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In vivo effects of Maillard reaction products derived from biscuits

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ABSTRACT

The antioxidant activity, antihypertensive effect and prebiotic activity of Maillard reaction products (MRPs) derived from biscuits were investigated in Wistar rats. Animals were fed the following diets for 6 weeks: control (AIN-93 diet); Asc-diet (AIN-93 diet with ascorbic acid in the drinking water); HT-B diet (containing high amount of MRP derived from biscuits) and LT-B diet (containing negligible amounts of biscuit MRP). Serum antioxidant activity (FRAP, ABTS), as well as lipid peroxidation (TBARS) were determined at the end of the experiment. Results showed that dietary MRP reduced the food efficiency, increased the antioxidant activity of serum, increased the ratio between lactic and total aerobic bacteria, increased water-holding capacity of faeces and reduced blood pressure, but did not reduce mineral absorption. Therefore, the biscuit MRP functional claims could be demonstrated by an *in vivo* study.

1. Introduction

Nowadays there is an increasing demand for healthy products in the food industry. The antioxidant micronutrients are involved in many metabolic processes and block the effects of reactive oxygen species that can lead to the destruction of cells and DNA damage (Langner & Rzeski, 2014). Although different antioxidants are currently used in the food industry, the consumer sometimes questions their safety (Rufián-Henares & Morales, 2007a). Therefore, the use of natural antioxidants, such as α-tocopherol, β-carotene or ascorbic acid is of great concern. In addition, the possible benefits of antioxidants naturally formed during cooking procedure, like the Maillard reaction products (MRPs), are of particular interest.

The Maillard reaction (MR) is a non-enzymatic browning reaction that occurs between the amino groups of amino acids and the carbonyl groups, mainly of the sugars. The MRP is a heterogeneous group that includes low molecular weight compounds, which contribute to the product flavour, as well as polymeric products, which are linked to the colour and texture of the foodstuffs (Wang, Qian, & Yao, 2011). In particular, melanoidins, formed at the final stages of the MR, have attracted much attention in recent years. Numerous investigations have reported the possible antioxidant, antihypertensive, prebiotic and antimicrobial activity that these compounds exhibit in model systems (Borrelli & Fogliano, 2005; Kitrytė, Adams, Venskutonis, & De Kimpe, 2012; Rufián-Henares & Morales, 2007a). Furthermore, some works have evaluated these effects in real systems like bakery products and coffee with excellent results: Martín et al. (2009) described the ability of biscuit melanoidins to protect human Hep G2 cells against free radical damage; the results obtained by Monente et al. (2015) strongly suggested that a high content of coffee melanoidins could inhibit the growth of Gram-negative bacteria by metal-chelating mechanisms. Moreover, Lindenmeier, Faist, and Hofmann (2002) explored the modulating activity of bread crust melanoidins in intestinal Caco-2 cells. However, the effect of these compounds derived from real systems using *in vivo* assays has been barely studied.

The two major sources of dietary melanoidins are coffee and bakery products. Recent studies by Pastoriza and Rufián-Henares (2014) showed that coffee contributed most to the antioxidant activity exerted by melanoidins followed by biscuits, while bread only showed a slight contribution. What is more, biscuits consumption has grown steadily; the annual consumption *per capita* ranges from 2.5 kg in Asian countries to 7.5 kg in the USA (Filipčev, Šimurina, & Bodroža-Solarov, 2014). In spite of this, the impact on health of a diet with high amount of biscuit melanoidins remains unstudied and requires clarification.

The aim of the present work was to investigate the *in vivo* effects of MRP on rats. In order to simulate the consumption conditions, the MRP were not isolated from the biscuits and were administered as biscuits. The antioxidant activity, prebiotic effect, antihypertensive capacity, as well as some other biomarkers, were determined in different groups of animals in order to describe the effects of MRP derived from biscuits. One rat group was fed with a

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diet partially replaced by biscuits with a high amount of MRP. To achieve accurate results, three control groups were used: a group fed only with a control diet (negative control); a second group in which ascorbic acid was added in the drinking water (positive control), and a third group fed with the control diet partially replaced by biscuits with a negligible amount of MRP. This last group was used as a "negative diet control", to rule out any possible effect linked to the biscuit ingredients.

Since the *in vivo* effects of the biscuit melanoidins were investigated, this study provides valuable information about the health benefits of MRP.

2. Materials and methods

2.1. Materials

The biscuit ingredients were: wheat flour (Favorita 000; Molinos Río de la Plata, Buenos Aires, Argentina), corn starch (Maizena, Unilever de Argentina S.A., Buenos Aires, Argentina), skim milk powder (SanCor Sunchales, Santa Fe, Argentina), high oleic sunflower oil (Propia; Lezama, Buenos Aires, Argentina), sucrose (Ledesma, Jujuy, Argentina) and baking powder (Royal, Kraft Foods, Buenos Aires, Argentina).

2.2. Biscuit preparation

Biscuits used in the experimental diets were prepared in the laboratory as described by Patrignani, Conforti, and Lupano (2014) with slight modifications. Briefly, 20 g of the high oleic sunflower oil were mixed with wheat flour (35 g), corn starch (25 g), skim milk powder (20 g), sucrose (12.5 g) and baking powder (0.9 g) in a kneading Philips Cucina mixer (Sao Paulo, Brazil) for 60 s at low speed. Then 25 ml of tap water were added in two consecutive steps, mixing each time at high speed. Finally everything was mixed for 1 min at low speed to obtain a clay-like dough. Rectangles of dough (3.3 × 5.2 × 0.3 cm) were cut and baked in an oven (White Westinghouse, W-CG18). Biscuits were processed under two different conditions: 150 °C for 25 min (high temperature biscuits) or 100 °C for 80 min (low temperature biscuits) to reach final moisture contents lower than 10%.

