PAPER

Effects of the intake of white wheat bread added with garlic and resistant starch: action on calcium bioavailability and metabolic parameters of growing Wistar rats

Adriana R. Weisstaub, [ID] *[a] María Victoria Salinas,[b] María Jimena Correa, [ID] [b] Magalí Barchuk,[c] Gabriela Berg[c,d] and Angela Zuleta[a]

Wheat bread is a widely consumed food and is suitable for the introduction of functional ingredients. The aim of this work was to study the effects of bread with garlic and resistant starch as a fiber source on physiological, metabolic, and functional parameters using an *in vivo* Wistar rat model. Rats were fed with three diets: a control diet prepared according to the American Institute of Nutrition (C), and two semisynthetic diets containing wheat bread (B) and wheat bread with garlic, resistant starch and calcium citrate (BGR). Fresh feces were weighed and lactobacilli (L) and Enterobacteriaceae (E) were analyzed at different times: 1, 20, 45 and 60 days. The pH of the caecal content was recorded and at the end of the study changes in the bone mineral density of total skeleton (ts BMD), femur (F-BMD), spine (S-BMD) and tibia (T-BMD) were determined. Lipoprotein profile was assessed, atherogenic indexes were calculated and malonaldehyde content was measured in the serum and liver. In relation to gut microbiota, the BGR group showed an increase in the L/E ratio with respect to the other groups which was correlated with a lower cecal pH. Besides, the BGR group presented lower weight and a more favourable metabolic profile. In relation to bone measurements, the BGR group presented higher values of ts BMC, ts BMD, F-BMD, and T-BMD than the B group. Thus, bread with resistant starch, garlic and calcium citrate showed a prebiotic effect increasing calcium bioavailability and deposition in bones, compared with wheat bread. The observed beneficial health effects allow us to consider the design of healthier breads.

Introduction

At present, non-transmissible chronic diseases (NTCD) are the main cause of morbidity and mortality worldwide, an unhealthy diet being among the main factors that determine them.[1] High consumption of dietary fiber has been associated with reduced risk of cardiovascular disease, diabetes, hypertension, obesity and gastrointestinal disorders.[2] Fermentable fiber, like non-digestible oligosaccharides, can modify the colonic microbiota by increasing the proliferation and activity of beneficial flora producing prebiotic effects, like enhancing the absorption of minerals such as calcium (Ca), decreasing blood triglyceride and cholesterol content and reducing oxidative stress.[3]

Among the population, there are entrenched eating habits, such as daily consumption of white bread, and due to its high content of carbohydrates of rapid absorption from wheat flour,[4] it contributes to the development of NTCD. On the other hand, white bread may be a good vehicle for the introduction of other healthy components, such as garlic and resistant starch,[5,6] which have prebiotic properties, in order to design functional foods.

Garlic (*Allium sativum* L.) is widely used all over the world like a spice or condiment, and many studies have reported the role of alliums in the prevention of several human diseases including metabolic syndrome and cardiovascular disease, due to their effects on lowering lipids, blood pressure and glycemia, being mainly attributed to the presence of fructooligosaccharides (FOS).[7] Moreover, antioxidant effects have been described due to the high content of organosulfur compounds in garlic.[8–10]

[a] *Departamento de Nutrición y Bromatología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, Buenos Aires, Argentina. E-mail: arweiss@ffyb.uba.ar*

[b] *Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA) (Facultad de Ciencias Exactas-UNLP, CIC, CONICET), 47 y 116 (C.P 1900), La Plata, Argentina*

[c] *Instituto de Fisiopatología y Bioquímica Clínica (INFIBIOC), Departamento de Bioquímica Clínica, Laboratorio de Lípidos y Aterosclerosis, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires Buenos Aires, Argentina*

[d] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina*

Resistant starch type 4 (R4) is a resistant starch due to chemical modification[11] and has a behavior equivalent to dietary fiber. It can be added into cereal-based food formulations like white bread, to enhance the fiber content without a significant energy contribution to the diet.[12]

The R4 and FOS provided by garlic are not susceptible to enzymatic attack, arriving intact into the colon where they are fermented by bifidobacteria and lactobacilli, stimulating their growth and proliferation and producing final products such as short-chain fatty acids (SCFA), like propionic, butyric and acetic acids producing prebiotic effects.[3]

The aim of this work was to study the effects of wheat bread and wheat bread prepared with garlic and resistant starch on physiological, metabolic, and functional parameters using an animal model.

Materials and methods

Rats and diets

Twenty-four male weaning Wistar rats (40.9 ± 2.0 g) were obtained from the Animal Service Laboratory, Facultad de Farmacia y Bioquímica (FFyB), University of Buenos Aires (UBA) (Argentina). The rats were housed in individual stainless steel cages in a temperature (21 ± 1 °C) and humidity (60 ± 10%) controlled room with a 12 h light–dark cycle, and throughout the experiment the animals were allowed free access to deionized water and food. Three groups of rats ($n = 8$ per group) were fed for 60 days with the following diets, respectively (Table 1):

- Control group (C): Semi-synthetic diet prepared according to the American Institute of Nutrition diet (AIN 93) containing 5% of cellulose.[13]

- Wheat bread group (B): Semi-synthetic diet prepared according to the AIN 93 diet containing 5% of total fiber from white bread made with wheat flour and calcium citrate (Fluka, USA) (11.41 g of Ca salts per kg wheat flour).

