RESEARCH ARTICLE

Intake of Maillard reaction products reduces iron bioavailability in male adolescents

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The effects of diets with different Maillard reaction products (MRPs) content on biological iron utilization were compared using *in vitro*/*in vivo* assays. Diets were rich (brown diet, BD) or poor (white diet) in MRP. *In vitro* studies included iron solubility after *in vitro* digestion of diets and iron transport across Caco-2 cells. In the human assay 18 healthy adolescent males (11–14 years) participated in a 2-wk randomized two-period crossover trial. Subjects collected urine and faeces on the last 3 days of each dietary period, and fasting blood samples were obtained after periods. *In vitro* dietary iron availability was significantly lower with the BD than the white diet (9.52 and 12.92%, respectively), as a consequence of the lower iron solubility after the *in vitro* digestion, but not as a result of decreased transport of the remaining soluble iron. The BD consumption increased iron fecal excretion (~1.4-fold) and significantly decreased its bioavailability (~2.7-fold), mainly due to the effects found at digestive level. Serum biochemical parameters related to iron metabolism remained unaltered. It is concluded the presence of MRP in the diet negatively affects iron bioavailability. As iron deficiency may be related to learning impairment and to reductions of cognitive and physical functions, possible long-term effects of excessive MRP intake during adolescence warrant attention.

Keywords:
Caco-2 cells / Gastrointestinal digestion / Iron bioavailability / Iron metabolism / Maillard reaction products

1 Introduction

Total nutrient needs for growth and development are higher during adolescence than any other time in the lifecycle. Moreover, nutrition is also important in this time to prevent adult diet-related diseases. Iron is essential during this phase of life to support the increased rate of haematopoiesis and myoglobin generation necessary for new muscle formation and tissue accretion [1, 2], being heme iron better absorbed than non-heme iron. Thus, adolescents are especially vulnerable to iron deficiency which, moreover, may provoke deficits in cognitive or behavioral functions leading to learning impairment and physical performance [3, 4]. The prevalence of iron deficiency among adolescents [5] is not only due to their high physiological needs, but also to their dietary habits. It is well known that the dietary habits of adolescents have changed in recent decades, and there tends to be a higher consumption of snacks and fast food [6]. Such consumption is less common in Mediterranean countries than in the northern Europe or in the USA [7], but the changed dietary pattern of Spanish adolescents means that some of the most important values of the Mediterranean diet have been lost. The nutritional preferences of this population are changing toward less healthy options [8], and

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Abbreviations: BD, brown diet; **MRP,** Maillard reaction products; **WD,** white diet

hence it is clearly important to investigate the dietary factors that may affect iron absorption and utilization.

Processing of foods rich in protein and carbohydrates and/or fats favours the development of the Maillard reaction and the formation of browning products, which can improve food palatability [9]. The final products of the reaction are high-molecular-weight-colored compounds, melanoidins, with different physical–chemical properties [10]. The formation of these products depends directly on the temperature and time of processing; and is greatly heightened by long exposure to high heat [11]. These conditions can especially affect snacks and fast foods, since they are usually prepared by processes such as frying, roasting, grilling, baking and even reheated before being consumed. Thus, the Maillard reaction products (MRPs) content in foods is related not only to their composition, but also to the method and conditions of preparation, as well as with reheating [12]. Controlled browning is pursued through many food technology processes aimed at promoting consumer acceptance, and hence MRPs are widely consumed as part of the human diet [13], especially during adolescence [14].

MRP consumption is associated with certain positive biological actions, such as antioxidant activity, chemopreventive activity or antimutagenic actions [15], but at the same time MRPs are known to provoke negative consequences, including pro-mutagenic and genotoxic effects [15] or antinutritional properties, mainly related to protein damage [16], and also some alterations of vitamins [17] and mineral availability [18].

Effects derived of from the presence of MRPs on iron availability have been shown *in vitro* [10], but *in vivo* studies, mainly performed in rats, are scarce and revealed controversial results [19]. There are few assays carried out in humans [20] and studies on the influence of whole diets rich in MRP on iron bioavailability are still lacking.

The aim of this study was to investigate dietary iron bioavailability and iron status in a sample of male adolescents consuming MRP-rich diets, as are usually consumed by this population, and to compare the results with the corresponding values derived from an MRP-low diet. Moreover, experiments in cell cultures using Caco-2 cells were carried out to study dietary MRP effects on iron absorption. This cell line exhibits, in culture, many properties of normal intestinal epithelium and it has been widely used as a suitable model to study iron metabolism [21]. In addition, the usefulness of Caco-2 cells in assessing human iron absorption and in studying iron bioavailability from foods has been validated [22].

2 Materials and methods

2.1 Chemicals

All the chemical products and solvents, for all the analyses, were of the highest grade available and acquired from Sigma (Sigma-Aldrich, St. Louis, MO, USA) and Merck (Darmstadt, Germany).

