International Journal of Food Science +Technology

Institute of Food Science +Technology ifst

Original article

Calcium–inulin wheat bread: prebiotic effect and bone mineralisation in growing rats

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(Received 10 May 2017; Accepted in revised form 12 June 2017)

Summary The nutritional performance of wheat bread prepared with calcium carbonate and inulin was studied in growing rats fed with three diets (BD: diet containing bread with calcium and inulin, SD: diet with synergy1® and CD: diet control-AIN 93G-) up to 60 days. Animals fed with BD consumed less diet and presented a caecal pH (5.5) lower than that of the CD group (7.0) with the highest amount of anaerobic micro-organisms (1.68×10^{10} cfu g^{-1}) at 23 days. Microbiota profiles (DGGE) indicated that BD groups presented 66% of similarity and greater variability than the CD group, suggesting changes due to the prebiotic effect of inulin. In the BD group, calcium apparent absorption (AA_{Ca}: 83%), bone mineral content (18 g kg^{-1}), proximal tibia density (242 mg cm^{-2}) and bone volume (BV: 41%) were higher than in the CD group. The decrease in pH due to fermentation in the large intestine increased calcium bioavailability. Although all variables studied on animals fed with diets containing the prebiotic were improved with respect to a control without inulin, in the case of bread diet, many of them were similar to those of the positive control. Consequently, the prebiotic effect was not altered during the baking process. Results suggest that wheat bread, a highly consumed product throughout the world, is an adequate vehicle for including calcium and inulin in healthy food.

Keywords Anaerobic micro-organisms, calcium bone mineralisation, calcium carbonate, inulin, wheat bread.

Introduction

Wheat flour is deficient in several nutrients as result of the grain milling process. Therefore, flour fortification for its use in breadmaking is an important strategy to improve the nutritional status of the population. Calcium mineral is one of the most critical nutrients, especially in children and women (National Institutes of Health, 2007). Calcium deficiency may be due to a reduced intake and/or low bioavailability. This mineral should be available in sufficient amount to ensure good bone mineralisation to prevent osteopenia and osteoporosis (Harper, 2017). The positive effects of nondigestible oligosaccharides (oligofructose and fructooligosaccharides-FOS) on calcium absorption and of calcium content on bone structure were studied (Scholz-Ahrens & Schrezenmeir, 2007). In a more recent review (Scholz-Ahrens, 2016), it was reported that calcium bioavailability increases in the presence of prebiotics such as inulin. This increase is straightforwardly related to the higher activity of bifidobacteria and lactobacillus (Cilla *et al.*, 2016).

Inulin and fructans are considered as prebiotics because they resist gastric acidity and mammalian enzymes, are susceptible to fermentation by gut bacteria and enhance the viability and/or activity of beneficial micro-organisms, exerting a health-promoting effect (Vandeputte *et al.*, 2017).

Fortification of wheat flour with calcium is a useful strategy to increase the consumption of this mineral through bread. The technological quality parameters of breads formulated with calcium carbonate and inulin + FOS were studied (Salinas & Puppo, 2015). Breads with inulin (13%) and 1.8 g kg^{-1} Ca presented higher specific volume and lower firmness than the control. However, no studies on the nutritional quality of these breads were previously performed. Therefore, the aim of this work was to evaluate the effect of wheat bread fortified with calcium carbonate and

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doi:10.1111/ijfs.13531

inulin on calcium absorption, bone mineralisation and prebiotic activity in growing rats.

Materials and methods

Materials

A wheat flour (type 0000; Molino Campodónico Ltd., La Plata, Argentina) (AAC, 2016) suitable for breadmaking was utilised. Flour composition was as follows: protein, 9.92%; lipids, 0.86%; ash, 0.382%; and moisture, 11.8%. Alveographic parameters were 132 mm, 47 mm and 264 for tenacity, extensibility and deformation energy of dough, respectively. Other ingredients used (wheat flour basis) were sodium chloride (CELUSAL, Timbo S.A., Buenos Aires, Argentina), fresh yeast (Calsa S.A.I.C., Buenos Aires, Argentina), calcium carbonate (Anedra S.A., Buenos Aires, Argentina, PubChem CID 10112) and inulin enriched with FOS (70% inulin, 30% FOS) (Synergy1®; BENEO Orafti, Tienen, Belgium, 92.7% d.b.).

Breadmaking process

Dough was formulated with wheat flour (100 g), NaCl (2% wheat flour basis, w.f.b), fresh yeast (3% w.f.b.), 2.40 g kg^{-1} Ca and 12% Synergy1®. The farinographic parameters obtained were 51% water absorption and 20 min of mixing time for dough development (Salinas *et al*., 2012). Breads were prepared according to Salinas & Puppo (2015). After baking, bread was cooled at 20 °C, dried at 40 °C, milled and stored at −20 °C until its use in a rat diet.