- Wheat bread added with garlic and R4 group (BGR): Semi-synthetic diet prepared according to the AIN 93 diet containing 5% of total fiber from white bread made with wheat flour and calcium citrate (Fluka, USA) (11.41 g of Ca salts per kg wheat flour), with the addition of garlic (3 g per 100 g wheat flour) and R4 (20 g per 100 g wheat flour).

Considering the contribution of wheat flour to the total content of proteins, lipids, carbohydrates, minerals and vitamins, these nutrients were added to the B and BGR diets to reach equivalent amounts in the three diets, with final values in accordance with the requirements of AIN 93 for rats.

The analyses of the three diets confirmed that they were iso-caloric and supplied a similar amount of macronutrients, Ca (0.5 g per 100 g diet) and phosphorus (P) (0.3 g per 100 g diet), respectively.

The control diet (C) (AIN 93 G diet) contained 5 g per 100 g diet of cellulose. In order to make bread diets, white bread (B) (fiber: 5.56 g per 100 g bread) and white bread added with garlic and R4 (BGR) (fiber: 15.51 g per 100 g bread) were added in different amounts to provide a final dietary fiber concentration of 5 g per 100 g diet. Finally, dextrin was added as a carbohydrate source to achieve 1 kg of diet in the B and BGR diets (Table 1).

Body weight (BW) was recorded once a week throughout the study. Food intakes were recorded every three days throughout the experiment and total intake (g per 60 days) and daily intake (g per day) were calculated.

This study was approved by and carried out in accordance with the National Institute of Health 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 FFyB, UBA. All experiments complied with the current laws of Argentina.

Feces moisture

Feces were collected from the three groups every 15 days and dried to constant weight to calculate the percent moisture (% M), according to AOAC methods.[14]

Intestinal balance

Individual fresh feces samples were weighed and total lactobacilli (L) (beneficial microflora) and Enterobacteriaceae (E) (used as an index of pathogenic microbiota) were analyzed at the beginning (t1), 20 days (t20), 45 days (t45) and 60 days (t60) of the assay. The samples were diluted (1 : 10 w/v) with physiological solution (9 : 1000 w/v NaCl) and were homogenized under sterile conditions. Serial dilutions of the homogenized samples were obtained and aliquots (0.1 mL) of appropriate dilution were spread onto the surface of two agarized media (Britania, Argentina): Mann–Rogosa–Sharpe (MRS) for total L and MacConkey for E counts. The MRS media culture plates were anaerobically incubated at 37 °C for 48 h, while the MacConkey media culture plates were aerobically incubated at 37 °C for 24 h. All media were prepared using sterile distilled water. The results were expressed as log CFU per g feces. The intestinal balance was calculated as the ratio between the bacterial populations (L/E).

Table 1 Composition of the control (C), wheat bread (B) and wheat bread added with garlic and R4 (BGR) diets

Ingredient (g per kg diet)	C	B	BGR
White bread	—	868.0	—
White bread added with garlic (3%) and R4 (20%)	—	—	322.0
Casein	200.0	67.5	152.6
Soybean oil	70.0	39.0	63.3
Mineral mixture (AIN-93-M-MX)	35.0	10.3	27.2
Vitamin mixture (AIN-93-VX)	10.0	9.7	10.0
L-Cystine	3.0	3.0	3.0
Cellulose	50.0	—	—
Choline bitartrate (41.1% choline)	2.5	2.5	2.5
Dextrin	629.5	—	419.4
Total energy (kcal per kg diet)	3828	3829	3828

Apparent calcium absorption (%CaAbs)

Food intake and feces recorded during the last three days of the experiment were used to calculate the apparent Ca absorption as follows (eqn (1)):

$$[(Ca\ intake - fecal\ Ca)/Ca\ intake] \times 100. \quad (1)$$

Feces were dried under infrared light and pounded. Diets and feces were wet-ashes with nitric acid using Parr bombs.[15] The Ca concentration in the diets and feces was determined using an atomic absorption spectrophotometer.[16] Lanthanum chloride (6500 mg L^{-1} in the final solution) was added to avoid interferences. The NIST reference material RM 8435 (whole milk powder) was also subjected to identical treatment to verify the accuracy of the analytical procedures and treated with each batch of samples to ensure accuracy and reproducibility of the mineral analysis.

Bone measurements

At the end of the experiment (t = 60), total skeleton bone mineral content (ts BMC) and total skeleton bone mineral density (ts BMD) were determined *in vivo* under light anesthesia with a total body scanner by dual energy X-ray absorptiometry (DXA), provided with specifically designed software for small animals (DPX Alpha, Small Animal Software, Lunar Radiation Corp., Madison, WI) as previously described.[17–19] 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 coefficient of variation (CV) was 0.9% for ts BMD and 3.0% for ts BMC. The analysis of the different subareas (femur, proximal tibia and spine) was carried out on the image of the animal on the screen using a ROI for each segment. The BMD CV was 2.2% for the femur. To minimize interobserver variations, all analyses were carried out by the same technician.

