

Effect of a combination GOS/FOS[®] prebiotic mixture and interaction with calcium intake on mineral absorption and bone parameters in growing rats

Gabriel Bryk · Magalí Zeni Coronel · Gretel Pellegrini · Patricia Mandalunis ·
María Ester Rio · María Luz Pita Martín de Portela · Susana Noemí Zeni

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Abstract

Aim Increasing calcium intake is the most effective strategy for avoiding Ca deficit. However, if intake remains inadequate, improving Ca absorption becomes an important tool to optimize Ca homeostasis and bone health.

Purpose The effect of a mixture of GOS/FOS[®] 9:1 added to a normal- or low-Ca diets on Ca absorption and bone mineralization, density and structure was investigated, in a model of growing rats. Several colonic parameters to help support the findings were also evaluated.

Results Weanling Wistar rats received one of the four experimental AIN-93G diets: C5: 0.5 % Ca; C3: 0.3 % Ca; P5: 0.5 % Ca + 5.3 % GOS/FOS[®]; P3: 0.3 % Ca + 5.3 %

GOS/FOS[®] until 50 days ($T = 50$). At $T = 50$, lactobacillus and cecum weights were higher, whereas cecum pH was lower in P5 and P3 versus C5 and C3 ($p < 0.001$). At $T = 50$, fecal Ca, Mg and P were lower and their absorptions (mg/dL) were higher in P5 and P3 versus C5 and C3, respectively ($p < 0.05$). Ca, Mg and P absorption % was higher in P5 and P3 versus C5 and C3 ($p < 0.001$). Femur Ca and P content, bone mineral content, trabecular bone mineral density, tibia length, bone volume, osteoblast surface, stiffness and elastic modulus were higher in P5 and P3 versus C5 and C3 ($p < 0.05$). Despite the lower Ca content, P3 group reached similar values than C5 in all these latter parameters.

Conclusions Supplementing diets with the GOS/FOS[®] mixture increased bone mineralization, density and structure due to an increase in Ca, P and Mg absorptions. Thus, this prebiotic mixture may help to improve bone development in a period of high calcium requirements.

G. Bryk · G. Pellegrini · S. N. Zeni
General and Oral Biochemistry Department, School of Dentistry,
Buenos Aires University (UBA), Buenos Aires, Argentina

G. Bryk · M. Z. Coronel · G. Pellegrini · S. N. Zeni (✉)
Metabolic Bone Diseases Laboratory, Clinical Hospital,
Immunology, Genetic and Metabolism Institute (INIGEM),
National Council for Scientific and Technologic Research
(CONICET), Buenos Aires University (UBA), Av. Córdoba
2351-8vo. Piso, 1120 Buenos Aires, Argentina
e-mail: snzeni@hotmail.com

P. Mandalunis
Histology and Embryology Department, School of Dentistry,
Buenos Aires University (UBA), Buenos Aires, Argentina

M. E. Rio
National Council for Scientific and Technologic Research
(CONICET), Buenos Aires, Argentina

M. L. P. M. de Portela
Food Science and Nutritional Department, School of Pharmacy
and Biochemistry, Buenos Aires University (UBA),
Buenos Aires, Argentina

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Galacto-oligosaccharides · Fructo-oligosaccharides ·
Growing rats · Growth plate cartilage

Abbreviations

Ab %	Absorption percentage
Ab	Absorption
AIN	American Institute of Nutrition
BAP	Bone alkaline phosphatase
BL	Body length
BV/TV	Bone volume
BW	Body weight
Ca	Calcium
CaI	Calcium intake
CFU	Numbers of colony-forming units
DXA	Dual energy X-ray absorptiometry

ENNyS	National nutrition and health survey
F	Feces
FOS	Fructo-oligosaccharides
GOS	Galacto-oligosaccharides
GPC.Th	Whole thickness of the growth plate cartilage
HpZ.Th	Hypertrophic zone thickness
LS	Colonies of <i>lactobacillus</i>
Mg	Magnesium
NDO	Non-digestible oligosaccharides
P	Phosphorous
PTH	Parathyroid hormone
SCFAs	Short-chain fatty acids
TL	Tail length
tsBMC	Total skeleton bone mineral content
tsBMD	Total skeleton bone mineral density

Introduction

Calcium (Ca) homeostasis is vital to maximize net Ca retention, helping to achieve peak bone mass during growth and to reduce bone loss in adult life [1]. Despite the importance of an optimal Ca intake (CaI), a great percentage of the population, including children, do not meet recommendations [2, 3]. The National Nutrition and Health Survey (ENNyS) conducted in Argentina during 2004–2006 demonstrated CaI insufficiency in 16–21 % of the pediatric population [4, 5]. It is important to take into account that dairy products are the main source of Ca. The replacement of milk with beverages may influence Ca nutrition and peak bone mass acquisition in children and adolescents [6]. In this regard, the Committee of Nutrition has reported that CaI decreases from 80 to 50 % between the ages of 1–3 to 5 years [7]. Increasing CaI would be the most effective strategy for avoiding Ca deficit. However, if intake remains inadequate, the improvement of Ca absorption becomes an important tool to optimize Ca homeostasis and bone health. Any increment in Ca absorption during childhood and adolescence may eventually increase bone retention, which contributes to attain optimal peak bone mass.