Bread proximal composition

The composition of dried bread, determined according to AACC approved methods (AACC, 2000), was: 9.93% moisture, 9.35% proteins, 3.95% total dietary fibre, 3.64% lipids and 2.457% ash. The content of inulin-FOS, determined by HPLC (Zuleta & Sambucetti, 2001), was 9.15%.

Animals and feeding procedures

A total of 24 male *Wistar* rats in the growing stage (21 days old) were utilised for the experiment. This study was carried out in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals and approved by the Committee of Health Guide for the Care and Use of Laboratory Animals of the School of Pharmacy and Biochemistry, University of Buenos Aires.

Throughout the assay, animals ingested distilled water and food *ad libitum* and were housed in individual stainless steel cages in a room with controlled temperature (21 ± 1 °C) and humidity (60 ± 10%) under a 12-h light/dark cycle. Diets were prepared according to the American Institute of Nutrition-AIN 93G (Reeves *et al*., 1993; Table S1). Eight animals per group were randomly assigned and fed with semisynthetic diets according to the American Institute of Nutrition (AIN 93G; Reeves *et al*., 1993). A control diet (CD) prepared with cellulose, a diet containing Synergy1 (Orafti BENEO, Tienen, Belgium) (SD) and a diet with wheat bread formulated with Synergy1 (BD) were used. The wheat bread, included in BD, was formulated with 2.40 g kg^{-1} Ca as calcium carbonate and 12% of Synergy1. All diets supplied a similar amount of calcium (0.5 g per 100 g) and phosphorus (0.3 g per 100 g). The proximal composition and caloric values of diets are listed in Table S2.

Animals were fed with diets for a 60-day period. At the end of the experience, rats were placed under anaesthesia (0.1 mg/100 g body weight of ketamine hydrochloride + 0.1 mg/100 g body weight of acepromazine maleate) and killed. The caecum from each rat was excised, and the right tibia and femur were removed for biochemical analysis.

Calcium and phosphorous content

Calcium and phosphorus were determined after drying of the samples under infrared light and mineralisation with nitric acid using acid digestion vessels (Parr Instrument Company, Moline, IL, USA). Calcium was determined at 422.7 nm by atomic absorption spectrophotometry in a Perkin Elmer AAnalyst 400 spectrophotometer (Perkin Elmer, Waltham, MA, USA). Lanthanum chloride (6500 mg L^{-1} in the final solution) was added to avoid interferences. Phosphorus was measured according to Weisstaub *et al*. (2013).

NIST reference material RM 8435 (whole milk powder) was also subjected to identical treatment to verify the accuracy of analytical procedures and treated with each bath of samples to ensure accuracy and reproducibility of the mineral analysis.

Intake and body weight

Food intake was recorded every 2 days throughout the experiment: daily and total intakes were calculated. The initial and final body weights of animals were recorded.

Microbiological analysis

Anaerobic count

Serial dilutions in PBS buffer of fresh faecal samples of animals were collected at 0, 2, 23 and 50 days. A selective medium for anaerobic *Lactobacillus* and

Bifidobacterium such as agar MRS (Difco Laboratories, Detroit, MI, USA) supplied with cysteine 0.05% (w/v) was utilised. Plates were incubated at 37 °C for 48 h under anaerobic conditions using Anaero Pack-Anaero kit (Mitsubishi Gas Chemical CO Inc., Tokyo, Japan). The enumeration of anaerobic micro-organisms, expressed as colony-forming units per grams of stool (cfu g^{-1}), was performed.

Extraction, PCR amplification and analysis of DNA by DGGE

Stool (500 mg) collected at the different times was placed in 1 mL of PBS buffer. DNA was extracted and purified using the AccuPrep Stool DNA Extraction Kit (Bioneer, Daejeon, Korea) according to the manufacturer's protocol and stored in DNA-free Eppendorf tubes at −20 °C until use. The V3 region of the 16S rRNA gene (positions 341–534 in the *E. coli* gene) was amplified using 518R (ATTACCGCGGCTGCTGG) and 341F-GC (CCTACGGGAGGCAGCAG) primers for eubacteria, and the PCR reaction was performed. DNA amplified by PCR was analysed by denaturing gradient gel electrophoresis (DGGE) according to Hamet *et al.* (2016). The similarity between DGGE profiles was determined using GelCompar II software package (Applied Maths, Kortrijk, Belgium) and evaluated by Jaccard's coefficient and cluster analysis with the unweighted pair group method with arithmetic mean (UPGMA).

Weight of caecum and pH of caecal content

The weight of caecum from each rat and pH of caecal content were determined.

