

## Composition and functionality of bone affected by dietary glycated compounds

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Our aim was to investigate the effects of Maillard reaction products (MRPs) from bread crust (BC) on bone composition and its mechanical properties, determining whether any such effects are related to the molecular weight of different MRPs. For 88 days after weaning rats were fed a control diet or diets containing BC, or its soluble low molecular weight (LMW), soluble high molecular weight (HMW) or insoluble fractions. Animals' food consumption and body weights were monitored. After sacrifice, the femur, pelvic bone and tibia were removed for composition, physical and biomechanical properties analysis. It was found that body and femur weights, density, volume and organic matrix decreased, whereas pentosidine increased after consumption of experimental diets, especially in the HMW and insoluble groups (104.7 and 102.9 mmol mol<sup>-1</sup> collagen) vs. the control group (41.7 mmol mol<sup>-1</sup> collagen). Bone stiffness fell by 50% in the LMW, HMW and insoluble groups and failure load and energy to failure tended to decrease in the same animals after MRPs intake. Consumption of diets containing assayed MRPs during growth leads to lower bone size and introduces some changes in its mechanical behavior which appear to be related to an increase in the pentosidine level of bone.

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### Introduction

The ability of bone to resist fracturing depends on its quantity, architecture and turnover, but also on the intrinsic properties of its constituent material.<sup>1</sup> Bone is a sophisticated composite material consisting of a mineral phase (mainly hydroxyapatite), an organic phase and water with complex relationships among them, influencing bone strength. An imbalance between the two major constituents or an alteration in their quality modifies the mechanical behaviour.<sup>2</sup> The mineral phase, comprising approximately 60% (w/w) of the bone, essentially contributes its stiffness and strength; while the organic phase, consisting mainly of collagen type I, is primarily responsible for providing toughness and ductility.<sup>1,3,4</sup> Also, the collagen content and its cross-links could affect the mechanical properties.<sup>5</sup>

Specifically, non-enzymatic collagen cross-links, those formed by the generation of advanced glycation end products (AGEs) by the reaction of sugars within the extracellular space with the amino groups of protein collagen, have been associated with adverse effects on the mechanical and biological functions of bone.<sup>4,6</sup> It has been reported that the formation of AGEs in

ribosylated human bones provokes a decrease in the specific mechanical properties relating to post-yield behaviour;<sup>7</sup> moreover, the accumulation of collagen non-enzymatic cross-links in bones has been correlated with an increase in collagen stiffness.<sup>8</sup>

Pentosidine, a fluorescent AGE, is used as a specific biomarker of non-enzymatic cross-links in mechanical studies *in vivo* since it has been reported to accumulate in bones with aging.<sup>9</sup> Wang *et al.*<sup>4</sup> reported that the age-related increase in pentosidine was associated with a decrease in human femur toughness and post-yield properties. Saito *et al.*<sup>10</sup> demonstrated a decrease in bone strength in diabetic rats associated with an increase in non-enzymatic cross-links measured as pentosidine.

The formation of AGEs can take place in the organism but may also occur exogenously during food processing. These compounds used to be called Maillard reaction products (MRPs).<sup>11,12</sup> It has been demonstrated that dietary MRPs can be absorbed and therefore contribute to the AGEs formed *in vivo*,<sup>13</sup> which accumulate in some tissues, playing a physiopathological role in the development and progression of diseases such as diabetes, osteoporosis and other age-related diseases.<sup>14</sup> In this process dicarbonyl compounds ingested seem to be implicated, since they are highly reactive for reactions with proteins, leading to the formation of peptide-bound amino acid derivatives in the final stages of the Maillard reaction.<sup>15</sup>

The aim of the present study was to investigate the effects of the consumption of MRPs from bread crust, as one of the major sources of AGEs in the diet,<sup>16</sup> on the composition and the

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physical and biomechanical properties of bone, and also to determine whether these possible effects are related to the molecular weight of different MRPs present in the bread crust.