Most Ca absorption occurs in the small intestine; however, if the insoluble, unabsorbed Ca coming from the small intestine is maintained in an ionic form, about 5–10 % could occur in the colon [8]. Non-digestible fructo-oligosaccharides are not hydrolyzed enzymatically in the small intestine but are fermented by resident microbiota in the colon, producing short-chain fatty acids (SCFAs) that lower intraluminal pH and thereby may increase mineral

solubility [9]. In addition, the low pH modifies the colonic microbiota and increases proliferation and activity of beneficial flora, such as *lactobacillus* species [10, 11]. Butyrate, one of the bacterial metabolites, stimulates the intestinal epithelium and increases its absorptive capacity. These effects are responsible for the increase in Ca absorption [8, 12]; however, it was proposed that they are closely linked to the nature of the prebiotic carbohydrates and to the mineral concentrations in the lumen [13, 14].

Inuline, oligofructose and a mixture of short- and long-chain products (Synergy) are the most intensively investigated prebiotics with regard to functionality including mineral metabolism and bone health [15]. Studies reported in the literature showed that these prebiotics, mainly consumed as part of a semi-purified diet, stimulated Ca absorption and enhanced Ca content of bone under certain conditions, though results on phosphorus metabolism were less conclusive [15]. With the aim to mimic the prebiotic effect of human milk, a prebiotic mixture of 90 % of short-chain galacto-oligosaccharides (GOS) and 10 % long-chain fructo-oligosaccharides (FOS) (GOS/FOS[®]) was developed [16]. Supplementation with this mixture leads to a colonic flora similar to that of breast-fed infants [17]. Several studies have evaluated the effect the GOS/FOS[®] on the composition and metabolic activity of gut microflora in infants [18–20]; however, whether they exert a similar effect in older age groups needs further investigation. Most of the studies evaluated the beneficial effect of the mixture in modulating the immune system [21–23]. Another important factor to be determined during growth is the impact of this mixture on mineral absorption and bone health which, to our knowledge, has not been studied to date.

Experimental models are a powerful tool for nutritional studies, since they provide homogenized samples by controlling dietary and environmental factors, which are difficult to control in human studies. The weanling rat is a useful experimental model to evaluate peak bone mass development because it displays progressive gain in bone parameters during growth [24].

The aim of the present study was to investigate the effect of a GOS/FOS[®] 9:1 prebiotic mixture and its interaction with CaI, on Ca, P and Mg absorption in weanling rats. The effects on bone mineralization and bone density and structure were also investigated. The hypothesis was that as Ca intake influences peak bone mass acquisition, the beneficial impact of the prebiotic mixture may be particularly greater when the level of Ca in the diet is low. Several indicative colonic parameters (e.g., luminal pH, weight of the cecum and bacterial count) were also evaluated, to help support the findings of the present report.

Materials and methods

Diets

All experimental diets were isocaloric by formulation and were prepared according to the American Institute of Nutrition Rodent Diets Recommendations for growing animals settled in 1993 (AIN-93G) [25]. CaCO₃ (Analytical grade, Anedra, Argentina) was added to obtain the two dietary levels of Ca: 0.5 or 0.3 %. Diets containing prebiotics were prepared by adding 5.3 g % of a mixture of GOS/FOS[®] (9:1) (NV Nutricia) (batch No 110710 and HPPGJ1AGJ, respectively) (Table 1).

Animals

Male weanling Wistar rats (35–40 g) were obtained from the General and Oral Biochemistry Department, School of Dentistry, Buenos Aires University, Argentina. They were

Table 1 Composition of the experimental diets (g/100 g)

Diet	C5	P5	C3	P3
Energy (kcal)	395	395	395	395
Protein (g) ^a	17	17	17	17
Lipids (g) ^b	7	7	7	7
Ca-free salts mixture (g)	3.5	3.5	3.5	3.5
Vitamins (g) ^c	1	1	1	1
Choline (g) ^e	0.25	0.25	0.25	0.25
Cellulose (g)	5	5	5	5
Dextrin ^f	To complete 100 g			
Calcium (Ca) (g) ^d	0.5	0.5	0.3	0.3
Phosphorus (P) ^e	0.3	0.3	0.3	0.3
Magnesium (Mg) ^e	0.0513	0.0513	0.0513	0.0513
GOS/FOS [®] mixture (g) ^h		5.3		5.3

C5 and C3: control diets and P5 and P3: experimental diets containing 0.5 and 0.3 % of Ca, respectively

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

^b Commercial soy oil. Molinos Rio de la Plata. Argentina

^c Vitamins Prepared to 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), 5 % cellulose and 0.71 % choline citrate (food-grade, Anedra, Argentina)

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

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

^f Corn dextrin from corn refinery, provided by Food SA Argentina was added as carbohydrate source to achieve 100 g of diet

^g Choline citrate (food-grade, Anedra, Argentina)

^h GOS and FOS batch No 110710 and HPPGJ1AGJ, respectively

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, and the protocol was approved by the Bioethics Committee of Buenos Aires University.