2.2 Subjects, diets and study design

Selection of the subjects, composition of diets and study design have been described previously [16]. Briefly, 20 male adolescents (12.4 ± 0.34 years of age, mean $\pm$ SE) participated in a 2-wk randomized two-period crossover trial, in which they consumed two different diets, in opposite order, with a 40-day washout period. The diets were designed to take into account the eating patterns of the subjects, previously evaluated by nutritional survey, and the Recommended Intakes for the Spanish Population [23], to achieve diets that were balanced and adjusted to the nutritional requirements of this age group. Two 7-day menus were elaborated, each containing the same servings/day of the different food groups and with a similar content of energy and nutrients, as follows: white diet (WD), free, as far as possible, of foods in which the MR Maillard reaction develops during cooking practices (*i.e.* frying, toasting and roasting) or those usually containing MRPs, such as bread crust, or chocolate; brown diet (BD), rich, as far as possible, in processed foods with an evident development of browning and, thus, rich in MRPs. Lunch and dinner, the two main meals in the Spanish diet, were prepared by a local catering firm, always under the strict control of the researchers (Table 1) and were distributed daily to the homes of the participants. Each 7-day menu was repeated during the second half of the 14-day experimental period. The subjects and their parents were given instructions about what to eat at breakfast and in the afternoon snack, prepared at home, for each one of the diets. The food composition of the breakfast was whole milk with sugar, white bread without crust with margarine and fruit juice in the WD, whole milk with cocoa powder, breakfast cereals and fruit juice in the BD. The afternoon snack was composed of whole milk with sugar, sandwich of white bread without crust with pâté or cheese and margarine in the WD, whole milk with cacao powder and pastries in the BD.

The food composition of the diets was transformed into energy and nutrient values using the Spanish Food Composition Tables [24], under AYS44 Diet Analysis software supplied by ASDE, SA (Valencia, Spain). The overall daily content of the energy and nutrients in the study diets were as follows: energy 2530 kcal, fat 107.5 g, carbohydrate 316.9 g, protein 90.1 g, fiber 25.1 g, cholesterol 311.4 mg, sodium 1865 mg, potassium 3826 mg, calcium 1049 mg, phosphorus 1595 mg, magnesium 372 mg, iron 17.5 mg, zinc 8.9 mg, retinol 1.4 mg, ascorbic acid 117.4 mg znd α-tocopherol 11.4 mg.

To enable analysis of the iron content and Maillard reaction markers in the experimental diets, the *in vitro* digestion and the Caco-2 cell assays, the catering firm also provided the meals to the researchers; the breakfast and the

Table 1. Lunch and dinner, a 7-day menu for the diets used for dietary treatments

Diet	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
White Diet Lunch	Legumes (chickpeas) Salad (lettuce, tomato, *etc.*) Strawberry yoghurt Bread without crust	Salad of pasta Boiled chicken and potatoes Pears Bread without crust	Legumes (lentils) Tuna-filled eggs Rice with milk Bread without crust	Vegetable stew Baked loin of pork and boiled potatoes Syrup peach Bread without crust	Russian salad with tuna Legumes (beans) Strawberry yoghurt Bread without crust	Boiled potatoes, boiled eggs and ham Baked meat (veal) with vegetables Custard Bread without crust	Salad (lettuce, tomato, *etc.*) Stewed rice Apple Bread without crust
Dinner	Soup of pasta and chicken Sausages with mashed potatoes Bananas Bread without crust	Prawn cream Baked fish Custard Bread without crust	Tropical salad Fish with cream and rice Oranges Bread without crust	Spaghetti with tomato sauce, cheese and ham Bananas Bread without crust	Consommé with noodles Baked fish with boiled potatoes Pears Bread without crust	Soup of vegetables Pasta with tomatoes and cheese Bananas Bread without crust	Vegetables purée Fish pudding Custard Bread without crust
Brown Diet Lunch	Legumes (chickpeas) Salad (lettuce, tomato, *etc.*) Chocolate yoghurt Bread	Salad of pasta Fried chicken and fried potatoes Pears Bread	Legumes (lentils) Spanish omelette Rice with milk and cinnamon Bread	Sauté vegetables Griddle loin of pork and fried potatoes Torrija[a)] Bread	Empanadillas[b)] with salad Legumes (beans) Chocolate yoghurt Bread	Spanish omelette with ham Meatballs (veal) with vegetables Chocolate custard Bread	Salad (lettuce, tomato, *etc.*) Paella Apple Bread
Dinner	Soup of pasta and chicken Hamburger with fried potatoes Bananas Bread	Purée of prawns Fish croquettes Caramel custard Bread	Tropical salad Breaded fish and rice Oranges Bread	Gratin macaroni with béchamel sauce Bananas Bread	Consommé with noodles Breaded fish with fried potatoes Pears Bread	Soup of vegetables Pizza Bananas Bread	Vegetables cream with croutons Breaded hake fish-fingers Chocolate custard Bread

a) Fried bread with milk, sugar and cinnamon.
b) Small tuna-filled breaded pasties.