Then, the rats were placed under anesthesia (50 mg per kg BW of ketamine hydrochloride + 10 mg per kg BW of xylazine) and the right femur was excised at sacrifice for biochemical analysis. The femur was cleaned of any adhering soft tissue and dried at 100 °C for 72 hours, and fat was extracted by immersion for 15 days in a chloroform–methanol (3 : 1) mixture, which was removed and replaced every three days. Finally, it was dried for 48 hours at 100 °C. The fat-free and dried femurs were weighed, and ashes were obtained at 700 °C until they were white and crystalline. Thereafter, they were dissolved in HCl and diluted for Ca and P analysis. The amounts of Ca and P were calculated as percentage content of dried fat-free tissue and the femur Ca/P ratio was also calculated. The Ca concentration in the femur was determined with the same method as that for the diets and feces, and P concentrations were measured according to the Gomori method.[20]

Caecal pH

After the rats were killed, the caecum from each rat was excised, split open, and the pH recorded using a portable digital pH meter (HANNA HI-98103, USA) that was previously calibrated.

Serum samples

An abdominal incision was made; blood was withdrawn from the abdominal aorta and centrifuged at 3000–3500 rpm for 20 minutes at 4 °C. The obtained serum samples were stored at −80 °C for further determination of the lipoprotein profile.

Total cholesterol (TC) and triglyceride (TG) levels were measured using commercial enzymatic kits (Roche Diagnostics GmbH, Mannheim, Germany) in a Cobas C-501 autoanalyser; the intra-assay coefficient of variation (CV) was <1.9% and the inter-assay CV was <2.4% for all parameters. High density lipoprotein cholesterol (HDL-c) was determined by a standardized selective precipitation method using phosphotungstic acid/MgCl$_2$ as a precipitating reagent.[21] Given the naturally low serum concentration of low density lipoprotein cholesterol (LDL-c) in rats, non-HDL cholesterol (non-HDL-c) was calculated as the difference between TC and HDL-c as an approximation of atherogenic lipoprotein levels.

Besides, two atherogenic indexes of the serum were calculated as the risk index: (a) the Castelli index as the relation TC/HDL-c and (b) the TG/HDL-c index calculated to estimate insulin resistance.[22] The amount of malonaldehyde (MDA)[23] and proteins according to Lowry *et al.* (1951)[24] were determined in the serum and liver.

Statistical analysis

The results were expressed as mean ± standard deviation (SD). Differences were tested by one-way analysis of variance (ANOVA) and the statistical differences among the samples were determined using the LSD (least significant difference) test. The significance was established at $p < 0.05$.

Results and discussion

Effects of diets on intake and body weight gain (BWG)

Daily intake (g per day), total intake (g per 60 days), initial rat BW (g), final rat BW (g), and BWG (g per 60 days) for the three experimental groups are shown in Table 2.

Even though the total energy provided by each diet was the same (~3828 kcal per kg diet), the BGR group had a significantly lower daily intake than the C and B groups ($p < 0.05$) and a lower total intake than the B group ($p < 0.05$), without significant differences with the C group. These parameters were reflected in the final BW and BWG, because the animals fed with the BGR diet showed a lower BW and BWG than the C and B groups ($p < 0.01$).

Although the fiber concentration was the same in the three diets (5 g per 100 g diet), the kind of fiber was different for the three groups. The AIN 93 diet contained cellulose, an insoluble fiber, while the B diet was made with white bread. The latter had a mixture of soluble and insoluble fibers provided by the endosperm of wheat and a low proportion of resistant starch produced during bread making. On the other hand, the BGR

Table 2 Daily intake, total intake, initial body weight (BW), final body weight (BW), and body weight gain (BWG)

Diets	Daily intake (g per day)	Total intake (g per 60 days)	Initial BW (g)	Final BW> (g)	BWG> (g per 60 days)
C	16.5 ± 1.8^a	$937 \pm 62^{a,b}$	41.3 ± 2.2	303 ± 15^a	249 ± 14^a
B	16.0 ± 1.6^a	956 ± 60^a	41.6 ± 2.0	297 ± 14^a	250 ± 15^a
BGR	13.8 ± 1.1^b	868 ± 62^b	39.7 ± 2.0	261 ± 13^b	222 ± 14^b
p	0.0045	0.0227	0.1672	<0.0001	0.0010

Data are expressed as mean $\pm$ SEM (n = 8 per group). Different letters in the same column indicate significant differences ($p < 0.05$). Control diet (C); wheat bread diet (B); wheat bread added with garlic and R4 diet (BGR).

diet had the contribution of the fiber provided by wheat flour, the addition of R4, and the FOS provided by garlic, which implied a higher proportion of soluble fiber.

The significant differences in the BWG values obtained for the animal groups were probably due to the higher proportion of the soluble dietary fiber present in the BGR diet with respect to the C and B diets. Satiety produced by soluble dietary fiber accompanied by a subsequent reduction in food intake is the basis of many dietary treatments aimed at weight control.[25,26] Besides, it could also be attributed to a lower acceptability of the BGR diet.

Effects of the three diets on feces moisture

Fig. 1 shows the evolution of feces moisture (%) from the 15th day to the 60th day of diet intake in the three experimental groups.