Experimental design

A total of 32 rats were randomly assigned to the 4 studied groups (8 rats per group) to receive one of the four experimental diets until 50 days of age ($T = 50$). As one remodeling cycle in rats lasts about 21 days and, in order to evaluate changes in bone parameters, the diets were supplied during a 28-day period.

C5 AIN 93-G diet containing 0.5 % of Ca (normal-Ca diet),

C3 AIN 93-G diet containing 0.3 % of Ca (low-Ca diet)

P5 AIN 93-G diet containing 0.5 % of Ca + 5.3 % of the mixture of GOS/FOS[®]

P3 AIN 93-G diet containing 0.3 % of Ca + 5.3 % of the mixture of GOS/FOS[®]

The diets were offer as powder. Food consumption and body weight (BW) were determined twice a week. Body and tail length (BH, TH, respectively) were measured once a week under moderate CO₂ sedation.

Fresh fecal samples were obtained weekly. Densitometry analysis was performed at $T = 50$. 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 CO₂ 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 % CO₂). The colonies of lactobacillus (LS) were counted and the numbers of colony-forming units (CFU) were expressed as log CFU per gram of feces.

At the end of the study, the cecum from each rat was 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/d). Apparent absorption, expressed as a percentage of intake (Ab %), was calculated according to the following equation: $Ab \% = (I - F/I) \times 100$. Feces were dried under infrared light and pounded. Diets and feces were digested with nitric acid using Parr bombs to evaluate Ca, P and Mg content [26].

Femur analysis

The left femurs were cleaned of soft tissue, dried in an oven at 100 °C for 72 hrs 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–HNO₃ (1:1) to evaluate Ca, Mg and P.

Biochemical determination

Ca concentration in serum, feces, diet and femur was determined by atomic absorption spectrophotometry [27]. Lanthanum chloride (6,500 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 [28]. Serum CTX (ng/mL) was assessed employing immunoassay (ELISA) (Rat Laps. Osteometer. BioTech, Herlev, Denmark).

Densitometry

At $T = 50$ 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) was used as described in a previous report [29]. 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 [30].

Histology

Immediately after euthanasia, the right tibia was resected, cleaned of soft tissue and weight and length were recorded. The tibiae were fixed in 10 % buffered formaldehyde solution for 48 h, decalcified in ethylene diamine tetraacetic acid (EDTA, Sigma), pH 7.4, for 30 days, and embedded in paraffin. One 8- to 10- μ m thick longitudinally oriented section of subchondral bone was obtained at the level of the middle third, including primary and secondary spongiosa, and was 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). The following static histomorphometric parameters were measured according to Parfitt et al. [31]: Bone volume fraction (BV/TV) (%): the percentage of cancellous bone within the total measured area; osteoblast surface (Ob.S/BS) (%): the fraction of trabecular bone surface covered with osteoblasts; eroded surface (ES/BS): the fraction of trabecular surface covered with lacunae (including “active” lacunae with osteoclasts and lacunae in reversal phase); osteoclast number (Oc.N/B.Ar, mm²): the number of osteoclasts in the total studied area; trabecular number (Tb.N, 1/mm); thickness (Tb.Th, μ m) and spacing (Tb.Sp, μ m).

Growth plate cartilage height was determined by measuring the whole thickness of the growth plate cartilage (GPC.Th) and the hypertrophic zone thickness (HpZ.Th) of the entire growth plate section along an axis oriented 90° to the transverse plane of the growth plate and parallel to the longitudinal axis of the tibia. The thickness of the growth plate and of the hypertrophic zone was calculated as the mean of twelve different randomly chosen measurements performed on each 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 \pm 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 one-way 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. Histologic 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 $p < 0.05$ was considered significant.

Results

Zoometric determination and food consumption

All the 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 the weight of the cecum in prebiotic fed groups, independently of the dietary Ca content.

At $T = 0$, no significant differences in BW (g) were observed among the four studied groups (C5: 43.4 ± 4.8 ; P5: 42.6 ± 6.2 ; C3: 45.3 ± 3.5 ; P3: 42.2 ± 4.8) (mean \pm SD). The changes in BW (g), BH (cm) and TH (cm) between $T = 50$ and $T = 0$ were no significant different among the four studies groups (Table 2).

Tibia length was significantly higher in P5 and P3 than in C5 and C3 ($p < 0.05$); P3 reach a value similar to C5, despite the lower diet Ca content (Table 2).

Fresh feces and cecum analysis

At $T = 0$, no differences in the CFU counts of LS in fresh feces were observed among the four studies groups. However, this parameter was significantly higher in P5 and P3 than in C5 and C3, respectively ($p < 0.001$) at $T = 50$.

Cecum weight was significantly higher and cecum pH was significantly lower in P5 and P3 than in C5 and C3, respectively ($p < 0.001$) (Table 2).

Biochemical determinations

At $T = 50$, no significant differences were observed in serum Ca, P and Mg or BAP levels among the four studied groups. CTX levels were lower in prebiotic-containing diets than in their control diets; however, only P3 versus C3 reached statistical significance ($p < 0.01$) (Table 2).