Biscuits were milled (Philips Cucina mixer, HR 7633, Sao Paulo, Brazil) and the following components were determined: protein by Kjeldahl method (AACC 46-11), total lipids by Soxhlet (AACC 30-25); total dietary fibre (AACC 32-07.01); moisture content (AACC 44-01); ash (AACC 08-03) and carbohydrates by difference. Diets were ashed in a furnace at 550 °C, dissolved in HNO_3 and the obtained solutions were used to determine Zn and Ca concentration by atomic absorption spectroscopy and Na by atomic emission spectroscopy (Perkin Elmer AAnalyst 400) using standard solutions.

Biscuit final composition was 77.8 g of carbohydrates (14.5% of lactose from milk; 67.6% of starch from flour, baking powder and corn starch and 18.1% of sucrose), 11.5 g of proteins (70% of proteins from milk and 30% from flour), 8.2 g of lipids (from high oleic sunflower oil), 2.3 g of dietary fibre, 1.72 mg of Zn, 351.7 mg of Ca and 213.4 mg of Na per 100 g of biscuit (dry basis). The moisture content of the final products was lower than 10%.

2.3. Experimental diets

Three different experimental diets were prepared according to the American Institute of Nutrition Rodent Diets Recommendations (Reeves, Nielsen, & Fahey, 1993):

- Control: AIN-93 diet.
- HT-B diet: AIN-93 diet replaced by high temperature biscuit (39.1 g of dried weight biscuit per 100 g of diet).
- LT-B diet: AIN-93 diet replaced by low temperature biscuits (39.1 of dried weight biscuit per 100 g of diet).

The composition of each diet is detailed in Table 1. All the diets were accurately prepared and supplied the same amounts of proteins, minerals, fibre, lipids, and energy (372 ± 8 kcal/100 g). Dextrin was added as a carbohydrate source to achieve 1000 g of diet.

2.4. Diet analysis

2.4.1. Sample extraction

Diets (0.2 g) were extracted in 1.5 mL of warm deionised water (45 °C). Then, the tubes were shaken vigorously for 10 min (25 g) and left for 30 min at 4 °C. After that time, the mixtures were centrifuged 10 min at 10,000*g* (5415 R; Eppendorf, Hamburg, Germany). The supernatants were collected, filtered (0.45 μm pore size) and stored at −20 °C until analysis (Morales, Martin, Açar, Arribas-Lorenzo, & Gökmen, 2009). The aqueous solutions were used for determination of the browning intensity and antioxidant activity.

2.4.2. Measurement of the browning intensity

The amount of Maillard reaction products was measured by absorbance at 420 nm (Monente et al., 2015). Appropriate dilutions (1/4) of the aqueous extracts of diets were made using distilled water, and the absorbance was measured at 420 nm using a UV-mini 1240 spectrophotometer (Shimadzu, Kyoto, Japan).

2.4.3. Antioxidant activity of diets

The antioxidant activity was measured by the FRAP assay according to the method of Benzie and Strain (1996). Briefly, 1.8 mL of freshly prepared FRAP reagent were mixed with 50 μL of the diet aqueous solution and 150 μL of distilled water. After 4 min, the sample absorbance was measured at 593 nm. A Trolox solution was used as standard. Results were expressed as μg of Trolox/mg of dried sample.

The DPPH assay was used in order to measure the free radical scavenging of 50 μL of the diet aqueous solution (1 mL final volume), as described by Brand-Williams, Cuvelier, and Berset (1995). Results were expressed as μg Trolox/mg of dried sample.

All determinations were performed in triplicate for each sample.

Table 1
Composition, A_{420} and antioxidant activity (FRAP and DPPH) of control, HT-B and LT-B diets.

Composition (g/kg of diet)*	Diet		
	Control	HT-B	LT-B
Casein (85% protein)	140	87	87
Mineral mix (AIN-93M-MX)	35	35**	35**
Vitamin mix (AIN-93-VX)	10	10	10
L-Cysteine	1.8	1.8	1.8
Soybean oil	40	7.8	7.8
Choline bitartrate (ml)	7.1	7.1	7.1
Cellulose	50	40.9	40.9
Dried biscuit (g)	–	391	391
Diet parameters			
A_{420}	0.02 ± 0.00^{c}	0.13 ± 0.00^{a}	0.03 ± 0.00^{b}
FRAP (μg Trolox/mg dried sample)	0.05 ± 0.01^{b}	0.62 ± 0.04^{a}	0.11 ± 0.05^{b}
DPPH (μg Trolox/mg dried sample)	0.20 ± 0.07^{b}	0.77 ± 0.02^{a}	0.29 ± 0.03^{b}

Results expressed as means ± SD. Values in the same row with different superscript letter are significantly different ($p \leqslant 0.05$).

* Dextrin was added as carbohydrate source to achieve 1000 g of diet.

** Composition according to AIN-93 with the necessary adjustments to ensure an adequate mount of Zn, Ca and Na.

2.5. Animals, maintenance, and experimental design

All the experiments described in the present work were conducted in accordance with the Laboratory European guidelines for the care and use of laboratory animals (2010-63), and it was approved by the Committee for Animal Care of the Medical School of the Universidad Nacional de La Plata (Protocol No. T04-01-2014). Moreover, the number of animals used was reduced to the minimum necessary to fulfil the scientific objectives of the study.

Twenty-four male Wistar rats (8 weeks old; 250–300 g body weight) were obtained from the Experimental Animal Centre of Centro Atómico Ezeiza (Buenos Aires, Argentina). Rats were housed in stainless steel cages and acclimatised for a week in laboratory conditions (23 ± 5 °C and 12 h light/dark cycles). After that period, rats were weighed and divided into four homogeneous groups of 6 rats each: control (fed with control diet); Asc (fed with control diet); HT-B (fed with HT-B diet) and LT-B (fed with LT-B diet). The animals in the Asc group were administered 300 mg/kg/day of ascorbic acid as antioxidant in their drinking water (Sönmez, Türk, & Yüce, 2005). All the groups had *ad libitum* access to food and drink.

