic mixture studied here increased bone mineralization, density and structure during normal growth due to an increase in Ca, P and Mg absorption [20]. The same parameters were evaluated in the present work to study the effect of those same experimental diets on rats recovering from early protein malnutrition.

The GOS/FOS® 9:1 mixture has also been shown to stimulate *Bifidobacteria* and *Lactobacilli* growth, resulting in the same pattern of short-chain fatty acid (SCFA) production and reduction in fecal pH as that observed in breast-fed infants [33, 34]. *Bifidobacteria* were not determined in the present report; however, an increase in *Lactobacilli* growth was observed, independently of the dietary Ca content. In addition, β-glucosidase activity increased, while the activity of β-glucuronidase, tryptophanase and urease enzymes decreased in fresh feces. Such changes in enzyme activities suggest a stimulatory effect of the prebiotic mixture on lactic acid bacteria and a concomitant decrease in the risk of toxicological events [12, 35].

Even though the production of SCFA was not evaluated in the present report, the decrease in cecum pH indirectly indicates an increment in SCFA due to prebiotic fermentation [12]. The decrease in colonic pH favors the formation of soluble complexes that benefit mineral absorption. A twofold increase in cecum weight was also observed, suggesting an increment in the surface area of the cecum. This effect greatly increases contact between the digested foods and the mucosa, increasing mineral absorption [36]. These two mechanisms would account for the ability of the assayed prebiotic mixture to increase Ca, P and Mg absorption percentage, independently of the two levels of dietary Ca. Such effect on mineral absorption might result in an extra supply of minerals associated with bone growth and quality.

Prebiotic consumption exerted a beneficial effect on body and tibia length. In this regard, recovery groups fed the prebiotic-free diet evidenced a 10-day delay in body and tibia length for age, remaining as low as the nourished groups at $T = 40$. Instead, the recovery groups fed the prebiotic mixture showed lower stunting in body and tibia length, which remained lower than the well-nourished counterparts at $T = 50$, but they were higher than the nourished group at $T = 40$. The partial effect of prebiotic consumption on these two parameters might be a consequence of the age of the rats subjected to growth retardation as well as the short period of time for nutritional recovery. Regarding the age of the animals, the stage of linear growth of weaning rats is similar to linear growth in childhood. As the re-feeding period lasted 22 days, by the end of the study, the rats were entering puberty and had therefore not yet attained peak bone mass. Although catch-up growth began immediately after re-feeding a normal diet, recovery in terms of achieving the prestress predicted total length of long bones is a prolonged process, even if the period of insult was of very short duration [30].

Tibia epiphyseal cartilage growth is more sensitive to nutrition than bone length [37, 38]. Postnatal linear growth occurs by endochondral ossification in the EGP at the ends of long bones. Consequently, analysis of EGP during

catch-up growth is particularly interesting because EGP controls the rate and extent of linear growth by changes during chondrocyte proliferation and/or hypertrophy [5]. In the present study, and independently of the dietary Ca content, the growth rate in the proximal tibia GP region of the recovery group fed the free-prebiotic diet remained as low as the nourished group at $T = 40$; instead, prebiotic consumption enhanced total GP and hypertrophic zone thickness, which reached levels observed in the nourished group at $T = 50$. Therefore, the prebiotic mixture could be considered a positive mediator in catch-up response.

Any influence on growth plates affects bone development with marked changes in bone quality, shape and size [38, 39]. The increase in endochondral bone growth typically leads to more Ca accretion in the primary spongiosa and thus higher bone volume and proximal tibia BMD levels. In the present report, and irrespective of the dietary Ca content, consumption of the prebiotic mixture increased both parameters, as well as total body bone mass and spine BMD. These results are consistent with the observed decrease in bone resorption evaluated by CTX levels. Conversely, the studied prebiotic mixture had no effect on total skeleton BMD. This result was not unexpected because this last area is mostly composed of cortical bone (80 %), whereas the proximal tibia and spine are mainly composed of trabecular bone. The latter bone has a much higher surface-to-volume ratio than cortical bone and is consequently metabolically more active. Our studied re-feeding period lasted only one remodeling cycle of the rat (22 days), which would not be long enough to improve the density of a low metabolically active bone, as is the case of cortical bone.

The mechanical properties of bone also affect bone quality. We evaluated these properties at the center point of the femur, which consists of cortical bone. The results of the present report evidenced an increase in bone fragility, in the recovery group re-fed the prebiotic-free diets. Indeed, even in the group fed the NCa, the load required for fracture and the mechanical and material properties continued to decrease during the re-feeding period, reaching values lower than those of the nourished group at $T = 40$. This deterioration led us to conclude that returning to normal nutrition failed to compensate for the fragility of cortical bone caused by early protein malnutrition. These results, though with some differences regarding species, ages, diets, extent of the study, duration of the restriction and severity of the depletion, are in agreement with previous reports in young mice and rats [39, 40]. Prebiotic mixture consumption prevented some of these disturbances in the biomechanical properties of bone, depending on the dietary Ca content. While 0.5 %Ca content diet improved bone stiffness and material properties, suggesting normal mineralization and structure of the femur, the 0.3 %Ca content diet failed to improve stiffness. Nonetheless, prebiotics had no effect on bone breaking strength. This parameter indicates bone resistance to failure and derives from the contribution of cortical bone structure, in terms of quality and quantity. The lack of effect on breaking strength and on total skeleton BMD indicates that, under our experimental conditions, prebiotic consumption during the catch-up growth period affected trabecular bone more dramatically than cortical bone.

The positive effects of the prebiotic mixture on mineral absorption might have contributed to improving Ca, P and Mg bioavailability. The extra supply of minerals associated with bone growth and quality might improve catch-up response, preventing the possible negative effect resulting from fast bone growth. It is important to point out that the beneficial effect of the prebiotic mixture on almost all the studied bone parameters was greater in the recovery group fed the low Ca diet. This effect clearly shows that the prebiotic mixture studied here might prevent the well-known negative effect of feeding a low Ca diet on bone health [7, 32].

One limitation to the present study was that the rats were still growing at the end of the study. Had the study continued to the end of puberty (60 days of age), the effect of prebiotic consumption on tibia length and bone quality in recovery groups might have been great enough to allow attaining nourished control values. The second limitation is that changes in pro-inflammatory cytokines were not evaluated. The latter might have helped to evidence the rapid stimulatory effect of prebiotic consumption on the gut-associated immune system. It must be pointed out that we only evaluated ultrasensitive C-reactive protein, which did not show changes in any of the studied groups (data not shown).

Conclusion

Under the present experimental conditions, it is possible to conclude that supplementing diets with the GOS/FOS® mixture increases bone mineralization, density and structure due to an increase in Ca, P and Mg absorption and thus may help to improve bone development during recovery from protein malnutrition in the growing rat. Although the data of the present study need to be confirmed with human intervention trials, the present results suggest that this prebiotic mixture would be a useful tool to ensure optimal peak bone mass acquisition during the catch-up growth period.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest associated with this manuscript.

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