Table 2 Effect of the GOS/FOS[®] mixture in food consumption, body weight and length gain throughout the experience; lactobacillus colonies at $T = 0$ and at $T = 50$ and cecum weight and pH, serum biochemical parameters and tibia length at $T = 50$

	C5	P5	C3	P3
Food consumption (g/d)	13.6 ± 1.7	13.5 ± 0.5	12.1 ± 0.9	12.2 ± 1.1
Body weight gain (g/d)	4.6 ± 0.8	4.6 ± 0.5	4.2 ± 0.4	4.2 ± 0.4
Body length gain (cm)	8.3 ± 0.9	8.3 ± 0.5	7.7 ± 0.8	7.8 ± 0.8
<i>Bacteriological analysis, cecum weight and pH</i>				
Lactobacillus at $T = 0$ (log CFU/g feces) ^a	5.5 ± 0.1	5.4 ± 0.3	5.7 ± 0.7	5.6 ± 0.2
Lactobacillus at $T = 50$ (log CFU/g feces) ^a	5.6 ± 0.2	$7.1 \pm 0.2^{***}$	5.5 ± 0.3	$7.2 \pm 0.3^{***}$
Cecum pH	7.1 ± 0.1	$6.4 \pm 0.2^{***}$	7.1 ± 0.2	$6.3 \pm 0.2^{***}$
Cecum weight (g)	1.51 ± 0.14	2.19 ± 0.13	1.54 ± 0.18	2.16 ± 0.14
<i>Biochemical determinations at $T = 50$</i>				
Calcium (Ca) (mg/dL)	10.3 ± 0.3	10.3 ± 0.4	10.2 ± 0.3	10.3 ± 0.3
Phosphorus (P) (mg/dL)	10.2 ± 0.3	10.1 ± 0.2	10.9 ± 0.9	10.6 ± 0.8
Magnesium (Mg) (mg/dL)	$2.2 \pm 0.1^{**}$	$2.2 \pm 0.2^{**}$	2.8 ± 0.2	$2.5 \pm 0.3^{**}$
Bone alkaline phosphatase (BAP) (IU/L)	70 ± 13	67 ± 7	84 ± 19	86 ± 11
C-terminal telopeptide collagen type I (CTX) (ng/ml)	123 ± 19	$96 \pm 21^{***\#}$	$188 \pm 38^*$	$147 \pm 20^{**}$

Results were expressed as mean \pm SD ($n = 8$). Data were analyzed by ANOVA two factors and Bonferroni as a post hoc test

* $p < 0.05$ compared to C5; ** $p < 0.05$ compared to C3; # $p < 0.05$ compared to P3

^a Lactobacillus were analyzed by Kruskal–Wallis followed by a multiple comparisons test

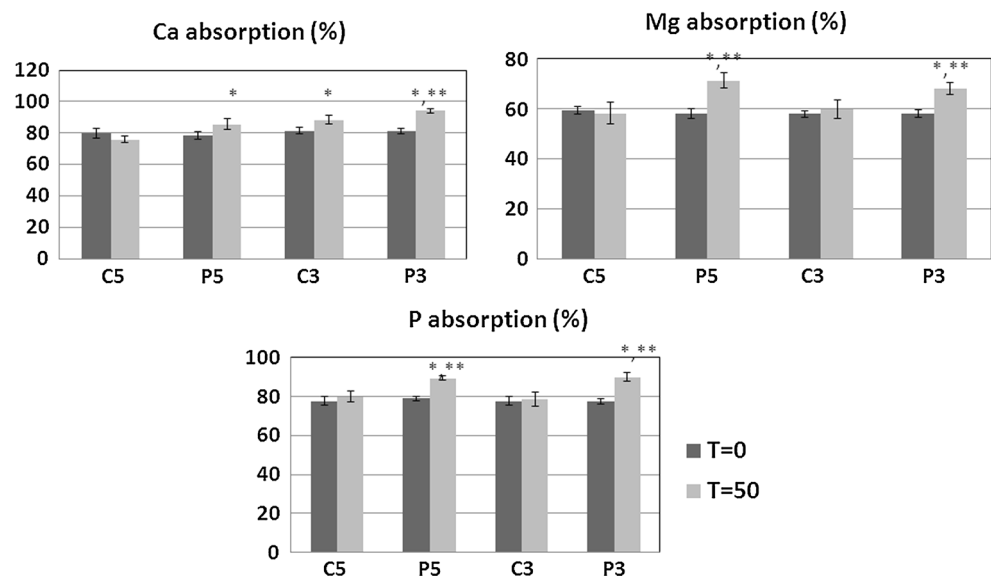
Table 3 Effect of the GOS/FOS[®] mixture in calcium (Ca), magnesium (Mg) and phosphorus (P) absorptions during the last 3 days of the study (from $T = 47$ to $T = 50$)