The food intake and the body weight were monitored weekly during the 6 weeks of the assay. The food efficiency was calculated each week as follows (Delgado-Andrade et al., 2013):

$$\text{food efficiency} = \text{weight gained } (g)/\text{food intake (g dry matter)}$$

At the end of the experimental period, rats were anaesthetised with diazepam (0.05 mg/kg of body weight) and pentobarbital (25 mg/kg of body weight). Then, animals were sacrificed by exsanguination from the abdominal aorta. Blood samples were collected into tubes and serum was separated by low speed centrifugation (1000*g*, 10 min). The samples were frozen by liquid nitrogen and kept at −80 °C until analysis.

2.6. Thiobarbituric acid reactive substances (TBARS) assay

The TBA determination was performed by the procedure optimised by Estepa, Ródenas, and Martín (2009) with slight volume corrections. Each determination was performed in triplicate for each sample and 1,1,3,3-tetramethoxypropane was used as standard. The results were expressed as nmol MDA/mL of serum.

2.7. Antioxidant potential in serum

In order to evaluate the serum total antioxidant activity, two different assays were performed: $ABTS^{\cdot+}$ radical cation (ABTS) and ferric reducing antioxidant power (FRAP) (Jimenez-Escrig, Dragsted, Daneshvar, Pulido, & Saura-Calixto, 2003).

The reducing power of the electron-donating antioxidants present in serum samples was determined by the FRAP assay as previously described. Results were expressed as μg Trolox/μL of serum.

The radical scavenging activity of samples was determined as described by Re et al. (1999) (ABTS assay). As recommended by these authors, ABTS solution was diluted with phosphate buffered saline (PBS) to an absorbance of 0.70 (±0.02) at 734 nm. Determinations were performed at least in duplicate for each sample. External calibration was performed with a Trolox standard solution, and results were expressed as μg Trolox/μL of serum.

2.8. Faecal microbiota

At the beginning and at the end of the experiment (weeks 1 and 5) freshly voided faeces were homogenised in peptone water (0.1 g/L). Then, serial decimal dilutions were carried out in the same medium. Aliquots of 0.1 mL of the appropriate dilutions were spread onto MRS agar media. MRS agar is a medium for the enumeration of mesophilic lactic acid bacteria and has been used for the detection of these bacteria from faeces (Jiménez-Escrig, Gómez-Ordóñez, Tenorio, & Rupérez, 2013). The plates were anaerobically incubated at 37 °C for 48 h. Similarly, for total aerobes count, 0.1 mL of the adequate dilution were spread onto plate count agar (PCA) and was aerobically incubated at 37 °C for 48 h.

After the incubation time, the colony count was performed in the Petri dishes with a growth range of 20–200 colony forming units (CFU). Results were expressed as log CFU per gram of faecal dry weight. Each determination was performed in duplicate in two different pools of rat faeces.

Besides the ability to growth on the selective media, the presence of lactic acid bacteria from the colonies isolated in MRS medium was confirmed by their phenotypic characteristics (catalase-negative and gram-positive) (Albesharat, Ehrmann, Korakli, Yazaji, & Vogel, 2011).

2.9. Apparent mineral absorption

During the third week of the experience faeces samples of each rat were collected. Faeces samples and diets were ashed in a furnace at 550 °C. The ashes were weighed and the apparent mineral absorption (AMA) was calculated as follows (Jiménez-Escrig et al., 2013):

$$\text{AMA } (\%) = [(\text{mineral intake} - \text{mineral faecal excretion})/\text{mineral intake}] * 100$$

Then ashes were dissolved in HNO_3 and the obtained solutions were used to determine Zn concentration by atomic absorption spectroscopy using standard solutions (Shimadzu AA-6650 spectrophotometer). The apparent Zn absorption (AZnA) was calculated according to the following equation:

$$\text{AZnA } (\%) = [(\text{Zn intake} - \text{Zn faecal excretion})/\text{Zn intake}] * 100$$

2.10. Blood pressure measurements

The systolic blood pressure (SBP) was measured indirectly (weeks 1, 2, 4 and 6) by the tail cut off method described by Fritz and Rinaldi (2008). The values of SBP were obtained by averaging at least three successful measurements without disturbance of the signal.

2.11. Statistical analysis

The results were expressed as means ± standard deviation. Data were statistically tested by analysis of variance (ANOVA), at the 0.05 significance level. The least significant differences (LSD) were calculated by comparing the means at a level of 95% ($p \leqslant 0.05$) using the Fisher test (InfoStat, 2012; Córdoba, Universidad Nacional de Córdoba, Argentina). When required, Pearson's correlation coefficients were calculated between the variables to evaluate the strength of the relations between them (Delgado-Andrade et al., 2013).

3. Results and discussion

3.1. Diet analysis

According to different authors MRP increase with increased reaction temperatures and time (Morales et al., 2009; Vhangani & Van Wyk, 2013). The objective of the present work was to evaluate the effect of the MRP in a real system; therefore three different diets were prepared: a control diet and two diets with the

addition of biscuits prepared with the same formulation but processed under different conditions (high temperature and low temperature). In order to ensure that the HT-B diet presented the highest amount of MRP different assays were performed.

The production of high molecular weight products (melanoidins) during the MR can be followed by an increase in the absorbance at 420 nm. Therefore, in the present study, A_{420} was used as a non-specific index to assess the extent of the MR (Vhangani & Van Wyk, 2013). As detailed in Table 1, A_{420} of the HT-B diet was four times higher than that of the others ($p \leqslant 0.05$).

It is generally accepted that melanoidins have antioxidant activity. In the present work significant differences were found in the antioxidant activity of the diets (Table 1) ($p \leqslant 0.05$). The HT-B diet showed the highest antioxidant activity, while no significant differences were found between the control and the LT-B diet.

According to these results it could be concluded that HT-B diet presented a high amount of MRP, while the LT-B and control diets showed only negligible amounts of these compounds.