Calcium apparent absorption

The calcium content consumed by diet and eliminated with stool, between days 45 and 47 of the experience, was determined by atomic absorption spectrophotometry. Calcium apparent absorption (AA_{Ca}) was calculated as (Eq. 1):

$$AA_{Ca}(\%) = [(I_{Ca} - S_{Ca})/I_{Ca}] \times 100 \qquad (1)$$

where I_{Ca} is the calcium intake and S_{Ca} is calcium in stool.

Bone mineral density

At the end of the experience ($t = 60$), bone mineral density (BMD) was measured *in vivo* on anaesthetised rats throughout the whole-body scan. A scanner by dual energy X-ray absorptiometry provided with specifically designed software for small animals (DPX Alpha, Small Animal Software, Lunar Radiation Corp., Madison, WI, USA) was utilised, as described by Zeni *et al.* (2002). The total skeleton bone mineral content was determined. The bone mineral density of femur, proximal tibia and spine was determined according to Albarracín *et al.* (2014).

Bone volume

Right tibiae of rats were cleaned by removing soft tissue. Bones were fixed in neutral formalin (10%), decalcified in EDTA (pH 7.2 for 48 h) and embedded in paraffin. An 8- to 10-μm-thick longitudinally oriented section of subchondral bone was obtained at the level of the middle third, including primary and secondary spongiosa. It was stained with haematoxylin–eosin and microphotographed (AXIOSKOP; Carl Zeiss, Oberkochen, Germany). The bone volume as a percentage of total bone volume (BV) of trabecular bone in the tibiae was determined on the central area of metaphyseal bone (Friedman *et al.*, 2001).

Bone mineral content

Femurs were cleaned of any adhered soft tissue, dried at 100 °C for 72 h. Fat was extracted by immersion in chloroform:methanol (3:1) mixture and dried for 48 h at 100 °C. The length and weight of fat-free and dried femurs were determined. Femur ash was obtained at 700 °C and then dissolved in HCl and diluted for Ca and P analysis (Weisstaub *et al.*, 2013).

Statistical analysis

Results were expressed as mean ± standard deviation. Differences were tested by one-way analysis of variance (ANOVA) using the Statgraphics Plus software. When ANOVA presented statistical differences ($P < 0.05$), intra-group comparisons were tested by LSD test.

Results and discussion

Results of this work focused on the prebiotic effect of a wheat bread fortified with calcium and inulin and therefore on the health properties of animal bones. Nevertheless, two control diets were utilised, a standard one (CD) (AIN 93G) and a positive control with Synergy1 (SD).

Effect of calcium–inulin wheat bread on intake and body weight of growing rats

The different parameters of diet intake are summarised in Table 1. Animals fed with CD consumed more diet than those fed with diets containing prebiotic, and no significant differences were observed in diet intake between BD and SD. These results suggest that diets with inulin provoked satiety. Parnell & Reimer (2012) evaluated the dose-dependent effects of prebiotics (inulin and oligofructose) on gut satiety hormones on obese and lean rats (ghrelin, proglucagon, and peptide YY). These authors found that ghrelin

Table 1 Effect of diets on intake and body weight

	Intake				Body weight	
Group	Diet (g day^{-1})	Ca (mg day^{-1})	Inulin (g day^{-1})	Total intake (g/60 days)	Weight*(g)	Δweight/100 g rat (g%)
CD	19 ± 1 b	94 ± 8 b	0.00	1108 ± 96 b	327 ± 33 b	87 a
SD	15 ± 2 a	76 ± 9 a	1.50	899 ± 110 a	269 ± 68 a	86 a
BD	16 ± 1 a	80 ± 7 a	1.12	940 ± 79 a	228 ± 54 a	84 a

*Measured at the end of the experience (60 days). Different letters in the same column indicate significant differences ($P < 0.05$).

O-acyltransferase mRNA levels were higher in obese rats and decreased when these animals were fed with 20% of prebiotic fibre. Plasma ghrelin response was attenuated in lean rats fed with 20% of fibre. In addition, proglucagon and peptide YY mRNA levels increased with prebiotic intake.

Considering that all diets supplied a similar amount of calcium (0.5 g per 100 g) and phosphorus (0.3 g per 100 g), a low consumption of diets with inulin (BD and SD) would represent a low intake of calcium. Therefore, total diet intake and final body weight were significantly lower in BD and SD than in CD group; nevertheless, the relative increment in body weight (Δweight) among groups did not significantly change (Table 1). This similar increment in body weight among all groups was achieved in spite of the lower energy intake of inulin-based diets (Table S2).