	C5	P5	C3	P3
Food consumption (g/d)	17.9 ± 1.7	18.2 ± 1.1	17.2 ± 0.7	17.9 ± 0.9
Ca intake (mg/d)	89.5 ± 8.3	90.8 ± 5.5 ^{*,#}	51.6 ± 2.0 [*]	53.8 ± 2.8 [*]
Fecal Ca (mg/d)	21.5 ± 2.9 ^{**}	12.9 ± 2.2 ^{*,**,#}	5.9 ± 1.1 [*]	3.2 ± 0.8 ^{*,**}
Ca absorption (mg/d)	68.0 ± 6.2 ^{**}	77.9 ± 7.4 ^{*,**,#}	45.7 ± 3.0 [*]	50.7 ± 2.2 ^{*,**}
P intake (mg/d)	53.7 ± 5.0	54.5 ± 3.3	51.5 ± 2.3	53.3 ± 3.7
Fecal P (mg/d)	10.6 ± 1.5	5.7 ± 0.6 ^{*,**}	10.8 ± 1.6	5.2 ± 1.0 ^{*,**}
P absorption (mg/d)	43.0 ± 2.6	48.8 ± 2.1 ^{*,**}	40.7 ± 3.4	48.1 ± 2.1 ^{*,**}
Mg intake (mg/d)	9.1 ± 0.8	9.3 ± 0.6	8.8 ± 0.5	9.0 ± 0.7
Fecal Mg (mg/d)	3.8 ± 0.3	2.6 ± 0.2 ^{*,**}	3.5 ± 0.3	2.9 ± 0.3 ^{*,**}
Mg absorption (mg/d)	5.3 ± 0.3	6.6 ± 0.4 ^{*,**}	5.2 ± 0.5	6.2 ± 0.4 ^{*,**}

Results were expressed as mean ± SD ($n = 8$). Data were analyzed by ANOVA two factors and Bonferroni as a post hoc test

* $p < 0.05$ compared to C5; ** $p < 0.05$ compared to C3; # $p < 0.05$ compared to P3

Fig. 1 Effect of the GOS/FOS[®] mixture in mineral absorptions expressed as percentage of the intake at $T = 0$ and at $T = 50$. Results were expressed as mean ± SD ($n = 8$). * $p < 0.01$ compared to C5; ** $p < 0.01$ compared to C3. Data were analyzed by ANOVA two factors and Bonferroni as a post hoc test



Mineral absorption

No differences in food consumption were observed during the balance periods either at $T = 0$ or at $T = 50$ (data not shown). CaI was directly related to dietary Ca content, whereas P and Mg intake was similar in the four studied groups.

At $T = 0$, CaAb expressed as mg/d was higher in 0.5 % groups compared to their respective 0.3 % group (C5 vs. C3: 53.8 ± 4.3 vs. 32.1 ± 3.0; P5 vs. P3: 50.7 ± 3.7 vs. 32.7 ± 3.8) (mean ± SD) ($p < 0.05$); no differences in MgAb or PAb were observed among groups (data not shown). At $T = 50$, fecal Ca, Mg and P excretions were significantly lower and their corresponding Abs, expressed as mg/dL, were significantly higher in P5 and P3 as compared to C5 and C3, respectively ($p < 0.05$) (Table 3).

At $T = 0$, Ca, Mg or P Abs expressed as percentage did not show significant differences among the four studied groups; at $T = 50$, they were significantly higher in P5 and P3 as compared to C5 and C3, respectively ($p < 0.001$) (Fig. 1).

Bone analyses

As expected, femur Ca, P and Mg content was significantly lower in groups fed the 0.3 % Ca diets as compared to their respective 0.5 % Ca groups ($p < 0.001$). In addition, femur Ca and P content was significantly higher in P5 and P3 as compared to C5 and C3, respectively ($p < 0.001$); however, no significant differences were observed in Mg content. Femur Ca, P and Mg content was similar in P3 and C5, despite the lower diet Ca content (Table 4).

Table 4 Effect of GOS/FOS[®] on femur mineral retention, total skeleton (ts) bone mineral content (BMC), ts, lumbar spine and proximal bone mineral densities (BMDs), on total and hypertrophic growth plate cartilage thickness (GPC.Th and HpZ.Th, respectively) and on biomechanical parameters related to bone structure at $T = 50$

	C5	P5	C3	P3
<i>Femur mineral content</i>				
Ca content (mg/g)	126.7 ± 1.8	175.2 ± 1.4***.#	113.5 ± 1.9*	125.1 ± 2.3**
Mg content (mg/g)	1.45 ± 0.06	1.43 ± 0.05	1.39 ± 0.06	1.39 ± 0.06
P content (mg/g)	75.7 ± 1.4**	99.5 ± 1.9***.#	67.6 ± 1.9*	76.2 ± 2.0**
<i>Densitometric parameters</i>				
tsBMC/BW (g/100 g BW)	2.24 ± 0.52	3.04 ± 0.12***.#	1.17 ± 0.19***	1.60 ± 0.18***
tsBMD (mg/cm ²)	261 ± 17	257 ± 7	245 ± 5	249 ± 6
Lumbar spine BMD (mg/cm ²)	189 ± 10	200 ± 7**.#	161 ± 15*	164 ± 10
Proximal tibia BMD (mg/cm ²)	184 ± 29**	201 ± 27#	142 ± 20	164 ± 25**
<i>Growth plate cartilage assessment^a</i>				
GPC.Th (µm)	512 ± 43	577 ± 68***.#	424 ± 36*	492 ± 17**
HpZ.Th (µm)	297 ± 23**	341 ± 58***.#	216 ± 17	278 ± 17**
Tibia length at $T = 50$ (cm)	3.41 ± 0.09	3.52 ± 0.10***.#	3.18 ± 0.08*	3.37 ± 0.08**
<i>Biomechanical parameters</i>				
Bone-breaking strength (N)	57.5 ± 8.7	64.1 ± 10.4**	41.7 ± 7.0*	52.4 ± 6.9**
Stiffness (N/mm)	119.0 ± 14.0	154.1 ± 14.4***.#	90.5 ± 6.3*	116.7 ± 12.8**
Elastic modulus (Mpa)	760.2 ± 193.5	1,113.2 ± 133.1***.#	454.9 ± 106.5*	682.6 ± 131.5**