3.2. Rat weight, food intake and food efficiency

As can be seen in Fig. 1, the different animal groups reached their final weight during the last 2 weeks of assay ($p > 0.05$). Significant differences were found in the weight increase of the rats during the experiment: the HT-B group showed the least weight increase in comparison to the other groups ($p \leqslant 0.05$). At the end of the experiments rats fed with the HT-B diet showed a body weight (383.1 ± 14.1 g) significantly lower than the other groups (407.6 ± 19.0 g; 410.6 ± 12.7 g and 427.4 ± 17.3 g for control, Asc and LT-B groups, respectively). Delgado-Andrade et al. (2013) studied the effect on the weight of rats of diets supplemented with MRP from bread crust. They found that rats fed with the high molecular weight (HMW) bread crust fraction displayed lower body weight than rats fed with the control diet. According to these authors, this difference could be attributed to the lower diet consumption of the HMW diet. However, results in the present work showed that, even when rats fed with control diet presented a higher food intake than rats fed with HT-B and LT-B diets (data not shown, $p \leqslant 0.05$), no good correlation was obtained between the diet consumption and the weight increase ($r = 0.14$).

On the other hand, a good correlation was found between food efficiency and the weight increase ($r = 0.98$). As can be seen from Fig. 1, HT-B group showed the least food efficiency ($p \leqslant 0.05$), while no significant differences were found amongst the other groups ($p > 0.05$). During heating, the quality of the proteins could decrease because of the condensation of the ε-amino of lysine with the reducing carbohydrates. This is the beginning of the Maillard reaction, which leads to the formation of a physiologically unavailable product. With a more pronounced heat treatment, protein cross linking makes them less soluble and less susceptible to the digestive enzymes; therefore the protein digestion process is reduced (Oste & Sjödin, 1984). Then, it could be inferred that the high temperature used in the HT-B reduced the food efficiency by lowering the protein digestibility and by rendering the lysine residues unavailable, leading to a significant reduction in the body weight of rats.

3.3. Antioxidant activity and lipid peroxidation

Ascorbic acid is a well-known water soluble antioxidant, and its capacity in preventing oxidative damage *in vivo* is highly recognised (Sönmez et al., 2005). Furthermore, over the past years, the possible benefits of naturally generated antioxidants like MRP have grown in popularity (Vhangani & Van Wyk, 2013). Morales et al. (2009) indicated that the antioxidant activity of biscuits increased as the baking temperature and time increased. This could be attributed to MRP ability to scavenge free radicals (hydroxyl, superoxide, and peroxyl radicals) and chelate metals (Langner & Rzeski, 2014). As detailed in Table 1, the antioxidant activity of HT-B diet was significantly higher than that of LT-B diet ($p \leqslant 0.05$). As expected, results in Fig. 2a indicate that the antioxidant activity in the serum samples was significantly higher in the Asc and HT-B groups ($p \leqslant 0.05$), compared to the control and LT-B groups by the FRAP assay. The antioxidant activity determined by the ABTS assay goes in line with this finding; nevertheless, slight differences were observed. According to Fig. 2b, no significant differences were found by the ABTS assay in the serum antioxidant activity of rats

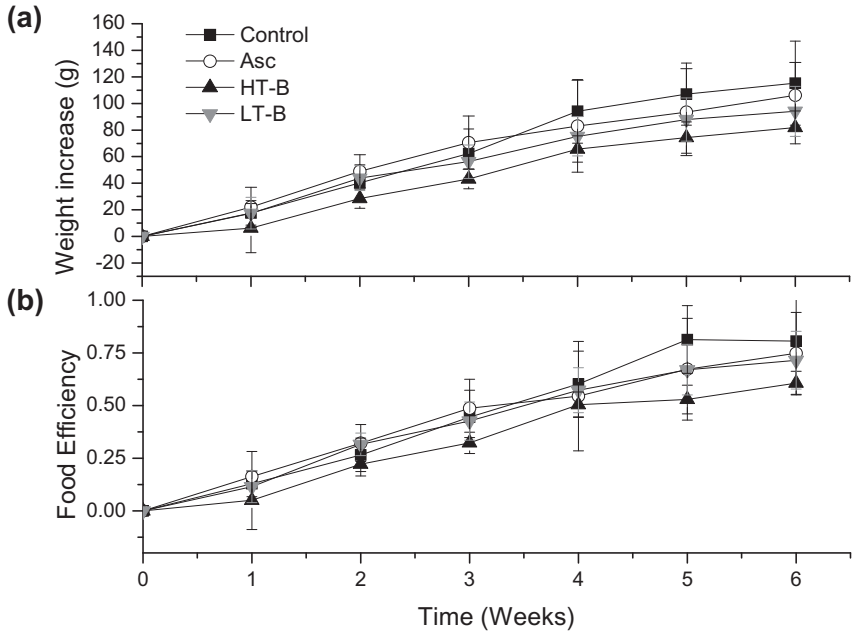

Fig. 1. Rat weight increase (a) and food efficiency (b) of animals fed with experimental diets (control, Asc, HT-B and LT-B) during the period of the assay.

fed with Asc and control diets ($p > 0.05$), but rats fed with HT-B diet showed a higher serum antioxidant activity compared to control ($p \leqslant 0.05$).

A detailed inspection of the results reveals that, although the antioxidant activity of HT-B diet is considerably higher than LT-B and control diet, the serum antioxidant activity of the HT-B group was not as high as expected. This could be attributed to the low absorption rate of melanoidins and their metabolites. At present, the bioavailability of MRP is still not well understood, but it is generally accepted that melanoidins are mainly recovered in faeces. According to Wang et al. (2011) only 30% of the low molecular weight melanoidins (LMW) (<10 kDa) are absorbed and could be taken up in the blood stream. On the other hand, high molecular weight melanoidins (>10 kDa) are absorbed to a much lesser extent than LMW compounds. Hence, they may exert only limited *in vivo* beneficial effects, such as antioxidant activity.