## Materials and methods

### 1 Preparation of diets

The AIN-93G purified diet for laboratory rodents (Dyets Inc., Bethlehem, PA) was used as the control diet.<sup>17</sup> The bread crust (BC) was supplied by a Spanish manufacturer of cereal-derived food products. The process by which the BC was removed is described in a previous work by Roncero-Ramos *et al.*<sup>18</sup> Briefly, the BC was submitted to a pronase E digestion, obtaining soluble and insoluble fractions. The soluble part was subjected to ultrafiltration using a Biomax polyethersulphone membrane (0.5 m<sup>2</sup> size, 17.8 cm width × 21 cm length, Millipore, MA, USA) with 5 kDa NMWL. The fraction containing compounds with a molecular weight higher than 5 kDa was retained (retentate, high molecular weight, HMW) whilst the fraction containing compounds with a weight below 5 kDa was filtered (filtrate, low molecular weight, LMW). BC and all the fractions obtained were lyophilized, powdered, homogenized and used for the formulation of diets. BC was added to the AIN-93G diet to reach a final concentration of 10% (w/w). This diet was termed BC. Since MRPs absorption can be affected by the molecular weight,<sup>19</sup> the soluble LMW, soluble HMW and insoluble fractions were used to prepare other diets aimed to determine the possible formulation responsible for the observed effects. Fractions were individually added to the AIN-93G diet in the same proportion as they were present in the 10% of BC, being calculated from the recovery of each fraction after pronase E digestion and the ultrafiltration process. These diets were termed LMW, HMW and insoluble, respectively.

The individual analysis of the different diets revealed no modification of the overall nutrient composition, compared with the control diet (AIN-93G). The mean ± SD nutrient content of the diets was: moisture (%) 7.9 ± 0.4; protein (g kg<sup>-1</sup>) 168.4 ± 4.0; fat (g kg<sup>-1</sup>) 77.9 ± 1.6; Ca (g kg<sup>-1</sup>) 4.86 ± 0.05 and P (g kg<sup>-1</sup>) 3.22 ± 0.08.

The higher MRPs content in the diets prepared, with respect to the control diet, was established by analysing the furosine and hydroxymethylfurfural (HMF) contents as described in the study by Roncero-Ramos *et al.*<sup>18</sup>

### 2 Biological assays

Thirty weanling (21 day old) Wistar female rats weighing 40.15 ± 0.16 g (mean ± SE) were used in the study. They were randomly distributed into five groups (6 animals per group) and each group was assigned to one of the dietary treatments. The animals were individually housed in metabolic cages in an environmentally controlled room under standard conditions (temperature: 20–22 °C with a 12 h light–dark cycle and 55–70% humidity). The rats had *ad libitum* access to their diets and demineralised water (Milli-Q Ultrapure Water System, Millipore Corps., Bedford, MA, USA).

On day 88, after being starved overnight, the animals were anaesthetised with sodium pentobarbital (5 mg per 100 g of body weight) (Abbott Laboratories, Granada, Spain) and terminal exsanguination was performed by a cannulation of the carotid artery. The right femur, tibia and pelvic bone were removed, weighed and frozen at –80 °C until analysis.

All management and experimental procedures carried out in this study were in strict accordance with the current European regulations (86/609 E.E.C.) regarding laboratory animals. The Bioethics Committee for Animal Experimentation at our institution (EEZ-CSIC) approved the study protocol.

### 3 Analytical techniques

#### 3.1 Analysis of the physical parameters of the femurs.

Whole femurs were removed from the animals by breaking the joints that bound them to adjacent bones and preserving their epiphysis and diaphysis. Before composition and mechanical testing, the bone specimens were cleared of external soft-tissue adhered to the surface and then weighed. To determine their water content, avoiding the possible promotion of the Maillard reaction in the tissue by applying high temperatures, bones were also weighed after an overnight drying at room temperature.

The femur length was measured using an analog Vernier caliper and the density of each femur was determined in triplicate, using a water picnometer (25 cm<sup>3</sup>, Pobel, Madrid, Spain). Briefly, this value was calculated gravimetrically, as the ratio between the femur weight in air and in water. The femur volume was estimated by dividing the bone weight by its density.

**3.2 Bone densitometry determination.** Bone mineral density (BMD) and bone mineral content (BMC) were measured by dual-energy X-ray absorptiometry (DEXA). BMD and BMC measurements were performed with a bone densitometer Norland Stratec (Norland Corp., Fort Atkinson, WI, USA) using special software for small animals, providing data as a function of the area considered and yielding comparable data between animals. The BMD and BMC were measured in the entire right femur and pelvic bone.