After 15 days of diet intake, feces moisture of the BGR group was significantly higher than those obtained in the B and C groups. Moreover, at 30 days the value of the B group is the highest. The feces moisture of the BGR group presented a significant increase from day 45 until the end of the experiment ($p < 0.0001$). The control diet is made with cellulose, an insoluble fiber, and did not show an increase in feces volume or humidity. The B diet, containing a mixture of soluble and insoluble dietary fibers, showed similar water content to that of the C diet but lower than that of the BGR diet.
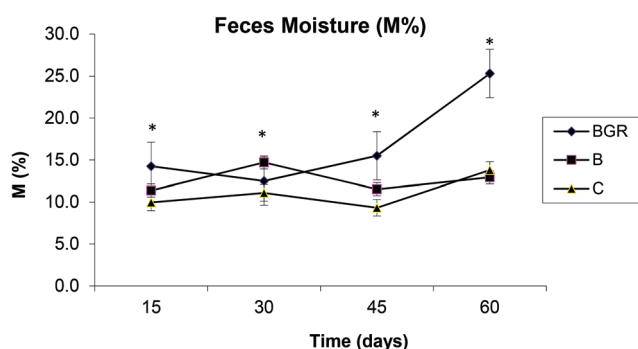
Fig. 1 Evolution of feces moisture (M%) from the 15th day to the 60th day of diet intake in the experimental groups. Control diet (C); white bread diet (B); white bread added with garlic and R4 diet (BGR). The symbol * indicates significant differences ($p < 0.05$).

On the other hand, the BGR diet contained a high amount of fermentable fiber from garlic and resistant starch. The fiber present in this diet is highly fermentable by intestinal microbiota which causes great retention of water and increase in stool volume, while the insoluble fiber present in the C diet and the mixture of soluble and insoluble fibers present in the B diet lead to low stool volume and water retention. It is well known now that the increase in both factors could improve intestinal transit.[27]

Effects of diets on fecal counts of total lactobacilli (L) and Enterobacteriaceae (E) and intestinal balance

The fermentation by the intestinal microbiota is one of the criteria to classify a food ingredient as a prebiotic. Besides, prebiotics have to promote selectively the growth and activity of gut beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*.[28,29]

Table 3 shows the total L and E fecal counts during the 60 days of intake of the control and experimental diets. The L population was considered an indicator of beneficial flora while the E population was selected as an indicator of pathogenic flora.

The results showed a constant count in the L population during the 60 days of feeding of the C and B groups and an increase in the BGR group ($p < 0.05$). However, during the whole assay, the B and BGR groups presented a higher count of the L population in feces than the C group ($p < 0.0001$).

With respect to the E population, the C group showed an increase in the E count ($p < 0.0001$) while the B group exhibited a constant count of E during the entire experiment. On the other hand, in the case of the BGR group, a reduction of this population was observed ($p < 0.0001$). The comparison of the three groups at the same time of feeding showed that the BGR group always presented the lowest E counts ($p < 0.0001$).

Besides, the intestinal balance of the rats was calculated as the ratio between the bacterial populations (L/E)[30] to evaluate the prevalence of beneficial bacteria with respect to pathogenic bacteria. The L/E ratio was significantly higher for the BGR group (3.82 ± 0.44) than the values presented by the other two groups (-0.89 ± 0.44 for C and 0.83 ± 0.54 for B) ($p < 0.0001$). Several authors showed that caloric restriction may beneficially affect the indicators of colonic health in aging mice.[31] The reduction of food intake by the BGR group led to an increase of the L population and lower counts of the E population.

Table 3 Total lactobacilli and Enterobacteriaceae fecal counts (log CFU g^{-1}) of Wistar rats fed for 60 days with the control and experimental diets

Time (days)	Diet			
	C	B	BGR	p
	Lactobacili (L)			
t1	8.78 ± 0.37[b]	9.95 ± 0.43[a]	9.10 ± 0.20[b]	<0.0001
t20	8.52 ± 0.10[b]	9.64 ± 0.77[a]	9.10 ± 0.22[a]	0.0004
t45	8.51 ± 0.15[b]	9.74 ± 0.43[a]	9.51 ± 0.31[a]	<0.0001
t60	8.55 ± 0.13[b]	9.26 ± 0.29[a]	9.54 ± 0.17[a]	<0.0001
	Enterobacteriaceae (E)			
t1	8.03 ± 0.26[a]	8.01 ± 0.60[a]	6.70 ± 0.39[b]	<0.0001
t20	8.74 ± 0.17[a]	7.58 ± 0.75[b]	6.75 ± 0.40[c]	<0.0001
t45	9.06 ± 0.23[a]	7.85 ± 0.27[b]	4.78 ± 0.64[c]	<0.0001
t60	9.44 ± 0.26[a]	8.73 ± 0.83[a]	4.92 ± 0.72[b]	<0.0001
	Intestinal balance (L/E)			
t60	−0.89 ± 0.44[c]	0.83 ± 0.54[b]	3.82 ± 0.44[a]	<0.0001

Data are expressed as mean ± SEM (n = 8 per group). Different letters in the same row indicate significant differences ($p < 0.05$). Control diet (C); white bread diet (B); white bread added with garlic and R4 diet (BGR).