As can be seen in Fig. 2c, significant differences were found in the thiobarbituric acid reactive substances (TBARS), which are considered to be markers of lipid peroxidation. The TBARS values found were slightly higher than those reported by Da Silva et al. (2013) but agree with those reported by Matsumura, Ohno, Miyawaki, Shimizu, and Morimoto (1982). The differences among the TBARS concentration could be attributed to the different techniques used in these studies. Results from the present work indicate that rats from the HT-B group presented the lowest amount of TBARS ($p \leqslant 0.05$) while no significant differences were observed among the other groups ($p > 0.05$). The low amount of TBARS in the HT-B group seems to suggest that biscuit MRP were more efficient in protecting against oxidation damage than the ascorbic acid under the conditions of the assay, although the antioxidant activity did not show appreciable differences. The molecular mechanisms involved in the melanoidin antioxidant effects as well as in their bioavailability are still not well understood (Martín et al., 2009) and they are beyond the scope of this study. Future works should be performed in order to understand this aspect of melanoidins mechanisms.

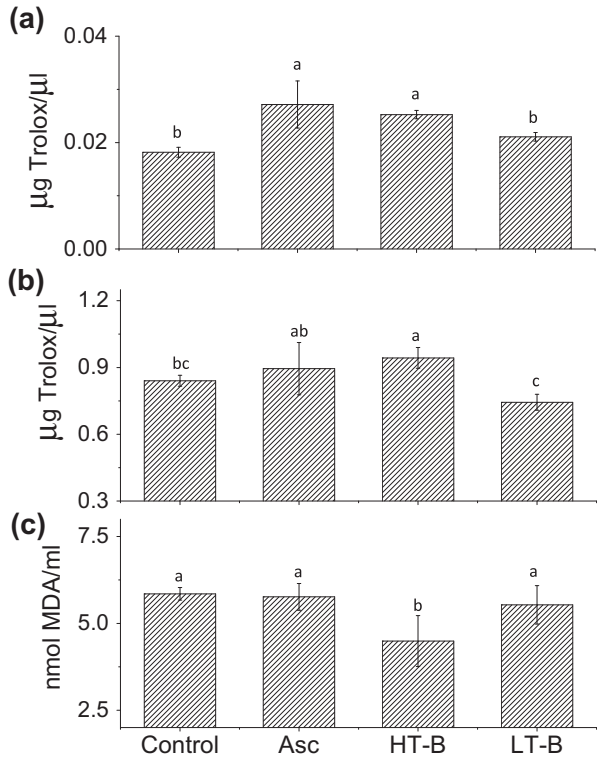

Fig. 2. Effect of diet consumption (control diet, Asc diet, HT-B diet, LT-B diet) on rat serum antioxidant status (a) FRAP, (b) ABTS, and lipid peroxidation (c) TBARS. Different superscript letters indicate significant differences at $p \leqslant 0.05$.

3.4. Faecal microbiota

The potential health benefits of lactic acid bacteria have attracted much attention. Evidence suggests that these bacteria promote desirable changes in the gastrointestinal tract: they reduce the severity/frequency of diarrhoea, alleviate lactose intolerance, stimulate the immune system, and prevent colonic cancer, among other benefits (Masood, Qadir, Shirazi, & Khan, 2011). Prebiotics are dietary carbohydrates which are not digested in the small intestine, but undergo bacterial fermentation in the large intestine. These compounds beneficially affect the intestinal microbiota like lactic bacteria (Jiménez-Escrig et al., 2013). Even though melanoidins are not digested in the upper gastrointestinal tract, only a few studies have focused on their potential prebiotic effects (Borrelli & Fogliano, 2005; Wang et al., 2011).

In the present work rats were fed with a high MRP diet (HT-B diet) and with low MRP diet (control and LT-B diets). At the beginning of the assay no significant differences ($p > 0.05$) were found among lactic and total aerobic bacteria counts for the different groups (9.0 and 8.3 log CFU per gram of faecal dry weight, respectively). However, at the end of the assay, significant differences were found in the faecal microbiota among groups. Rats fed with the HT-B diet showed the highest lactic bacteria count ($p \leqslant 0.05$), while no significant differences were found among rats fed with LT-B diet or control diet. Moreover, no significant differences were observed in total aerobic bacterial count on faeces among the different rat groups at the end of the experiment ($p > 0.05$). However, a tendency to lower the total amount of these bacteria was observed in the HT-B group ($p = 0.28$). What is more, the anaerobic/aerobic ratio in the HT-B group was significantly higher than in the other groups ($p \leqslant 0.05$). This suggests that there was a greater predominance of anaerobic bacteria in rats which consume the HT-B diet. Previous studies had indicated that a high proportion of these bacteria could change the intestinal environment favourably and ultimately lead to a lower incidence in colorectal cancer (Vargo, Moskovitz, & Floch, 1980). Results of bacterial faecal count at the end of the experimental period can be seen in Table 2.

In the main, these results suggest that the intake of HT-B for 6 weeks was capable of significantly modifying the composition of colonic microbiota in healthy rats. It could be inferred that melanoidins from the HT-B that escape from the digestion in the upper gastrointestinal tract appeared to selectively enhance the growth of beneficial bacteria in the gut. In good agreement with our results, Borrelli and Fogliano (2005) studied the potential prebiotic

Table 2
Faecal microbiota, faeces moisture and apparent mineral absorption of rats fed with different diets (control, HT-B, LT-B).