#### 3.3 Determination of the main constituents of the femur.

The water content was determined as described above. The femurs were then dry-ashed in a muffle furnace (Selecta, Mod.366, Barcelona, Spain) at 450 °C after which the white ashes obtained were weighed; the weight of the organic phase was calculated as the difference between the weights of the ashes and the bones. The white ashes were dissolved with HCl–HNO<sub>3</sub>–H<sub>2</sub>O (1 : 1 : 2). The calcium analyses in all samples were carried out by flame atomic absorption spectroscopy (AAS) in a PerkinElmer Analyst 700 Spectrophotometer (Norwalk, Conn., USA). Standard solutions were prepared from a stock Tritisol solution of calcium (Cl<sub>2</sub>Ca in 6.5% HCl, 1000 mg Ca). Lanthanum chloride was added to the samples and standards to reach a final concentration of 0.3%. Phosphorus was determined colorimetrically at 820 nm in a spectrophotometer (Shimadzu UV-1700, Model TCC-240A, Columbia, USA) by the vanadomolybdate procedure (AOAC, 1990).

#### 3.4 Analysis of the biomechanical properties of the bones.

Tibias were cleaned in the same way as described for femurs

once removed from the animals and fat inside was not extracted for the tests. Mechanical properties of the whole tibia were determined using a three-point bending test. The bones were loaded perpendicularly to their long axis with a Warner–Bratzler device adapted to a universal testing machine (Model 4501, Instron Engineering Corp., Canton, MA, USA) connected to a Vectra ES/12 computer (Hewlett Packard Company, WA, USA). Load–displacement curves were derived applying a cell of 5 kN of total load capacity at a crosshead speed of 20 mm min<sup>-1</sup> to the bone diaphysis.

The following parameters were determined from the load–displacement curve: displacement to failure (mm) as the elongation to the failure load; the failure load (N) as the maximum load sustained by the specimen at breaking; the bending stiffness (N mm<sup>-1</sup>) as the slope of the linear portion of the curve; the energy to failure (mJ) as the area under the curve to failure load.

**3.5 Pentosidine content in tibia.** Pentosidine was determined following the method described by Takahashi *et al.*<sup>20</sup> for sample hydrolysis and the method of Scheijen *et al.*<sup>21</sup> for the chromatographic separation. Briefly, 150 mg of powdered rat tibia was hydrolysed with 3 mL 6.0 M HCl at 110 °C for 23 h in a Pyrex screw-cap vial with PTFE-faced septa. High-purity N<sub>2</sub> gas was bubbled through the solution for 2 min. A 500 µL portion of the hydrolysate was evaporated under vacuum and the dried sample dissolved in 200 µL of 25 mM citric acid. Finally, 50 µL of the sample was injected into a liquid chromatograph (Jasco LC Pump, Model PU-2089, Jasco Corporation, Madrid, Spain) coupled to a fluorescence detector (Jasco, Model FP-2020) and a computing integrator connected to a PC. Pentosidine was separated on a ODS-B 5 µm column (Tracer Excel 120 ODS-B 5 µm, 250 mm × 4.0 mm i.d., Tecknokroma, Barcelona, Spain) thermostatted at 32 °C. Solvent A was 25 mM citric acid and solvent B was (50/50, v/v) ACN–25 mM citric acid. A linear gradient was started at 99% solvent A which was changed after 15 minutes to 90% solvent A. After cleaning the column with 100% solvent B for 5 minutes the column was equilibrated for 8 min at the initial composition. The flow rate was 1 mL min<sup>-1</sup> and the fluorescence detector was set at λ<sub>ex</sub> = 335 nm and λ<sub>em</sub> = 385 nm. Duplicate samples were analysed. The external standard method was used for the quantification. A standard stock solution containing 245 pmol mL<sup>-1</sup> of pentosidine was used to prepare the working standard solution. The calibration was performed with a pentosidine standard curve (r<sup>2</sup> = 0.9994).