Thus, the B and BGR diets promoted a selective growth of beneficial flora, but only the BGR diet could inhibit or delay the growth of pathogenic flora in the gut of these rats.

Effects of diets on cecal content pH and apparent Ca absorption (%CaAbs)

Table 4 shows the cecal content pH and apparent Ca absorption in the three experimental groups.

The pH of the cecum content of the BGR group was significantly lower than that of the B and C groups ($p < 0.0001$). The

Table 4 Cecal content pH, daily Ca intake (Ca I), daily fecal Ca excretion and percentage of apparent calcium absorption (%CaAbs)

Diets	Cecal content pH	Ca I (mg per day)	Fecal Ca excretion (mg per day)	%CaAbs (%)
C	7.47 ± 0.17[a]	73.0 ± 11.9[a]	20.9 ± 2.6	71.1 ± 3.3[a]
B	7.15 ± 0.19[b]	51.9 ± 9.8[b]	23.3 ± 2.6	53.9 ± 8.8[b]
BGR	6.78 ± 0.06[c]	61.9 ± 0.1[c]	20.8 ± 6.3	66.6 ± 6.2[a]
p	<0.0001	0.001	0.4169	0.0001

Data are expressed as mean ± SEM (n = 8 per group). Different letters in the same column indicate significant differences ($p < 0.05$). Control diet (C); wheat bread diet (B); wheat bread added with garlic and R4 diet (BGR).

cecum content of the BGR diet group was more acidified due to fiber fermentation by intestinal microflora.

The control group exhibited higher calcium intake (CaI) and similar fecal excretion compared to the B and BGR groups leading to a higher %CaAbs. These results suggested that the Ca present inside the bread matrix has lower availability. In spite of this, when garlic and resistant starch were added to the bread formulation, an increase in %CaAbs occurred with respect to the C group. According to Cashman and other authors (2003),[32] the decrease of pH promotes the growth of beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus* and improves the absorption of some minerals like Ca, maintaining it in solution.[17,33,34] The decrease in the cecal pH of the BGR group is consistent with an increase in the absorption of Ca with respect to the B group ($p < 0.001$).

Effects of diets on the mineral content and density of bones

Table 5 presents the values for the total skeleton bone mineral content (ts BMC) and the bone mineral density of total body (ts BMD), femur (F-BMD), spine (S-BMD), and proximal tibia (T-BMD) at the end of the experiment ($t = 60$), in the three experimental groups.

With respect to ts BMC, the BGR group exhibited the highest value ($p < 0.0001$). The total skeleton bone mineral density (ts BMD) of the BGR group was higher than that of the B and C groups ($p < 0.0001$). Moreover, the BGR group had higher F-BMD and T-BMD compared with the B group, without differences with the C group ($p < 0.05$). The values of S-BMD, F-BMD and T-BMD of the B group were lower than those of the C group. However, in the BGR group the addition of resistant starch and garlic compensated for this negative effect of the bread matrix reaching similar values to that of the control group. These results suggest that the Ca in bread (B) is not available as in the control diet for mineralization probably due to the presence of fitates from wheat flour. On the other hand, the incorporation of resistant starch and FOS from garlic contributed to the increase of apparent calcium absorption probably as a consequence of the acidification of the caecal content.

Femur is a representative bone tissue because it is considered a parameter for bone strength against fracture, and is subject to fair remodeling by the ongoing exercise stimulus. Bone matrix is formed by organic and inorganic components. The inorganic component is mainly comprised of hydroxyapatite crystals [$Ca_5(PO_4)_3(OH)$] because the ratio of Ca/P close to 2 is associated with adequate bone mineralization.[35] Table 6 shows the right femur parameters at the end of the experiment: ash content, organic content (OC), ash/organic content ratio, calcium content (Femur Ca), phosphorus content (Femur P) and Ca/P ratio.

In agreement with the higher value of F-BMD for the BGR group, its femur had higher content of ashes, Ca and ash/OC, and lower content of OC than those of the other groups ($p < 0.0001$). Furthermore, the Ca/P ratio was significantly the highest in the BGR group ($p < 0.0001$).

Q6

Table 5 Total skeleton bone mineral content (ts BMC), bone mineral density of total body (ts BMD), bone mineral density of femur (F-BMD), bone mineral density of spine (S-BMD), and bone mineral density of proximal tibia (T-BMD) (mg cm^{-2}) at the end (t = 60) of the experiment

Diets	ts BMC (mg)	ts BMD (mg cm^{-2})	F-BMD (mg cm^{-2})	S-BMD (mg cm^{-2})	T-BMD (mg cm^{-2})
C	3693 ± 205[c]	237 ± 10 [b]	263 ± 16 [a]	249.0 ± 23.1[a]	232.2 ± 3.7[a]
B	3950 ± 203[b]	238 ± 9 [b]	226 ± 21[b]	222.1 ± 19.7[b]	212.7 ± 11.2[b]
BGR	4566 ± 200[a]	269 ± 10[a]	249 ± 9 [a]	226.6 ± 12.0[a,b]	226.1 ± 11.4[a]
p	<0.0001	<0.0001	0.0006	0.0209	0.015

Data are expressed as mean ± SEM (n = 8 per group). Different letters in the same column indicate significant differences ($p < 0.05$). Control diet (C); wheat bread diet (B); wheat bread added with garlic and R4 diet (BGR).