Effects of calcium–inulin wheat bread on caecum pH and microbiota of growing rats

The type of diet consumed by growing rats will influence the fermentation process in gut, leading to differences in caecum content and pH. Both the BD and SD groups presented the highest values of caecum weight; besides, the lowest value of caecal pH (pH 5.5) was observed in the BD group (Fig. 1). A decrease in pH together with an increase in caecum weight could

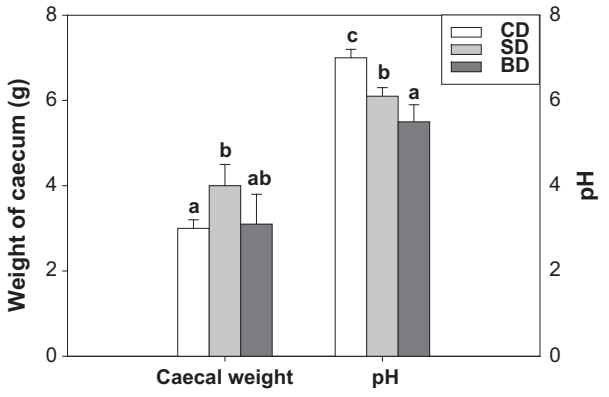

Figure 1 Weight of caecum and pH of caecal content. Different letters from the same group indicate significant differences ($P < 0.05$).

be associated with prebiotic activity of the diet. Oligosaccharides are not absorbed in the upper gastrointestinal tract; they are fermented by microbiota of the large intestine. The fermentation process, conducted by probiotic bacteria such as *Bifidobacterium* and *Lactobacillus*, decreases the pH due to the production of lactic acid and short-chain fatty acids, mainly propionic, acetic and butyric acids (Coudray *et al.*, 2005). These acids constitute a barrier to the growth of pathogenic bacteria, such as *Clostridium*, because they are sensitive to acid conditions; while anaerobic bacteria such as *Bifidobacterium* and *Lactobacillus* are resistant. A small number of clostridia due to consumption of oligofructose + inulin was previously informed (Portune *et al.*, 2017).

Anaerobic micro-organisms decreased two orders of magnitude between 0 and 50 days of the experience in CD group (Fig. 2a); nevertheless, enumeration in the SD group remained constant. The BD group presented a significant increment in anaerobic micro-organism content at day 23 with a significant decrease at day 50. These differences in the viability of anaerobic micro-organisms could be attributed to the nature of fibre present in the diet, cellulose (CD) or inulin (BD and SD). In a review, Portune *et al.* (2017) reported the results from various authors that demonstrated the bifidogenic effect of rats fed with oligofructose as well as the increase in lactobacilli in animals that consumed a mix of oligofructose and inulin.

Denaturing gradient gel electrophoresis profiles of the PCR amplicons belonging to the V3 regions of bacterial 16S rDNA are shown in Fig. 2b. The number of DNA bands found for the different profiles varied between 21 and 48. This number is not indicative of the number of species present in each community, because a species may yield more than one band in the electrophoretic profile (Salles *et al.*, 2002). Nevertheless, the analysis of electrophoretic profiles allows comparing variations that occur in microbial communities.

At 0 day, the electrophoretic profiles for different group (SD0, BD0 and CD0) were different. This behaviour is expected, as the microbiota of each rat is unique. In addition, there were also differences in the number of bands of each electrophoretic profile of the