Collagen determination was performed by quantitation of the released 4-hydroxyproline in hydrolysates of samples using the technique described by Jamall *et al.*<sup>22</sup> Briefly, a 7 point curve

of standard hydroxyproline was constructed ranging from 0 to 1.6 µg of hydroxyproline in 1.2 mL of 50% isopropanol. 50 µL of a 1 : 80 hydrolysate sample dilution was added to each point. The final volume was adjusted to 1.2 mL with isopropanol : water (50 : 50) and then 200 µL of 0.58% chloramine T solution (in citrate buffer pH 6.0) was added. After 10 minutes, 1 mL of Ehrlich reagent was added and the sample was then incubated for 90 minutes at 50 °C in a water bath. Finally, the samples were cooled in water at room temperature for 15 minutes and their absorbances at 558 nm were read using water as a reference. The collagen content was calculated assuming that the collagen mass was 6.0 times the hydroxyproline mass, as determined from the composition of rat dentin collagen.<sup>23</sup>

#### 4 Statistical analysis

All data were statistically tested by one-way analysis of the variance (ANOVA), followed by Duncan's test to compare means that showed a significant variation (*P* < 0.05). Analyses were performed using Statgraphics Plus, version 5.1, 2001. Evaluation of the relationship between the different variables was carried out by computing the relevant correlation coefficient (Pearson's linear correlation), whereas the multiple relationships among variables were evaluated by a multiple regression test (Durbin–Watson's correlation), both at the *P* < 0.05 confidence level. All data were also analysed by one-way analysis of the variance (ANOVA) considering the final body weights of animals as covariate. When differences among the treatments were significant (*P* < 0.05) as determined by the *F* test in the ANOVA, means were separated using the Duncan's test.

#### Results

Physical properties of the femurs are shown in Table 1. The femur weight was significantly less in all animals fed the BC diet and its fractions with respect to the control diet. Bone density also fell in all animals fed the experimental diets, although only slightly within the BC group. The femur length remained unchanged in all groups, but the volume decreased significantly.

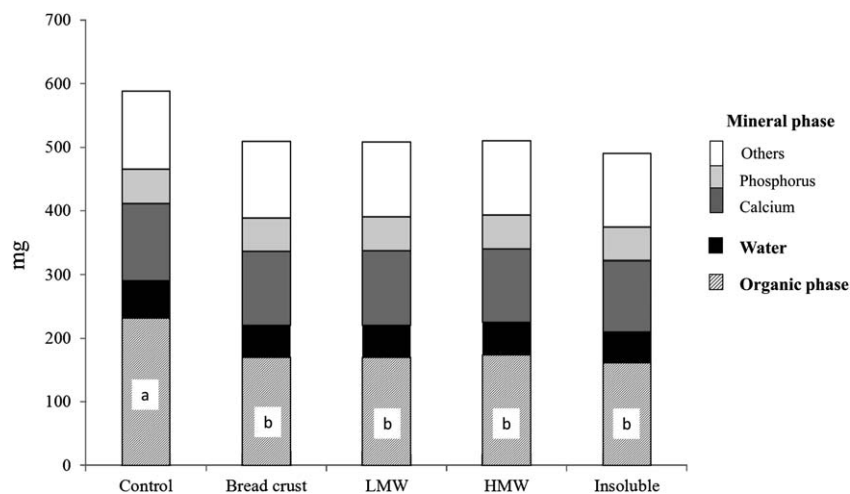
Regarding bone composition (Fig. 1), the mineral phase content in the femurs did not vary and no significant differences were detected in calcium and phosphorus contents. Nevertheless, the organic phase was significantly lower in the animals fed diets containing BC derivatives (Fig. 1).

The femur BMD tended to decrease in all experimental groups, compared with the control one, but the difference was

**Table 1** Physical parameters of femurs after feeding rats the different diets<sup>a</sup>

Group	Weight (g)	Length (cm)	Density (g cm <sup>-3</sup> )	Volume (cm <sup>3</sup> )
Control	0.59 ± 0.05 <sup>A</sup>	3.39 ± 0.05	1.49 ± 0.03 <sup>A</sup>	0.44 ± 0.02 <sup>A</sup>
BC	0.51 ± 0.02 <sup>B</sup>	3.26 ± 0.08	1.43 ± 0.04 <sup>AB</sup>	0.38 ± 0.01 <sup>B</sup>
LMW	0.51 ± 0.02 <sup>B</sup>	3.30 ± 0.04	1.36 ± 0.03 <sup>B</sup>	0.39 ± 0.02 <sup>B</sup>
HMW	0.51 ± 0.02 <sup>B</sup>	3.31 ± 0.04	1.37 ± 0.04 <sup>B</sup>	0.39 ± 0.01 <sup>B</sup>
Insoluble	0.49 ± 0.01 <sup>B</sup>	3.28 ± 0.04	1.38 ± 0.02 <sup>B</sup>	0.37 ± 0.01 <sup>B</sup>

<sup>a</sup> Values are means ± SE, *n* = 6. Different letters within a column indicate significant differences between groups (*P* < 0.05).