Prebiotics such as inulin, oligofructose and galactooligosaccharides are fermented in the gut by beneficial flora, producing short chain fatty acids (SCFA). These molecules decrease the cecal pH and stimulate Ca absorption and its retention.[36] The prebiotic effect of the BGR diet was evidenced by the higher bone mineral density (ts BMD, F-BMD and T-BMD) compared with the B diet. Furthermore, an improved Ca/P ratio was shown by the BGR group which is associated with appropriate bone mineralization.

Effects of diets on lipid metabolism

Lipid metabolism was evaluated by using several biomarkers in the serum such as TC, HDL-c, non-HDL and TG (Table 7). The results showed that the BGR group had significantly lower TC and non-HDL than the B group. However, there were no significant differences in both parameters with respect to the C group ($p < 0.01$). Besides, the BGR and B groups presented higher HDL-c levels than the rats under the control diet ($p < 0.05$), suggesting a favorable effect on lipoprotein metabolism. The rats fed with the control diet presented 25 mg dL^{-1} of HDL-c while the rats fed with the BGR and B diets had significantly higher values ($p < 0.05$), suggesting that it is enhanced the transport of excess cholesterol from peripheral tissues to the liver. Furthermore, HDL has been associated with antioxidant, anti-inflammatory, antithrombotic, and antiapoptotic activities which contribute to the inhibition of atherosclerosis.[37]

On the other hand, the BGR and B groups presented lower TG levels than the C group, without significant differences between them (Table 7). A reduction of the TG level is related to a lower cardiovascular heart disease risk. The rats fed with the BGR diet showed similar values of non-HDL to that of the C group while the B group showed the highest value ($p < 0.05$). The lower levels of non-HDL observed in the BGR group are in agreement with the decrease in TC and TG and the less atherogenic profile observed in this group. The inclusion of garlic and resistant starch in bread caused a significant decrease in TC and non-HDL serum contents with respect to the rats fed with the B diet (27% and 34%, respectively).

These results could be related to the intestinal fermentation of the FOS present in garlic and the resistant starch of the BGR diet, generating SCFA that may act in the reduction of cholesterol in the serum. Our experimental data are in agreement with the studies carried out by different authors: Delzenne *et al.* described that inulin and oligofructose were able to exert a systemic effect by modifying the hepatic metabolism of lipids in animal models[38,39] and Daubioul *et al.* observed a decrease in serum lipids of rats fed with a diet supplemented with nondigestible carbohydrates (fructan type), compared to cellulose fed rats (control group).[40]

The traditional lipoprotein metabolism biomarkers, like HDL-c and TG, generally present good correlation with insulin resistance and diabetes type 2.[41] Moreover, the TG/HDL-c ratio is a very good surrogate marker of insulin resistance; Table 6 shows that the two semisynthetic diets led to a significant decrease in TG/HDL-c, through the decrease in TG and the increase in HDL-c.

Finally, Table 7 shows the MDA values from the serum and liver. The measured MDA is employed as a biomarker of peroxidation of polyunsaturated fatty acids since one of the secondary oxidation products of this process is MDA. The rats fed with the BGR diet showed lower serum and liver MDA content than the B group ($p < 0.05$). The decrease in this oxidative stress marker may be due to the high content of organosulfur compounds, like allicin derivative compounds such as ajoene, vinyldithiins and alkenyl-sulphides provided by garlic, which have shown antioxidant effects.[8–10]

Table 6 Right femur parameters at the end of the experiment: ashes, organic content (OC), ash/organic content ratio, Ca and P contents and Ca/P ratio

Diets	Ashes (mg per 100 g)	OC (mg per 100 g)	Ash/OC (mg per mg)	Femur Ca (mg per 100 g)	Femur P (mg per 100 g)	Ca/P
C	52.6 ± 3.2[b]	47.4 ± 3.2[a]	1.12 ± 0.14[b]	14.9 ±.11[b]	9.1 ± 0.4	1.63 ± 0.09[b]
B	51.0 ± 3.9[b]	49.0 ± 3.9[a]	1.05 ± 0.15[b]	15.1 ± 2.1[b]	9.1 ± 0.9	1.65 ± 0.16[b]
BGR	59.6 ± 0.7[a]	40.4 ± 0.7[b]	1.48 ± 0.04[a]	21.7 ± 3.0 [a]	9.1 ± 0.4	2.38 ± 0.29[a]
p	<0.0001	<0.0001	<0.0001	<0.0001	0.842	0.0004

Data are expressed as mean ± SEM (n = 8 per group). Different letters in the same column indicate significant differences ($p < 0.05$). Control diet (C); wheat bread diet (B); wheat bread added with garlic and R4 diet (BGR).