Data are expressed as mean ± SD and were analyzed by ANOVA two factors and Bonferroni as a post hoc test

* $p < 0.05$ compared to C5;

** $p < 0.05$ compared to C3;

$p < 0.05$ compared to P3

^a Analyzed by a single-point factorial analysis

The lowest tsBMC/BW was observed in C3 ($p < 0.05$), while P5 and P3 presented significantly higher values than C5 and C3, respectively ($p < 0.05$). No significant differences were observed in tsBMD among the four studied groups. Lumbar spine and proximal tibia BMDs were higher in P5 and P3 as compared to C5 and C3; however, only tibia BMD reached statistical significance ($p < 0.05$) (Table 4).

Bone strength was higher in P5 and P3 compared with C5 and C3, respectively, but only the difference between P3 and C3 reached statistical significance ($p < 0.001$). P5 and P3 showed a significantly higher stiffness and elastic modulus than C5 and C3, respectively ($p < 0.001$). Again these three biomechanical parameters were similar in P3 and C5 despite the lower diet Ca content (Table 4).

Table 4 showed that GPC.Th and HpZ.Th were higher in P5 and P3 as compared to C5 and C3, respectively; however, only P3 versus C3 reached statistical significance ($p < 0.01$).

BV/TV and Ob.S/BS % were significantly higher in P5 and P3 as compared to C5 and C3, respectively ($p < 0.01$) (Table 5). The prebiotic mixture decreases the eroded surface %; however, only P3 versus C3 reached statistical significance ($p < 0.01$). No significant differences were found in Tb.N, Tb.Th and Tb.Sp among the four studied groups. Despite the lower diet Ca content, P3 showed similar BV/TV, Ob.S/BS % and eroded surface % values than C5 (Table 5). Figure 2 evidenced the higher trabecular number of the zone of the prebiotic groups as compared to C3 and C5, respectively, and the similar values of P3 and C5 groups.

Discussion

The results of the present study evidenced that the mixture of short-chain GOS and long-chain FOS (GOS/FOS[®]) enhanced Ca, P and Mg absorption and bone retention and also improved the development of the growth cartilage plate and the mechanical properties of bone in normal growing rats. Nevertheless, the main finding of the present work was the positive effects of a combination GOS/FOS[®] prebiotic mixture when added to the low-Ca diet (0.3 %). Indeed, despite the limited amount of Ca supplied in this diet, in almost all, the studied bone parameters reached the values observed in the group fed the 0.5 % Ca diet without the GOS/FOS[®] mixture.

All studied groups showed a similar BW gain rate. This result was not unexpected because the four studied diets were isocaloric, and food consumption was similar in all groups. According to a previous study, this prebiotic mixture resembles the neutral fraction of human milk oligosaccharides with regard to molecular size distribution [16]. The mixture has also been shown to stimulate Bifidobacteria and Lactobacilli growth and metabolic activity in preterm, term and weaning infants. As a result, feeding the mixture results in the same pattern of SCFA production and reduction in the fecal pH as that observed in breast-fed infants [32–34]. However, to our knowledge, there are no previous experimental or clinical studies evaluating the effect of this mixture on mineral absorption and bone health. Therefore, the importance of the present experimental study is that it shows the beneficial effect of another prebiotic on mineral absorptions and their effects on bone.

Table 5 Histological parameters obtained at the level of the middle third: osteoblast surface (Ob.S/BS), osteoclast number (Oc.N/B.TA); trabecular number (Tb.N), trabecular thinner (Tb.Tn) and trabecular separation (Tb.Sp.) at $T = 50$

	C5	P5	C3	P3	p
Bone volume (BV/TV)	32.9 ± 3.7	38.3 ± 2.1 ^{***#}	20.3 ± 3.0 [*]	29.3 ± 5.6 ^{**}	0.01
Osteoblast surface (Ob.S/BS) %	50.8 ± 2.9	55.3 ± 3.8 ^{***#}	44.0 ± 2.2 [*]	48.8 ± 5.4 ^{**}	0.001
Eroded surface (ES/BS) %	4.8 ± 0.8	4.5 ± 0.5 ^{**}	5.3 ± 0.2 [*]	4.6 ± 0.6 ^{**}	0.05
Total osteoclast number (Oc.N/B.Ar) (mm ²)	2.5 ± 0.7	2.3 ± 0.1	2.2 ± 0.3	2.3 ± 0.1	0.5
Tb.N (1/mm)	2.3 ± 0.2	2.4 ± 0.2	2.2 ± 0.1	2.3 ± 0.1	0.4
Tb.Th (mm)	0.16 ± 0.02	0.15 ± 0.01	0.16 ± 0.01	0.15 ± 0.02	0.5
Tb.Sp (mm)	0.29 ± 0.03	0.27 ± 0.02	0.29 ± 0.01	0.28 ± 0.01	0.5