**Fig. 1** Major constituents of femur in rats fed the different diets (mean  $\pm$  SE,  $n = 6$ ). Different letters within a parameter indicate significant differences between groups ( $P < 0.05$ ).

only significant in the insoluble group, with a reduction of 7.3% (Fig. 2). The BMC of this bone did not experience any significant change, and neither did the BMD and BMC in the pelvic bone (Fig. 2).

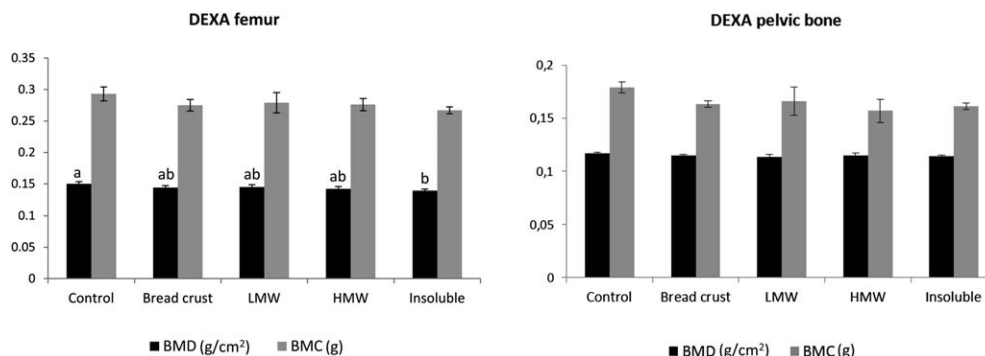
Data corresponding to mechanical properties of the tibia are shown in Table 2. No modification was observed in bone displacement to failure, but there was a general reduction in bone diameter after consumption of BC and its derivatives. Bone bending stiffness significantly decreased, by almost 50% in the tibias from the LMW, HMW and insoluble groups compared with the control and the BC groups. There were no changes in the failure load or the energy to failure in any of the experimental groups, but there was a marked decline in the latter two parameters in the BC group.

The pentosidine concentration in the tibia increased significantly in all the animals fed the experimental diets (Fig. 3), with respect to the control group. The highest values were observed in the HMW and insoluble groups (104.7 and 102.9  $\text{mmol mol}^{-1}$  collagen, respectively vs. 41.7  $\text{mmol mol}^{-1}$  collagen in the control animals).

## Discussion

### 1 Composition and physical properties

During growth, a maturation process takes place in the bones, involving changes in the matrix composition and in the physical and chemical characteristics of the bone mineral.<sup>24</sup> Burnell *et al.*<sup>24</sup> described an initially rapid increase in the bone matrix (organic phase) in a normal maturation process, during which the mineral phase follows a progressive but diminishing increase. The most intense period of bone maturation in rats takes place between 4 and 22 weeks, when the mineral density increases slowly up to 22 weeks, whilst at the same time there is a faster organic growth phase that slows down from 8 weeks. Since animals of the present study took part in other metabolic assays,<sup>18,25</sup> they began the trial at weaning (weighing  $40.15 \pm 0.16$  g; mean  $\pm$  SE). At the end they reached the age of 15 weeks and so these normal physiological changes should be taken into account when interpreting the data. In previous works we have already reported that global food intake of our experimental animals was lower than in the control ones, as were body

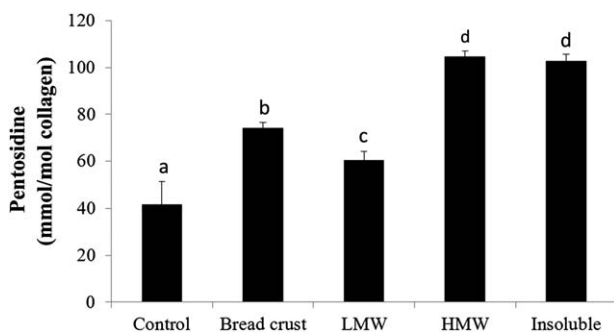


**Fig. 2** Bone mineral density (BMD) and bone mineral content (BMC) measured by dual-energy X-ray absorptiometry (DEXA) in femur and pelvic bones (mean  $\pm$  SE,  $n = 6$ ). Different letters within a parameter indicate significant differences between groups ( $P < 0.05$ ).