Table 7 Total cholesterol (TC), HDL-cholesterol (HDL-c), non-HDL cholesterol (non-HDL), triglyceride (TG) content, atherogenic indexes (TC/HDL-c and TG/HDL-c) and malondialdehyde (MDA) level from serum and liver

Diet	TC (mg dL^{-1})	HDL-c (mg dL^{-1})	Non-HDL (mg dL^{-1})	TG (mg dL^{-1})	TC/HDL-c	TG/HDL-c	MDA serum (nmol per g protein)	MDA liver (nmol per100 g protein)
C	88 ± 10[b]	25 ± 13[b]	63 ± 21[a,b]	96 ± 30[a]	4.6 ± 2.4[a]	2.6 ± 1.1[a]	31 ± 5[a,b]	36 ± 7[a]
B	118 ± 18[a]	41 ± 12[a]	81 ± 12[a]	29 ± 11[b]	3.3 ± 1.1[ab]	0.6 ± 0.2[b]	37 ± 8[a]	26 ± 2[a]
BGR	86 ± 9 [b]	37 ± 10[a,b]	53 ± 11[b]	32 ± 10[b]	2.5 ± 0.6[b]	1.0 ± 0.3[b]	25 ± 4 [b]	9 ± 3[b]
p	<0.0001	0.03	0.006	<0.0001	<0.05	<0.001	0.01	0.0009

Data are expressed as mean ± SEM (n = 8 per group). Different letters in the same column indicate significant differences ($p < 0.05$). Control diet (C); wheat bread diet (B); wheat bread added with garlic and R4 diet (BGR).

Conclusions

The physiological effect of the intake of wheat bread and wheat bread with resistant starch and garlic (BGR) in an experimental model of growing Wistar rats was studied. The group of rats fed with BGR showed potential health benefits compared to the B group, associated with the prebiotic effects of the intake of resistant starch and the fructo-oligosaccharides provided by garlic. This phenomenon was observed by the reduction of cecal content pH and an increase in the beneficial gut microbiota.

Besides, in the BGR group a lower body weight gain and an increase in calcium retention were observed. Moreover, the lipid profile was improved since the rats fed with the BGR diet presented a decrease in TC and non-HDLc. In the same sense, atherogenic indexes and MDA showed a protective role of bread with garlic and resistant starch against oxidative stress. The addition of bioactive compounds in foods of daily consumption, such as wheat bread, could bring health benefits. On the other hand, bread during its cooking process offers benefits other than those produced by the addition of functional ingredients. Baking leads to an increase in resistant starch, and the fermentation process leads to a decrease in calcium absorption inhibiting factors, such as phytates. From the nutritional point of view, bread is an optimal carrier because during its elaboration, the effects of the addition of bioactive compounds are enhanced.

The results obtained in this work regarding the physiological and metabolic parameters measured in growing Wistar rats led us to affirm that bread with garlic and resistant starch could be considered as a *functional food*.

Abbreviations

%CaAbs	Apparent calcium absorption
%M	Percent moisture
B	Wheat bread group
BGR	Wheat bread added with garlic, resistant starch and calcium citrate group
BW	Body weight
BWG	Body weight gain
C	Control group
Ca	Calcium
Ca/P	Femur Ca/P ratio
CV	Coefficient of variation
DXA	Dual energy X-ray absorptiometry
E	Enterobacteriaceae
F-BMD	Femur bone mineral density
Femur Ca	Femur calcium content
Femur P	Femur phosphorus content
FOS	Fructooligosaccharides
HDL-c	High density lipoprotein cholesterol
L	Lactobacilli
L/E ratio	Lactobacilli/Enterobacteriaceae ratio
LDL-c	Low density lipoprotein cholesterol
LSD	Least significant difference
MDA	Malonaldehyde
MRS	Mann–Rogosa–Sharpe
Non-HDL-c	Non-HDL cholesterol
NTCD	Non-transmissible chronic diseases
OC	Organic content
P	Phosphorus
R4	Resistant starch type 4
S-BMD	Spine mineral density
SD	Standard deviation
t1	Day 1
t20	Day 20
t45	Day 45
t60	Day 60
T-BMD	Tibia mineral density
TC	Total cholesterol
TG	Triglycerides
ts BMC	Total skeleton bone mineral content
ts BMD	Total skeleton bone mineral density

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

and Ricardo Orzuza and Cecilia Mambrin for their technical assistance and collaboration.

References

1 FAO/OMS- Diet, Nutrition and Prevention of Chronic Diseases, *WHO Thecnical Report Series 916 Geneva*, 2003.

2 J. M. Anderson, P. Baird, R. H. Davis Jr., S. Ferreri, M. Knudtson, A. Koraym, V. Waters and C. L. Williams, *Nutr. Rev.*, 2009, **67**, 188–205.

3 M. B. Roberfroid, *Br. J. Nutr.*, 2002, **87**(Suppl 2), 139–143.

4 ECNT FAO/OMS- Diet, Nutrition and Prevention of Chronic Diseases, *WHO Thecnical Report Series 916 Geneva*, 2003.

5 M. Sabater-Molina, E. Larqué, F. Torrella and S. Zamora, *J. Physiol. Biochem.*, 2009, **65**(3), 315–328.

6 Z. Ma and J. I. Boye, *Crit. Rev. Food Sci. Nutr.*, 2018, **58**(7), 1059–1083.

7 A. Zuleta, A. R. Weisstaub, M. Bauzá and M. Sance, *La Consulta. Ed Inst Nac Tec Agr*, 2007, **10**, 207–208.

8 I. Krest, J. Glodek and M. Keusgen, *J. Agric. Food Chem.*, 2000, **48**(8), 3753–3760.

9 Y. Zhu, R. Anand, X. Geng and Y. Ding, *Neurol. Res.*, 2018, (Mar 20), 1–5.

10 M. A. Vazquez-Prieto and R. M. Miatello, *Mol. Aspects Med.*, 2010, **31**(6), 540–545.

11 F. M. Nunes, E. S. Lopes, A. S. Moreira, J. Simões, M. A. Coimbra and R. M. Domingues, *Carbohydr. Polym.*, 2016, **141**, 253–262.