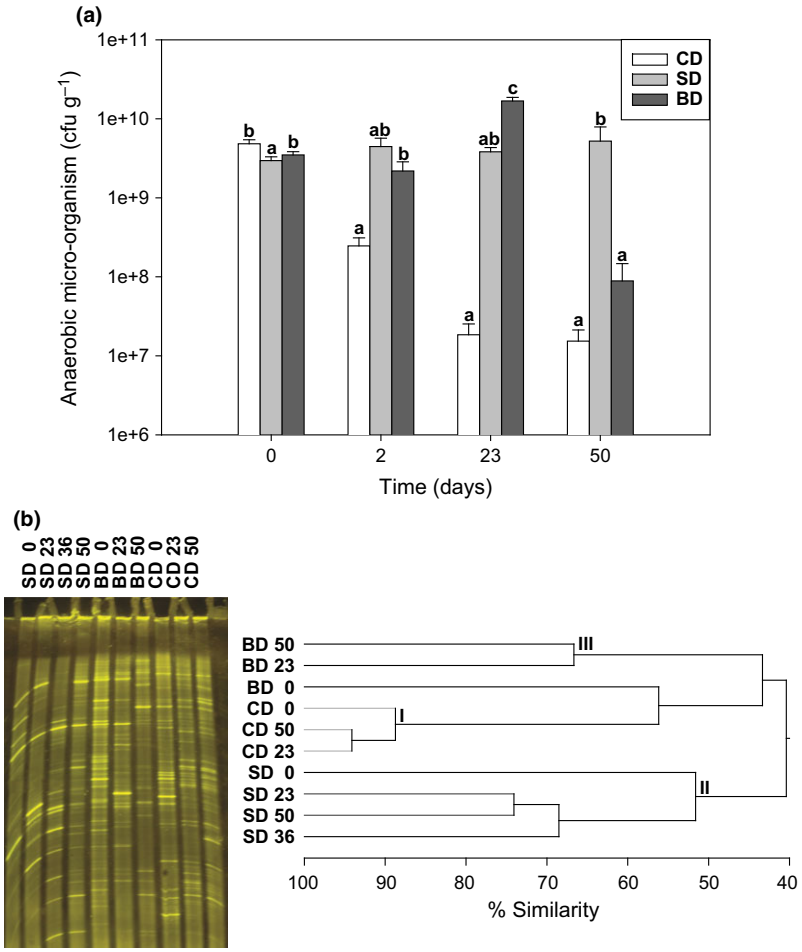

Figure 2 (a) Enumeration of anaerobic micro-organisms. Different letters from the same group indicate significant differences ($P < 0.05$). (b) DGGE profiles resulting from the amplification of DNA obtained from rat stool. Dendrogram obtained from the DGGE profiles.

individual rat over time as a consequence of diet. This variability between individuals' microbiota was widely described in mice (Hufeldt *et al.*, 2010). The variability of DNA bands can be analysed through dendrograms that adequately describe microbial similarity between different groups; low values of similarity suggest a high variability of micro-organisms. The dendrogram obtained from microbial profiles of CD presented 87% of similarity (subcluster I), while the SD group presented 52% of similarity (subcluster II) throughout all the experience (Fig. 2b). On the other hand, microbial profiles of BD groups presented 66% of similarity (subcluster III). It can be observed that the diet with Synergy1 within wheat bread (BD) presented values of similarity percentage comparable to those of the diet containing only this prebiotic (SD) (<70%). Differences between groups fed with prebiotic could be due to the fact that SD contains only inulin as fibre source, while BD contains a certain proportion of nonprebiotic fibre provided by wheat flour.

Effect of calcium–inulin wheat bread on bone parameters of growing rats

Apparent calcium absorption (AA_{Ca}) values were significantly different among groups: 71 ± 3%, 91 ± 5%, and 83 ± 4% for CD, SD and BD, respectively. These values suggest that although rats fed with prebiotic consumed less diet than the CD group (Table 1), they presented higher significant values of AA_{Ca}. The lower AA_{Ca} of the BD group compared to the SD one could be due to the lower quantity of inulin present in this diet (Table S2). Moreover, this difference could be attributed to bread processing (kneading and baking) and/or to the presence of antinutritional components in wheat flour such as phytates that could complex the calcium ion. There is strong evidence of the adverse effects of phytic acid and/or dietary fibre on calcium bioavailability. The reduction in phytic acid content in different grain wheat varieties increases calcium dialysability (Akhter *et al.*, 2012).

Krupa-Kozak *et al.* (2016) studied the effect of inulin and/or FOS on calcium uptake and absorption on Caco-2 cells from calcium-enriched gluten-free bread. They found that cellular calcium uptake and synthesis of organic acids (butyric, valeric and lactic acids) from bread digests incremented significantly with the presence of short-chain FOS.

Adequate mineralisation is needed to maintain healthy bones. Insufficient calcium uptake results in diseases such as rickets in children and osteoporosis in elderly people (National Institutes of Health, 2017). The bone mineral content is a relevant parameter for determining risk of woman fracture incidents (Curtis *et al.*, 2016). The values of total skeleton bone mineral content for BD and SD groups were 18 ± 1 and 20 ± 2 g kg^{-1}, respectively. These values were significantly higher than in rats of the CD group (12 ± 2 g kg^{-1}). This behaviour was similar to that described for AA_{Ca}, and it was influenced by the type of fibre. Although animals consumed less amount of calcium with diets containing inulin (Table 1), high absorption and mineralisation of this mineral were resulted.

Another important parameter that describes bone health is mineral density. Box plots of data obtained from spine, proximal tibia and femur bone mineral density (BMD) are shown in Fig. 3. The BMD of femur was higher than that of spine and proximal tibia in the CD group. No significant differences in the BMD of femur among groups were observed (Fig. 3). Nevertheless, animals fed with inulin (BD and SD) presented high values of BMD in spine and proximal tibia; in addition, these values were similar to that obtained from femur, suggesting that the prebiotic exerted a positive effect on bone mineralisation. Kruger *et al.* (2003) found that the BMD of femur and spine increased significantly in growing rats fed with 5% inulin.