	Control group	HT-B group	LT-B group
Lactic acid bacteria (log CFU/g faecal dry weight)	8.83 ± 0.15^{b}	9.32 ± 0.06^{a}	8.74 ± 0.33^{b}
Total aerobic bacteria (log CFU/ g faecal dry weight)	8.27 ± 0.95^{a}	7.66 ± 0.52^{a}	8.25 ± 0.25^{a}
Ratio between lactic and total aerobic bacteria	1.07 ± 0.09^{b}	1.21 ± 0.08^{a}	1.06 ± 0.04^{b}
Faeces moisture (%)	52.8 ± 1.8^{b}	65.3 ± 1.9^{a}	57.38 ± 2.0^{b}
Percentage of apparent mineral absorption	61.69 ± 8.54^{a}	51.86 ± 4.57^{a}	29.90 ± 5.86^{b}

Results expressed as means ± SD. Values in the same row that do not share the same superscript letter are significantly different at $p \leqslant 0.05$.

activity of bread crust melanoidins using an *in vitro* system. Their data demonstrate that beneficial bacteria such as bifidobacteria could use melanoidins as a carbon and nitrogen source more efficiently than other bacteria (*Clostridia*, *Bacteroides* spp., *Streptococci* and *Enterobacteriaceae*). Hence, results obtained in the present *in vivo* study support the idea that melanoidins may have a potential prebiotic activity similar to that of dietary fibre.

3.5. Faeces moisture and apparent mineral absorption

The moisture content of faeces from HT-B, LT-B and control groups is displayed in Table 2. It is evident from these results that the intake of HT-B diet significantly increased the water retention in rat faeces ($p \leqslant 0.05$). It has been well-documented that dietary fibre could increase the moisture content in faeces because of its water-holding capacity (Eastwood, 1992). Therefore, this evidence supports the previously mentioned idea that MRP behave like dietary fibre. Besides, in good agreement with this, Pérez-Jiménez, Díaz-Rubio, Mesías, Morales, and Saura-Calixto (2014) studied the MRP contribution to dietary fibre *via* an *in vitro* assay. According to these authors, a high development of MR could increase the content of dietary fibre in bakery products, although their structure is quite different from those generally considered as dietary fibre (non-starch polysaccharides, lignin, resistant starch and others). Several works have indicated that MRP have the ability to chelate metal ions. This is a major issue, as it could have negative consequences on health, especially for children who are particularly vulnerable to such conditions (Eastwood, 1992). Different investigations confirmed this effect, performing *in vitro* assays in model systems or in coffee brews (Morales, Fernández-Fraguas, & Jiménez-Pérez, 2005; Rufian-Henares & de la Cueva, 2009). In the present study no significant differences were found between the mineral absorption of the HT-B and the control group ($p > 0.05$) (Table 2). Therefore, it could be assumed that a high intake of biscuit MRP did not significantly reduce the mineral absorption. By contrast, significant differences were found in the mineral absorption of the LT-B group compared to HT-B group and control group ($p \leqslant 0.05$). Other authors have already indicated that there is not a linear relationship between the browning developed during thermal processing and the mineral binding ability of MRP in model systems (Kim & Lee, 2009; Ruiz-Roca, Navarro, & Seiquer, 2008). Nevertheless, it should be considered that biscuits constitute a complex system and many different reactions, apart from MR, can take place during thermal processing. Therefore, it may be reasonable to suppose that, although LT-B did not contain high amounts of MRP, the prolonged heating procedure used to cook them (100 °C for 80 min) could have led to a significant decrease in the mineral bioavailability.

3.6. Blood pressure

The *in vitro* assays performed by Rufián-Henares and Morales (2007a, 2007b) confirmed the antihypertensive effect of melanoidins in Maillard reaction model systems and different beverages (coffee, beer, and sweet wine). Their results suggested that the antihypertensive activity of melanoidins could be explained by the metal chelating properties of these compounds, as the angiotensin converting enzyme is Zn-dependent. However, little is known about the effects of MRP on Zn absorption *in vivo* and many times results are contradictory. Sarriá and Vaquero (2001) studied the effect of MRP on bioavailability of zinc using suckling rats. Their results showed that the consumption of an in-bottle-sterilised formula with high amount of MRP determined a lower zinc bioavailability, compared to a reconstituted powder formula with a low amount of MRP. By contrast, recent studies indicated that the intake of MRP derived from bread crust did not modify Zn balance in rats (Delgado-Andrade, Roncero-Ramos, Haro, Pastoriza, & Navarro, 2015).

Results in the present work support the idea that a diet with high amounts of MRP could reduce blood pressure. As can be observed in Fig. 3, HT-B group presented a lower systolic blood pressure than the other groups during the period of the assay ($p \leqslant 0.05$). Besides, at the end of the experimental period the pressure of the Asc group was also significantly lower than the control and the LT-B groups ($p \leqslant 0.05$). To explain this result based on the metal chelating capacity of melanoidins, the apparent Zn absorption was measured (89.5 ± 6.3; 51.3 ± 13.0; 12.3 ± 13.2 for control, HT-B and LT-B groups, respectively), but no good correlation was found between this parameter and the blood pressure of the rats (Pearson's correlation coefficient $r = -0.14$). On the other hand, excellent correlations were found between the decrease of blood pressure and the antioxidant activity of plasma (the Pearson's correlation coefficients were $r = -0.86$ and $r = -0.93$ for FRAP and ABTS, respectively; Fig. 4). Therefore, it could be inferred that the antihypertensive effect of biscuit melanoidins was associated with their antioxidant activity. Consistent with this theory, Rodriguez-Iturbe, Zhan, Quiroz, Sindhu, and Vaziri (2003) found a significant reduction in systolic blood pressure of rats fed with

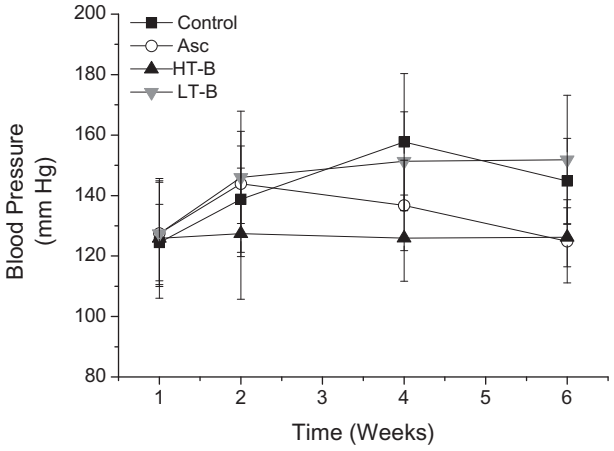

Fig. 3. Effects of different diets (control, Asc, HT-B and LT-B) on rat blood pressure during the period of the assay.