12 G. Arp, M. J. Correa, A. Zuleta and C. Ferrero, *Int. J. Food Sci. Technol.*, 2017, **52**, 550–558.

13 P. G. Reeves, F. H. Nielsen and G. C. Hahey, *J. Nutr.*, 1993, **123**(11), 1939–1951.

14 AOAC, *Official Methods of Analysis*, ed. W. Horwitz, Washington, DC, 17th edn, 2002.

15 R. E. Sapp and S. D. Davidson, *J. Food Sci.*, 1991, **56**, 1412.

16 Perkin Elmer Corp, Norwalk CT, 1971.

17 A. Weisstaub, V. Abdala, M. Gonzales Chaves, P. Mandalunis, A. Zuleta and S. Zeni, *Int. J. Food Sci.*, 2013, **2013**, 450794.

18 S. Zeni, S. Di Gregorio and C. Mautalén, *Bone*, 1999, **256**, 681–685.

19 S. Zeni, A. Weisstaub, S. Di Gregorio, P. Ronayre de Ferrer and M. L. Portela, *Calcif. Tissue Int.*, 2003, **73**, 594–600.

20 G. A. Gomori, *J. Lab. Clin. Med.*, 1942, **27**, 955–960.

21 G. Assmann, H. Schriewer, G. Schmitz and E. Hägele, *Clin. Chem.*, 1983, **29**, 2026–2030.

22 M. Dobiásová, *Clin. Chem.*, 2004, **50**(7), 1113–1115.

23 K. Yagi, *Biochem. Med.*, 1976, **15**, 212–216.

24 O. H. Lowry, N. I. Rosebrough, A. L. Farr and R. I. Randall, *J. Biol. Chem.*, 1951, **193**(1), 265–275.

25 J. L. Slavin, *Nutrition*, 2005, **21**, 411–418.

26 A. Brownlee, *Food Hydrocolloids*, 2011, **25**, 238–250.

27 X. Ge, H. Tian, C. Ding, L. Gu, Y. Wei, J. Gong, W. Zhu, N. Li and J. Li, *Arch. Med. Res.*, 2016, **47**(3), 236–242.

28 M. D. Collins and G. R. Gibson, *Am. J. Clin. Nutr.*, 1999, **69**, 1052–1057.

29 G. R. Gibson, H. M. Probert, J. Van Loo, R. A. Rastall and M. B. Roberfroid, *Nutr. Res. Rev.*, 2004, **17**, 259–275.

30 M. Castillo, S. M. Martín-Orúe, M. Roca, E. G. Manzanilla, I. Badiola, J. F. Perez and J. Gasa, *J. Anim. Sci.*, 2006, **84**, 2725–2734.

31 D. E. Kok, F. Rusli, B. van der Lugt, C. Lute, L. Laghi, S. Salvioli, G. Picone, C. Franceschi, H. Smidt, Vervoort and E. Kampman, *J. Nutr. Biochem.*, 2018, **56**, 152–164.

32 K. Cashman, *Curr. Issues Intest. Microbiol.*, 2003, **4**, 21–32.

33 J. Van Loo, J. Cummings, N. Delzenne, H. Englyst, A. Franck, M. Hopkins, N. Kok, G. Macfarlane, D. Newton, M. Quigley, M. Roberfroid, T. van Vliet and E. van den Heuvel, *Br. J. Nutr.*, 1999, **81**(2), 121–132.

34 K. E. Scholz-Ahrens and J. Schrezenmeir, *Br. J. Nutr.*, 2002, **87**, S179–S186.

35 V. Zaichicka and M. Tzaphlidou, *Appl. Radiat. Isot.*, 2002, **56**, 781–786.

36 M. V. Salinas, M. F. Hamet, J. Binaghi, A. G. Abraham, A. Weisstaub, A. Zuleta, P. Ronayne de Ferrer and M. C. Puppo, *Int. J. Food Sci. Technol.*, 2017, **52**, 2463–2470.

37 K. R. Feingold and C. Grunfeld, *Introduction to Lipids and Lipoproteins*, 2000.

38 N. M. Delzenne, C. Daubioul, A. Neyrinck, M. Lasa and H. S. Taper, *Br. J. Nutr.*, 2002, **87**(Suppl 2), S255–S259.

39 N. M. Delzenne and N. Kok, *Am. J. Clin. Nutr.*, 2001, **73**, 456–458.

40 C. Daubioul, N. Rousseau, R. Demeure, B. Gallez, H. Taper, B. Declerck and N. Delzenne, *J. Nutr.*, 2002, **132**, 967–973.

41 P. W. Wilson, J. B. Meigs, L. Sullivan, C. S. Fox, D. M. Nathan and R. B. D'Agostino, *Arch. Intern. Med.*, 2007, **167**(10), 1068–1074.