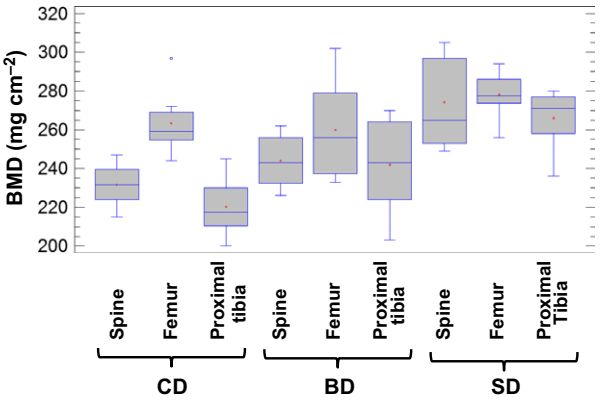

Figure 3 Box plot from data of bone mineral density at the end of the experience (60 days).

On the other hand, femurs were smaller in rats fed with diets containing prebiotics (<L and <W) than in the CD group (Table 2), a behaviour that could be attributed to the fact that these rats consumed less diet (Table 1). Nevertheless, W/L was higher, suggesting that a denser bone was formed when prebiotic was consumed (Table 1). No significant differences in calcium content and Ca/P ratio between SD and BD groups were detected. However, these Ca/*P* values were significantly lower than in the CD group. In addition, no changes in ash and in the ash-to-organic ratio were observed either (Table 2).

Figure 4 shows photographs of histological sections of the right proximal tibia of rats. It can be observed that the number of bone trabeculae of rats fed with prebiotics (BD and SD) was higher than that observed for the CD group. The greater number of trabeculae is associated with more calcium mineralisation. Also, differences in the metaphyseal growth cartilage (star) between tibiae of rats fed with prebiotic and the control ones were detected. Groups fed with prebiotic showed a strengthened bone structure. This finding is of importance as trabecular bone quality is associated with the biomechanical bone properties. Changes in the architecture of trabeculae could possibly be due to the increase in bioavailability of calcium (Macri *et al.*, 2012). Bone volume (BV) values (Fig. 4) attributable to a greater mineralisation followed the same tendency as the bone mineral content and BMD of proximal tibia values (Fig. 3). Scholz-Ahrens *et al.* (2001) attributed the increase in calcium absorption and bone mineralisation to several factors: quantity of FOS intake, time of administration, calcium quantity in the diet, part of the skeleton selected for the study and the age of the animal. Calcium is more soluble in intestinal lumen at acid pH, being more easily absorbed by mucosal cells. Instead, changes in the architecture of rat intestinal mucosa with an increase in cell and crypt number enhanced intestinal surface absorption.

However, no changes were detected in calcium mineralisation of the femur, represented by BMD and by the W/L ratio; calcium absorption and mineralisation of the tibia were higher in BD and SD groups. These results suggest that wheat bread formulated with calcium carbonate and inulin exerted the same healthy effect as the prebiotic alone, wheat bread being one of the most common foods consumed in Western countries.

Conclusions

Animals fed with calcium–inulin wheat bread consumed less diet; however, an increase in calcium absorption, bone mineral density of proximal tibia,

Table 2 Effect of diets on bone size and mineral content of femur

	Femur length L (cm)	Femur weight W (mg)	W/L (mg cm^{-1})	Ca (mg %)	P (mg %)	Ca/P (mg mg^{-1})	Ash (mg %)	Ash/organic ratio (mg mg^{-1})
CD	1.84 ± 0.08 b	504 ± 37 b	274 ± 5	23 ± 2 b	11.9 ± 0.9 a	1.9 ± 0.2 b	59.5 ± 0.7 a	1.47 ± 0.04 a
SD	1.64 ± 0.18 a	470 ± 73 ab	287 ± 18	20 ± 1 a	12.4 ± 0.6 a	1.6 ± 0.1 a	59.2 ± 0.6 a	1.48 ± 0.04 a
BD	1.53 ± 0.20 a	407 ± 86 a	285 ± 37	20 ± 1 a	12.5 ± 0.8 a	1.6 ± 0.1 a	59.7 ± 1.0 a	1.45 ± 0.06 a

Different letters in the same column indicate significant differences ($P < 0.05$).