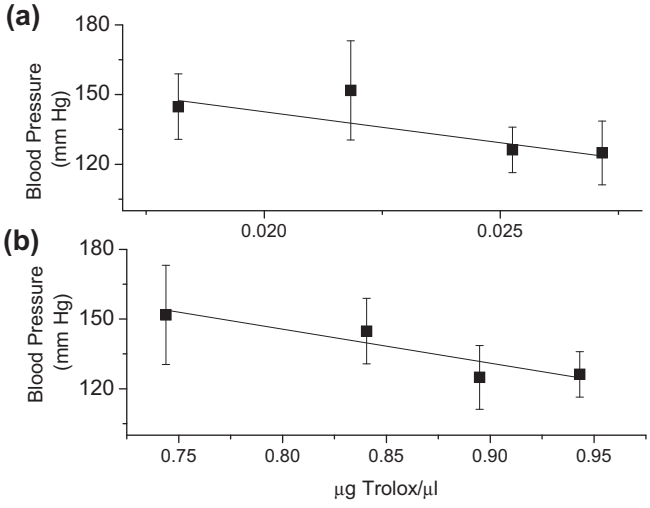

Fig. 4. Correlation of antihypertensive effect and the serum antioxidant activity (a) FRAP and (b) ABTS.

an antioxidant-rich diet. According to these authors, the oxidative stress raises arterial blood pressure by promoting nitric oxide (a vasodilator) deficiency. Hence, it is plausible that a diet rich in antioxidants like melanoidins could lower blood pressure.

4. Conclusions

According to the present results, the high temperature used in the baking of biscuits lowered the protein digestibility and reduced their food efficiency, leading to a significant reduction in the body weight of rats which consumed them.

The consumption of a diet with high amounts of MRP derived from biscuits increased the antioxidant activity of serum and reduced lipid oxidation. Besides, results seem to indicate that high amounts of MRP could reduce blood pressure. A good correlation between blood pressure and the antioxidant activity of plasma was found. Therefore, it could be inferred that the antihypertensive effect of MRP was associated with their antioxidant activity.

Finally, the present results are consistent with the idea that MRP behave like dietary fibre: they beneficially modulated the composition of the colonic microbiota, increasing the predominance of anaerobic bacteria and increased faeces water holding capacity. On the other hand, no reduction of the mineral absorption was observed in the rats fed with high amounts of MRP.

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References

AACC International (Ed.). (1983). *Approved methods of American association of cereal chemists* (8th ed.. St. Paul, Minnesota: AACC International.

Albesharat, R., Ehrmann, M. A., Korakli, M., Yazaji, S., & Vogel, R. F. (2011). Phenotypic and genotypic analyses of lactic acid bacteria in local fermented food, breast milk and faeces of mothers and their babies. *Systematic and Applied Microbiology, 34*, 148–155.

Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry, 239*, 70–76.

Borrelli, R. C., & Fogliano, V. (2005). Bread crust melanoidins as potential prebiotic ingredients. *Molecular Nutrition & Food Research, 49*, 673–678.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. L. W. T. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT – Food Science and Technology, 28*, 25–30.

Da Silva, J. K., Cazarin, C. B. B., Colomeu, T. C., Batista, Â. G., Meletti, L. M., Paschoal, J. A. R., ... de Lima Zollner, R. (2013). Antioxidant activity of aqueous extract of passion fruit (*Passiflora edulis*) leaves: In vitro and in vivo study. *Food Research International, 53*, 882–890.

Delgado-Andrade, C., Roncero-Ramos, I., Haro, A., Morales, F. J., Seiquer, I., & Navarro, M. P. (2013). Effects of diets supplemented with MRP from bread crust on the food intake and body weights in rats. *Food & Function, 4*, 1016–1022.

Delgado-Andrade, C., Roncero-Ramos, I., Haro, A., Pastoriza, S., & Navarro, M. P. (2015). Do bread-crust-derived Maillard reaction products affect the retention and tissue distribution of trace elements? *European Journal of Nutrition*, 1–9.

Eastwood, M. A. (1992). The physiological effect of dietary fiber: An update. *Annual Review of Nutrition, 12*, 19–35.

Estepa, V., Ródenas, S., & Martín, M. C. (2009). Optimización de un método para la determinación de la peroxidación lipídica en suero humano. *Anales de la Real Academia Nacional de Farmacia, 67*(3).

Filipčev, B., Šimurina, O., & Bodroža-Solarov, M. (2014). Quality of ginger nut type biscuits as affected by varying fat content and partial replacement of honey with molasses. *Journal of Food Science and Technology, 51*, 3163–3171.

Fritz, M., & Rinaldi, G. (2008). Blood pressure measurement with the tail-cuff method in Wistar and spontaneously hypertensive rats: Influence of adrenergic- and nitric oxide-mediated vasomotion. *Journal of Pharmacological and Toxicological Methods, 58*, 215–221.

Jimenez-Escrig, A., Dragsted, L. O., Daneshvar, B., Pulido, R., & Saura-Calixto, F. (2003). In vitro antioxidant activities of edible artichoke (*Cynara scolymus* L.) and effect on biomarkers of antioxidants in rats. *Journal of Agricultural and Food Chemistry, 51*, 5540–5545.

Jiménez-Escrig, A., Gómez-Ordóñez, E., Tenorio, M. D., & Rupérez, P. (2013). Antioxidant and prebiotic effects of dietary fiber co-travelers from sugar Kombu in healthy rats. *Journal of Applied Phycology, 25*, 503–512.