**Table 2** Mechanical properties of the tibia determined by a three-point bending test<sup>a</sup>

	Control	BC	LMW	HMW	Insoluble
Displacement to failure (mm)	0.94 ± 0.16	0.87 ± 0.09	0.87 ± 0.10	1.10 ± 0.13	0.98 ± 0.03
Diameter (mm)	2.68 ± 0.27 <sup>A</sup>	2.27 ± 0.10 <sup>B</sup>	2.30 ± 0.08 <sup>AB</sup>	2.15 ± 0.08 <sup>B</sup>	2.18 ± 0.06 <sup>B</sup>
Bending stiffness (N mm <sup>-1</sup> )	218.87 ± 21.62 <sup>A</sup>	238.73 ± 18.88 <sup>A</sup>	152.23 ± 18.50 <sup>B</sup>	139.90 ± 18.29 <sup>B</sup>	109.25 ± 10.50 <sup>B</sup>
Failure load (N)	114.95 ± 21.11	102.97 ± 6.38	90.77 ± 10.01	82.68 ± 3.60	85.23 ± 11.57
Energy to failure (mJ)	38.77 ± 12.77	21.95 ± 1.99	26.25 ± 4.22	26.05 ± 2.85	26.74 ± 3.13

<sup>a</sup> Values are means ± SE, *n* = 6. Different letters within a row indicate significant differences between groups (*P* < 0.05).



**Fig. 3** Pentosidine content in tibia of rats fed the different diets (mean ± SE, *n* = 6). Different letters indicate significant differences between groups (*P* < 0.05).

weights (247.6 ± 5.1; 235.0 ± 4.4; 236.3 ± 7.2; 227.5 ± 5.8 and 220.7 ± 4.0 g for control, BC, LMW, HMW and insoluble groups respectively),<sup>18</sup> leading to smaller bones, probably in the earlier stages of growth.

The physical characteristics of the femurs of the control rats were normal in terms of size, density and weight<sup>26–28</sup> (Table 1), whereas the femurs of the experimental rats were significantly smaller in weight and volume, but similar in length. A significant correlation between the femur weight and the final body weight of the animals ( $r = 0.650$ ;  $P < 0.001$ ) was found<sup>18</sup> which is in line with the observations of Fukuda and Iida<sup>29</sup> who reported that the growth period is the only stage during which body weight and BMD are closely correlated. Bone density significantly decreased in all groups fed diets containing MRPs, although the BC group showed only a tendency in this direction. Since total bone density includes both organic and mineral mass,<sup>30</sup> and the latter did not vary (Fig. 1), the significant decrease of the organic matrix, which correlated with the femur density ( $r = 0.490$ ;  $P = 0.007$ ), was responsible for the bone mass loss. In the study by Burnell *et al.*<sup>24</sup> performed on rats with lower food intakes and consequently lower body weight gains after 12 weeks (around 30%), a decrease in femur densities was also observed. Those bones contained lower amounts of mineral and hydroxyproline, but with a more pronounced reduction in hydroxyproline. In our assay, the decline in the bone organic matrix could have been caused by a delay in the bone growth related to the lower body weight. However this circumstance was not the only responsible factor but also MRPs intake through the diets, since a parallel statistical treatment considering final animal weights as covariate demonstrated that the bone organic matrix of animals fed MRPs diets was always

significantly lower than those fed the control diet ( $P = 0.004$ ). In this sense, it has been established that dicarbonyl compounds ingested coming from the Maillard reaction could be implicated in the formation of peptide-bound amino acid derivatives.<sup>16</sup> Thus, non-enzymatic glycation of bone collagen could be facilitated due to the existence of these highly reactive species, since the presence of mineral in bone does not prevent collagen glycation.<sup>8</sup>

The non-enzymatic cross-links which contribute to deteriorating biological and mechanical bone properties<sup>4</sup> increase with aging<sup>31</sup> and are exacerbated in diabetes-related pathologies.<sup>32</sup>