The effect of diets was performed by 2-way ANOVA (multiple comparisons test). Data are expressed as mean ± SD

* $p < 0.05$ compared to C5; ** $p < 0.05$ compared to C3; # $p < 0.05$ compared to P3

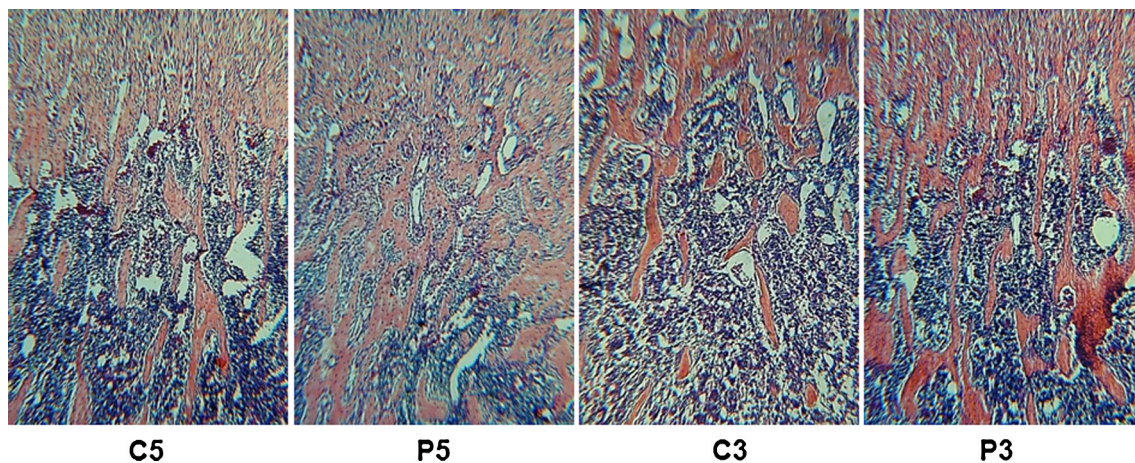


Fig. 2 History of decalcified sections of the proximal third of the right tibia in all studied groups at $T = 50$. Hematoxylin–eosin staining technique shows trabeculae stained in red. Note the decrease

in C3 group and the similarly in P3 and C5 groups in the number of trabeculae, respectively. $\times 50$

Independent of the dietary Ca content, the prebiotic mixture enhanced Ca, Mg and P absorption by $\sim 12\%$. Two mechanisms are considered to be responsible for such effect: first, the increase in the absorption surface area as a consequence of a trophic effect in the cecum and colon, and secondly, the formation of soluble complexes as a result of the low pH [35]. These effects were observed in the present study. Indeed, regardless of the dietary Ca content, the prebiotic mixture led to a twofold increment in cecal wall weight, an increase in lactobacilli growth and a considerable decrease in cecal pH content, as compared to the prebiotic-free diets. The greater effect of the prebiotic mixture observed in the low-Ca group compared with the high-Ca group, strongly suggest that the observed changes result from feeding the prebiotic mixture and confirm it has good fermentability.

There is general agreement that the decrease in cecum pH is a consequence of the increment in SCFA, acid-lactic bacteria fermentation products [35–37]. The production of

SCFA and its trophic effect on cecal cell proliferation were not evaluated in the present report. Nevertheless, the observed effects on cecal wall weight and pH indirectly suggest that both the above mechanisms are involved in the increase in Ca, P and Mg absorption found in the present study. It can be thought that the positive effects of the prebiotic mixture on mineral absorption contributed to the increase in Ca, P and Mg bioavailability. The increase in Ca and P absorption efficiency provided an extra supply of these minerals, the two main inorganic components of bone, inducing a beneficial increase in almost all the studied bone parameters associated with bone quality [24]. Indeed, not only bone content but also bone volume, some parameters of bone structure, total skeleton bone mass and tibia and spine BMDs were higher in all groups fed the prebiotic mixture. In addition, no changes in total skeleton BMD were observed. This finding could be explained by the fact that 80 % of the skeleton is cortical bone, whereas the proximal tibia and spine are mainly composed of