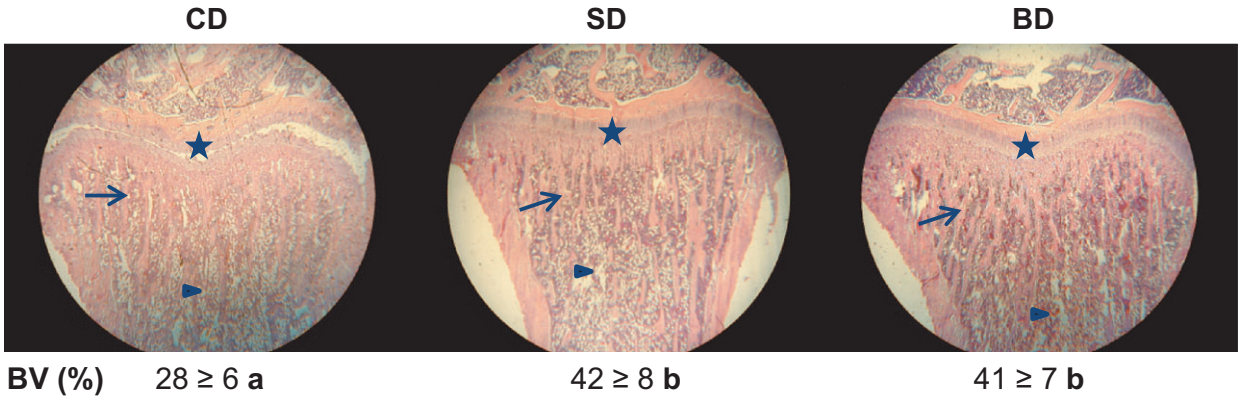

Figure 4 Right tibia bone histological section. CD, control diet; SD, Synergy1® diet; BD, bread diet and bone volume values.

total bone mineral content and bone volume was observed. Inulin in bread favoured calcium deposition in bone, suggesting that the technological process of baking did not affect the absorption or bioavailability of this mineral.

Higher values of total content of anaerobic microorganisms in stool of animals fed with bread were obtained, and these values were maintained throughout time. This behaviour was accompanied by changes in DNA band profiles. This growth of anaerobic bacteria, probably bifidobacteria and *Lactobacillus*, correlated with a decrease in caecal pH due to the rapid fermentation of inulin and FOS by the colonic flora resistant to acid medium. A consumption of 200 g day^{-1} of bread fortified with calcium contributes about 36% of the recommended calcium intake (1000 mg day^{-1}). Thus, bread with calcium and inulin as prebiotic constitutes a potential functional food that covers nutritional calcium deficiencies, mainly in lactose-intolerant patients, improving population health.

Acknowledgments

The authors thank technicians Ricardo Orzuza and Cecilia Mambrin for their technical assistance, PhD Patricia Mandalunis from Cátedra de Histología of Facultad de Odontología (UBA) for performing histological studies on bones and Diana Velasco for bibliographic assistance. Authors also thank MINCYT, CONICET, UBA and UNLP of Argentina for the financial support.

Conflict of interest

The authors declare no conflict of interest.

References

AAC (2016). *Argentinean Alimentarius Codex. Flours. Volume I-a. Chapter IX (Art. 661-Res.167)*. Buenos Aires: Argentinean Alimentarius Codex.

AACC (2000). *Approved Methods of the American Association of Cereal Chemists*, 10th ed. St. Paul: The Association.

Akhter, S., Saeed, A., Irfan, M. & Malik, K.A. (2012). In vitro dephytinization and bioavailability of essential minerals in several wheat varieties. *Journal of Cereal Science*, **56**, 741–746.

Albarracín, M., Weisstaub, A.R., Zuleta, Á., Mandalunis, P., González, R.J. & Drago, S.R. (2014). Effects of extruded whole maize, polydextrose and cellulose as sources of fibre on calcium bioavailability and metabolic parameters of growing Wistar rats. *Food & Function*, **5**, 804–810.

Cilla, A., López-García, G. & Barberá, R. (2016). In vitro bioavailability of iron and calcium in cereals and derivatives: a review. *Food Reviews International*, 1–33. https://doi.org/10.1080/87559129.2016.1210631.

Coudray, C., Feillet-Coudray, C., Tressol, J. *et al.* (2005). Stimulatory effect of inulin on intestinal absorption of calcium and magnesium in rats is modulated by dietary calcium intakes. *European Journal of Nutrition*, **44**, 293–302.