Pentosidine is one of the most commonly studied AGEs, due to its participation in non-enzymatic cross-links. Values of pentosidine found in the literature vary widely both intra- and inter-species. In this respect, very few studies have been performed with rats, and results are substantially different, ranging from 0.1–1 mmol mol<sup>-1</sup> collagen for Wistar rats<sup>10</sup> to 40–92 mmol mol<sup>-1</sup> collagen for F344 rats,<sup>33</sup> a similar interval to our results (Fig. 3). Pentosidine significantly increased in all groups compared to the control one. The consumption of BC and especially of isolated HMW and insoluble fractions increased pentosidine concentrations (104.7 and 102.9 mmol mol<sup>-1</sup> collagen, respectively), reaching the values reported by Silva *et al.*<sup>33</sup> for diabetic rats. Therefore, our data reveal that more advanced and higher molecular weight compounds could be the main factors responsible for the effect, although the LMW fraction also exerted a residual influence. It must be underlined that the effects do not seem to be additive, but rather are modulated when the compounds are consumed as a real food, BC, subjected to a normal digestive process. The covariate analysis establishing final body weight as a correction factor demonstrated that pentosidine increased as a consequence of MRPs intake, independently of the growth rate reached ( $P < 0.001$ ).

Although according to Kankova and Sebekova<sup>34</sup> ingested AGEs are absorbed in the small intestine and contribute to increasing endogenously formed AGEs, to date no studies have established the accumulation of dietary AGEs in the tissues, especially in bone. To the best of our knowledge, only the assay by Mikulikova *et al.*<sup>32</sup> using a diet with a high content of fructose, expected to increase plasma AGEs, has demonstrated a dietary-provoked increase in pentosidine levels in collagen-rich tissues.

The BMD values measured in our assay animals were in line with those reported in other studies for this stage of growth.<sup>35</sup> Consistent with the lack of changes in ash, calcium and

phosphorus contents, BMC in the femur and pelvic bone did not vary significantly (Fig. 2). BMD in the pelvic bone was also unchanged, but BMD in the femur did tend to decrease slightly, although it was only significant in the rats fed the insoluble diets, and these animals were also those which had the lowest food intake and body weight.<sup>18</sup>

The scientific literature relating BMD data measured by DEXA and AGEs accumulation is not available. In an earlier study by our research group with rats fed MRPs from a glucose-lysine model system, the pentosidine content was much higher than in the rats in the present assay. No change was found in BMC of the pelvic bone and femur, whereas a slight decrease was observed in BMD in the pelvic bone.<sup>36</sup> In assays performed in diabetic rats with the same age, BMD measured by pQCT decreased in several long bones, affecting trabecular rather than cortical bones.<sup>26,33</sup>

## 2 Biomechanical properties

In the load-displacement curve, no differences were observed among the displacement to failure, in the groups assayed (Table 2). Similarly, *in vitro* glycation assays on rat bones did not reveal changes in the displacement of tibia or femur using the same kind of test.<sup>37</sup> The diabetic pathology is a disorder whose progression and development is tightly linked with the *in vivo* levels of AGEs due to their involvement in the biochemical pathways related to this illness. Experiments with diabetic rats have shown no significant modifications in the displacement of tibia or femur, even at advanced stages of the disease.<sup>26</sup> Thus, although AGEs accumulation in bone has been related to the loss of elasticity, this was not observed in our assay.

Bone bending stiffness significantly decreased in animals fed LMW, HMW and insoluble diets with respect to control and BC diets. Stiffness mainly depends on bone mineral, which was unchanged in the present assay. Stiffness was positively correlated with density ( $r = 0.558$ ,  $P = 0.002$ ) and the amount of organic matrix ( $r = 0.424$ ,  $P = 0.022$ ), and negatively with the pentosidine content ( $r = -0.520$ ,  $P = 0.004$ ). This result appears to conflict with other findings describing increased stiffness due to AGE accumulation in bones.<sup>8,38</sup> The *in vitro* assays by Vashishth *et al.*<sup>8</sup> working with demineralised bones subjected to glycation by ribose incubation, demonstrated that this treatment enhances stiffness, although the effects were slighter with mineralised specimens. In spite of the higher pentosidine values found in our experiment, bone bending stiffness decreased, and so there should be other counteracted factors conditioning this effect. In fact, the multiple regression analysis (Durbin-Watson's correlation) performed indicated that 92% of the changes in bone stiffness were accounted for by the factors of density and pentosidine content, in accordance with the following equation:

$$[\text{stiffness (N mm}^{-1}\text{)} = -1.07\text{pentosidine (mmol mol}^{-1}\text{ collagen)} \\ + 179.7\text{density (g cm}^{-3}\text{)}]$$

in which bone density is the main factor responsible, and the participation of pentosidine is inversely effective, and to a minor degree. Prisby *et al.*<sup>26</sup> reported that advancing diabetes,

which is associated with high AGEs levels in serum,<sup>34</sup> leads to smaller bones, with lower weights and less stiffness, which is in line with our results for the LMW, HMW and insoluble diets, but not the BC group. According to the above-mentioned authors, the growth delay and the reduction in the cross-sectional dimensions of the large bones could be responsible for this outcome. This conclusion is consistent with our results, since the tibia diameters of the animals fed BC and its fractions were smaller than those of the control animals. Not only was the delayed growth the cause of the observed effect, but also the MRPs consumption, as established by the statistical covariate analysis. When the final animal weights were used as a correction factor, the stiffness data showed the same statistical significance as described in Table 2 ( $P < 0.001$ ).

Several studies have shown that bone strength is determined by bone mass and that its stiffness is mainly dependent on the mineral content.<sup>1</sup> In our work, in the tibias of animals fed BC derived fractions, the failure load tended to decrease, especially in groups consuming the higher molecular weight compounds (HMW and insoluble groups). This decline, although without statistical significance, was more than 25%, which is in agreement with the reduction rates described by Silva *et al.*<sup>33</sup> in diabetic rats. Non-enzymatic cross-links introduce structural changes which are a determinant of bone strength. Moreover, their presence may reduce strength by modifying osteoblastic and osteoclastic activity,<sup>39</sup> an effect that has been shown after *in vitro* treatment with sugars<sup>40</sup> or during aging.<sup>41</sup> Thus, rats fed the isolated fractions from BC had higher deoxyypyridinoline values in urine,<sup>18</sup> suggesting a situation of increased bone resorption or greater turnover. Only the study by Garnero *et al.*<sup>40</sup> has reported that a small accumulation of AGEs, measured as pentosidine, has a positive effect on bone strength.

Bones in the control rats tolerated higher energies to failure, although the difference was not statistically significant. This parameter correlated positively with the organic matrix ( $r = 0.549$ ,  $P = 0.002$ ) and it is known that the organic phase is an important contributor of bone resistance<sup>42</sup> and that bone resistance decreases with collagen deterioration.<sup>43</sup> As mentioned above, AGEs are inversely correlated with bone resistance<sup>38</sup> and products such as pentosidine would partially explain this fact, since their accumulation reduces the post-yield energy dissipation of bone tissue prior to fracture.<sup>44</sup> Nevertheless, and consistent with our own data, diabetic disease has usually not been found to produce severe effects on the energy at the point of failure.<sup>33</sup> In the cases where some effects have been detected, the changes take place after five<sup>26</sup> or eight<sup>10</sup> months of induced diabetes.

In general, consumption of the assayed diets, and especially in the case of the isolate fractions, led to smaller animals and bones, which modified certain mechanical properties, producing less stiff bones with a lower ability to withstand force and absorb energy to failure. Thus, although the covariate analysis showed a direct effect of the consumption of MRPs derived from BC on bone stiffness, differences in bone size and maturation appeared to account for almost all changes observed in the mechanical properties.

To sum up, our data suggest that the consumption of diets containing bread-derived products, especially those containing its isolate fractions, leads to a reduction in food intake, which seems to delay bone development at a crucial stage of growth, affecting bone characteristics.

Despite our results not being completely conclusive, we guess there is a more direct effect of the consumption of bread-derived MRPs on bone, especially related to the presence of the higher molecular weight compounds. However the manifestations were weaker when the compounds are consumed as a whole food, BC.

Although further study in this field is needed, our study suggests that dietary AGEs may influence bone health in healthy individuals, hence the importance of diet in preventing degenerative diseases.

## Conflicts of interests

The authors declare no conflict of interest.

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