trabecular bone, which is metabolically more active than cortical bone. The groups fed the GOS/FOS[®] mixture, irrespective of the Ca content, exhibited a decrease in bone resorption, as shown by the eroded surface % and CTX levels. This decrease, as well as the increment observed in the osteoblast surface %, would be responsible for the increase in bone mass and density observed at the two areas that are rich in trabecular bone. Despite the increase observed in bone retention and quality, the prebiotic mixture had no effect on total body length. However, the hypertrophic zone of the growth plate and tibia length increased. It is noteworthy that the beneficial effect of the prebiotic mixture on bone quality was accompanied by a beneficial effect on bone growth, especially when dietary Ca intake was inadequate. The observed benefits on bone health may be associated with the effect of the prebiotic mixture on Ca absorption, which depends on ionic Ca (Ca⁺²) solubility, the length of the intestinal segment and the rate of diffusion from the lumen to the blood [24]. The passive non-saturable process prevails in the small intestine at least, mainly when CaI is adequate or high [38]. Conversely, when CaI is inadequate several proteins involved in the transcellular route are upregulated to increase active Ca absorption. In this regard, several studies have shown that the SCFA derived from prebiotics contribute to enhance Ca absorption by increasing the expression of epithelial Ca channels, calbindins and Ca⁺² pumps and Na⁺/Ca⁺² exchanger in the colon [37, 39,40]. It should be kept in mind that the GOS/FOS[®] mixture supplies prebiotics of different chain length that improve fermentation along the entire colon increasing the mucosal surface through which absorption occurs [37].

The greater effect of the prebiotic mixture observed in the low-Ca group compared with the high-Ca group is in disagreement with previous reports using oligofructose [24]. The discrepancy between studies may partly be the result of differences in the experimental model of high-Ca demands (ovariectomized vs. growing rats), the type of prebiotic (oligofructose vs. GOS/FOS[®] mixture), the amount of Ca in the low-Ca diet (0.5 vs. 0.3 %) and the length of the experiment.

It has been reported that soluble Mg cecum levels are inversely related to Ca intake [41]. It has also been postulated that Ca in the gut may interact with Mg and may decrease Mg absorption [9, 42]. In the present report, however, Mg absorption was enhanced, irrespective of the dietary Ca content. It must be pointed out that the short-chain sugar induces selective bifidogenic fermentation in the proximal colon, whereas the long-chain sugar, which is fermented more slowly, can maintain the metabolic activity of the flora in more distal parts of the colon [43]. The prebiotic mixture used in the present study contains both types of sugar; thus, fermentation activity could take place

along the entire large intestine, enabling in turn the simultaneous increase in Ca and Mg absorption.

Unlike Ca and P, body Mg retention is not entirely related to bone. About 60 % of total Mg is stored in the bone. One-third of skeletal Mg resides in cortical bone, either on the surface of hydroxyapatite or in the hydration shell around the crystal [44]. Mg is important to maintain the biomechanical properties of the skeleton; however, intracellular Mg is also vital for numerous physiological functions. In this regard, it stabilizes enzymes in many ATP-generating reactions, antagonizes Ca in muscle contraction, modulates insulin signal transduction and cell proliferation and is important for cell adhesion and membrane transport [45]. All these metabolic functions are very important during normal growth, which is the period evaluated in the present study. Our results showed that the used prebiotic mixture was equally effective in improving Mg absorption in the groups fed the low- and normal-Ca diets. However, this extra Mg supplied by the gut improved retention in the femur in the group fed the low-Ca-prebiotic-contained diet, but not in the group fed the normal-Ca-prebiotic-contained diet. It is important to take into account that we used an experimental model of normal growth, during which Ca recommendations are high to ensure attaining optimal peak bone mass. The strength of the new-formed bone depends on the architectural disposition of bone material as well as on other factors that are unrelated to mineralization, such as crystal arrangement and size [46]. Moreover, Mg affects bone mineralization by inhibiting crystal growth and preventing brittle bone disease. We hypothesize that the increase in Ca retention observed in the low-Ca group must have been accompanied by an increment in Mg retention to maintain a bone Ca/Mg ratio that ensures the biomechanical properties of the skeleton. In contrast, as Mg metabolism is not as tightly regulated as Ca metabolism, it is likely that the increased Mg absorption observed in the normal-Ca group was balanced by an increase in urinary Mg excretion, which limited its retention in bone. In this regard, our data showed that structural bone strength and resistance to fracture in the mid-length region of the tibia increased by feeding the GOS/FOS[®] mixture, independently of dietary Ca content. This finding suggests a positive effect of Mg on the quality of the material and/or on diaphysis architecture.

One limitation of the present report is that urinary mineral output was not measured. This parameter may have clarified the effect of the normal-Ca diet on renal Mg reabsorption. The design of the present report did not include balance studies because they only evidence the effect of prebiotic intake on P–Ca metabolism during a short period of time. Bone retention, density and strength, however, are more adequate to observe the impact of long-term consumption. A low-Ca diet favors Pb absorption that

could be potentially improved by the prebiotic mixture. Although the presence of Pb in food is minimal, when absorbed it could displace Ca on the bone matrix because they are similar physical and chemical characteristics [47]. As no data are available, the effect of the prebiotic mixture added to a low-Ca diet on Pb bioavailability should be explored.

Conclusion

Under the present experimental conditions, it is possible to conclude that supplementing diets with the GOS/FOS[®] mixture increased bone mineralization, density and structure due to an increase in Ca, P and Mg absorptions and thus may help to improve bone development in a period of high calcium requirements, as is the case of the growing rat. It is important to point out that the beneficial effect of the prebiotic mixture was greater in the group fed the low-Ca diet, in almost all the studied bone parameters. Although the data of the present study need to be confirmed by human intervention trials, the present results suggest that this prebiotic mixture would be a useful tool to ensure optimal peak bone mass acquisition during growth.

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

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