Kim, J. S., & Lee, Y. S. (2009). Antioxidant activity of Maillard reaction products derived from aqueous glucose/glycine, diglycine, and triglycine model systems as a function of heating time. *Food Chemistry, 116*, 227–232.

Kitrytė, V., Adams, A., Venskutonis, P. R., & De Kimpe, N. (2012). Impact of lipid oxidation-derived aldehydes and ascorbic acid on the antioxidant activity of model melanoidins. *Food Chemistry, 135*, 1273–1283.

Langner, E., & Rzeski, W. (2014). Biological properties of melanoidins: A review. *International Journal of Food Properties, 17*, 344–353.

Lindenmeier, M., Faist, V., & Hofmann, T. (2002). Structural and functional characterization of pronyl-lysine, a novel protein modification in bread crust melanoidins showing in vitro antioxidative and phase I/II enzyme modulating activity. *Journal of Agricultural and Food Chemistry, 50*, 6997–7006.

Martín, M. Á., Ramos, S., Mateos, R., Rufián-Henares, J. A., Morales, F. J., Bravo, L., & Goya, L. (2009). Biscuit melanoidins of different molecular masses protect human Hep G2 cells against oxidative stress. *Journal of Agricultural and Food Chemistry, 57*, 7250–7258.

Masood, M. I., Qadir, M. I., Shirazi, J. H., & Khan, I. U. (2011). Beneficial effects of lactic acid bacteria on human beings. *Critical Reviews in Microbiology, 37*, 91–98.

Matsumura, Y., Ohno, Y., Miyawaki, N., Shimizu, T., & Morimoto, S. (1982). Effect of vitamin E-deficiency on renin release from renin granules. *Journal of Pharmacobio-Dynamics, 5*, 1030–1032.

Monente, C., Bravo, J., Vitas, A. I., Arbillaga, L., De Peña, M. P., & Cid, C. (2015). Coffee and spent coffee extracts protect against cell mutagens and inhibit growth of food-borne pathogen microorganisms. *Journal of Functional Foods, 12*, 365–374.

Morales, F. J., Fernández-Fraguas, C., & Jiménez-Pérez, S. (2005). Iron-binding ability of melanoidins from food and model systems. *Food Chemistry, 90*, 821–827.

Morales, F. J., Martin, S., Açar, Ö. Ç., Arribas-Lorenzo, G., & Gökmen, V. (2009). Antioxidant activity of cookies and its relationship with heat-processing contaminants: A risk/benefit approach. *European Food Research and Technology, 228*, 345–354.

Oste, R., & Sjödin, P. (1984). Effect of Maillard reaction products on protein digestion. In vivo studies on rats. *The Journal of Nutrition, 114*, 2228–2234.

Pastoriza, S., & Rufián-Henares, J. A. (2014). Contribution of melanoidins to the antioxidant capacity of the Spanish diet. *Food Chemistry, 164*, 438–445.

Patrignani, M., Conforti, P. A., & Lupano, C. E. (2014). The role of lipid oxidation on biscuit texture during storage. *International Journal of Food Science & Technology, 49*, 1925–1931.

Pérez-Jiménez, J., Díaz-Rubio, M. E., Mesías, M., Morales, F. J., & Saura-Calixto, F. (2014). Evidence for the formation of maillardized insoluble dietary fiber in bread: A specific kind of dietary fiber in thermally processed food. *Food Research International, 55*, 391–396.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine, 26*, 1231–1237.

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. *Journal Nutrition, 123*, 1939–1951.

Rodriguez-Iturbe, B., Zhan, C. D., Quiroz, Y., Sindhu, R. K., & Vaziri, N. D. (2003). Antioxidant-rich diet relieves hypertension and reduces renal immune infiltration in spontaneously hypertensive rats. *Hypertension, 41*, 341–346.

Rufian-Henares, J. A., & de la Cueva, S. P. (2009). Antimicrobial activity of coffee melanoidins: A study of their metal-chelating properties. *Journal of Agricultural and Food Chemistry, 57*, 432–438.

Rufián-Henares, J. A., & Morales, F. J. (2007a). Functional properties of melanoidins: In vitro antioxidant, antimicrobial and antihypertensive activities. *Food Research International, 40*, 995–1002.

Rufián-Henares, J. A., & Morales, F. J. (2007b). Effect of in vitro enzymatic digestion on antioxidant activity of coffee melanoidins and fractions. *Journal of Agricultural and Food Chemistry, 55*, 10016–10021.

Ruiz-Roca, B., Navarro, M. P., & Seiquer, I. (2008). Antioxidant properties and metal chelating activity of glucose–lysine heated mixtures: Relationships with mineral absorption across Caco-2 cell monolayers. *Journal of Agricultural and Food Chemistry, 56*, 9056–9063.

Sarriá, B., & Vaquero, M. P. (2001). Zinc and iron bioavailability in a powder or in-bottle-sterilized infant formula estimated by in vitro and in suckling rats. *The Journal of Nutritional Biochemistry, 12*, 266–273.

Sönmez, M., Türk, G., & Yüce, A. (2005). The effect of ascorbic acid supplementation on sperm quality, lipid peroxidation and testosterone levels of male Wistar rats. *Theriogenology, 63*, 2063–2072.

Vargo, D., Moskovitz, M., & Floch, M. H. (1980). Faecal bacterial flora in cancer of the colon. *Gut, 21*(8), 701–705.

Vhangani, L. N., & Van Wyk, J. (2013). Antioxidant activity of Maillard reaction products (MRP) derived from fructose–lysine and ribose–lysine model systems. *Food Chemistry, 137*, 92–98.

Wang, H. Y., Qian, H., & Yao, W. R. (2011). Melanoidins produced by the Maillard reaction: Structure and biological activity. *Food Chemistry, 128*, 573–584.