Curtis, E., Harvey, N., D'Angelo, S. *et al.* (2016). Bone mineral content and areal density, but not bone area, predict an incident

fracture risk: a comparative study in a UK prospective cohort. *Archives of Osteoporosis*, **11**, 39.
Friedman, S., Gamba, C.A., Boyer, P.M. *et al.* (2001). Growth deceleration and bone metabolism in nutritional dwarfing rats. *International Journal of Food Sciences and Nutrition*, **52**, 225–233.
Hamet, M., Medrano, M., Pérez, P. & Abraham, A. (2016). Oral administration of kefiran exerts a bifidogenic effect on BALB/c mice intestinal microbiota. *Beneficial Microbes*, **7**, 237–246.
Harper, L. (2017). Optimal nutrition for bone health in people with a learning disability. *Learning Disability Practice*, **20**, 31–35.
Hufeldt, M.R., Nielsen, D.S., Vogensen, F.K., Midtvedt, T. & Hansen, A.K. (2010). Variation in the gut microbiota of laboratory mice is related to both genetic and environmental factors. *Comparative Medicine*, **60**, 336–342.
Kruger, M.C., Brown, K.E., Collett, G., Layton, L. & Schollum, L.M. (2003). The effect of fructooligosaccharides with various degrees of polymerization on calcium bioavailability in the growing rat. *Experimental Biology and Medicine*, **228**, 683–688.
Krupa-Kozak, U., Świątecka, D., Bączek, N. & Brzóska, M.M. (2016). Inulin and fructooligosaccharide affect: in vitro calcium uptake and absorption from calcium-enriched gluten-free bread. *Food & Function*, **7**, 1950–1958.
Macri, E.V., Chaves, M.M.G., Rodriguez, P.N. *et al.* (2012). High-fat diets affect energy and bone metabolism in growing rats. *European Journal of Nutrition*, **51**, 399–406.
National Institutes of Health (2017). *Department of Health and Human Service*. *Calcium*. National Survey on Nutrition and Health (Survey) (2007). Argentina.
Parnell, J.A. & Reimer, R.A. (2012). Prebiotic fibres dose-dependently increase satiety hormones and alter Bacteroidetes and Firmicutes in lean and obese JCR: LA-cp rats. *British Journal of Nutrition*, **107**, 601–613.
Portune, K.J., Benítez-Páez, A., Pulgar, D., Gomez, E.M., Cerrudo, V. & Sanz, Y. (2017). Gut microbiota, diet, and obesity-related disorders—The good, the bad, and the future challenges. *Molecular Nutrition & Food Research*, **61**, 1–17.
Reeves, P.G., Nielsen, F.H. & Fahey, G.C. Jr. (1993). AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *The Journal of Nutrition*, **123**, 1939–1951.
Salinas, M.V. & Puppo, M.C. (2015). Optimization of the formulation of nutritional breads based on calcium carbonate and inulin. *LWT – Food Science and Technology*, **60**, 95–101.
Salinas, M.V., Zuleta, A., Ronayne, P. & Puppo, M.C. (2012). Wheat flour enriched with calcium and inulin: a study of hydration and rheological properties of dough. *Food and Bioprocess Technology*, **5**, 3129–3141.
Salles, J.F., De Souza, F.A. & van Elsas, J.D. (2002). Molecular method to assess the diversity of Burkholderia species in environmental samples. *Applied and Environmental Microbiology*, **68**, 1595–1603.
Scholz-Ahrens, K.E. (2016). Prebiotics, Probiotics, Synbiotics and foods with regard to bone metabolism. In: *Nutritional Influences on Bone Health: 9th International Symposium, Chap 2*. (edited by C.M. Weaver, R.M. Daly & H.A. Bischoff-Ferrari). Pp. 153–167 Cham: Springer International Publishing.
Scholz-Ahrens, K.E. & Schrezenmeir, J. (2007). Inulin and oligofructose and mineral metabolism: the evidence from animal trials. *The Journal of Nutrition*, **137**, 2513S–2523S.
Scholz-Ahrens, K.E., Schaafsma, G., van den Heuvel, E.G. & Schrezenmeir, J. (2001). Effects of prebiotics on mineral metabolism. *The American Journal of Clinical Nutrition*, **73**, 459s–464s.
Vandeputte, D., Falony, G., Vieira-Silva, S. *et al.* (2017). Prebiotic inulin-type fructans induce specific changes in the human gut microbiota. *Gut*, 1–7. https://doi.org/10.1136/gutjnl-2016-313271.
Weisstaub, A.R., Abdala, V., Gonzales Chaves, M., Mandalunis, P., Zuleta, Á. & Zeni, S. (2013). Polydextrose enhances calcium absorption and bone retention in ovariectomized rats. *International Journal of Food Science*, **2013**, Article ID 450794, http://doi.org/10.1155/2013/450794.
Zeni, S., Gregorio, S., Gomez, A., Somoza, J. & Mautalen, C. (2002). Olpadronate prevents the bone loss induced by cyclosporine in the rat. *Calcified Tissue International*, **70**, 48–53.
Zuleta, A. & Sambucetti, M.E. (2001). Inulin determination for food labeling. *Journal of Agricultural and Food Chemistry*, **49**, 4570–4572.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Ingredients used per kg diet.

Table S2. Percentage of protein, fat, total carbohydrate and fiber of diets and caloric value.