afternoon snack were prepared in the laboratory by following the instructions given to the participants, the ingredients being purchased at a local market. Every day, and for each diet, the edible portion of the foods was removed from all meals, weighed and homogenized with a hand blender (Taurus, vital CM, Spain). Aliquots of each meal were mixed to obtain the 1-day sample, and aliquots of each day were mixed to obtain the diet samples (WD and BD). Aliquots of meals and diets were stored at −20°C until the moment of analysis.

The analysis of Maillard reaction markers in the diets [16] confirmed the greater development of the Maillard reaction in the BD than in the WD, in accordance with the significantly higher values of hydroxymethylfurfural and the percentage of relative fluorescence intensity found in the BD (hydroxymethylfurfural: 0.94 ± 0.01 and 3.87 ± 0.03 mg/kg; fluorescence intensity: 7.31 ± 0.35 and $21.04 \pm 0.42\%$, in WD and BD, respectively). The analysis of furosine (ε-*N*-(furoylmethyl)-L-lysine) showed that the content of early MRPs was similar in both diets (6.99 ± 0.45 and 6.37 ± 0.15 mg/100 g in the WD and the BD, respectively), confirming that commonly consumed diets always contain some of the early Maillard reaction compounds.

Compliance with dietary treatments was assessed throughout the entire dietary treatments by daily records sheets, in which participants noted the details of their food consumption. If the prepared meals were not entirely consumed, the subjects were asked to weight and record every food that was left. They also weighted and recorded the food consumed at breakfast and afternoon snack. Data of food intake were transformed into energy and nutrient values (except iron) using the above-mentioned computer program.

To determine iron dietary utilization, the iron content was analyzed in the diets and in the urine and faeces samples. A 14-day period may be considered an adequate equilibration period that allows gastrointestinal clearance of unabsorbed minerals from the previous diet [25]. The balance comprised three 24-h urine and faeces collections at the end of each 14-day dietary treatment [26]. In total 24-h urine samples were collected on acidified recipients, beginning with the second voiding of the day and finishing with the first voiding of the following day, and the volume from each daily sample was measured. The subjects were asked to report any problem with the collections, such as spillages or missed specimens. Fecal samples were weighed, diluted with 6 N HCl and homogenized with a hand blender (Taurus). Aliquots were frozen at −20°C until analysis.

At the end of each dietary treatment and after a 12-h overnight fast period, blood samples were obtained from each child by venipuncture for biochemical and haematological screening test, using two types of vacutainers. EDTA-containing vacutainers were used for the collection of whole-blood samples, which were analyzed by a haematological autoanalyzer (Hitachi 917 autoanalyzer, Hitachi, Boehringer Mannheim, Mannheim, Germany) for the determination of haemoglobin (Hgb), transferrin, haematies (Hmt), haematocrit (Hct), mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration. Blood collected in vacutainers with no added anticoagulant was left to clot for 30 min, and centrifuged at $1700 \times g$ for 15 min (4°C) to obtain serum. Aliquots were frozen at −20°C for later analyses. Serum ferritine was measured by using a MicroELISA method and serum iron as described later.

Body weight and height were recorded at the beginning of the study and at the end of each diet period, and BMI (kg/m^2) was calculated.

This study was approved by the Ethics Committee of the San Cecilio University Hospital of Granada and was performed in accordance with the Helsinki Declaration of 2002, as revised in 2004. Moreover, the informed consent was obtained from the parents of all the children participating in the study. Of the 20 subjects recruited, 18 completed the study, one having dropped out because a surgical intervention and a second one that was found to be non-compliant.

2.3 *In vitro* digestion of diets

The technique of Miller *et al.* [27], modified to our requirements, was followed. It comprised two stages: gastric digestion and intestinal digestion. Shortly before use, 0.8 g of pepsin was dissolved in 5 mL of 0.1 M HCl. For intestinal digestion, 0.1 g of pancreatin and 78 mg of bile salts were dissolved in 25 mL of 0.1 M $NaHCO_3$. In total, 4 g of each diet sample was suspended in 10 mL of milli-Q water. pH was adjusted to 2 with 6 N HCl, a pepsin solution was added at a proportion of 0.05 g of pepsin/g of sample, and samples were incubated at 37°C in a shaking water bath at 110 oscillations/min for 2 h for the gastric digestion. For the intestinal digestion, the pH of the digest was raised to 6 with 1 M $NaHCO_3$ dropwise, and 2.50 mL of pancreatin+bile salts mixture was added. The pH was then adjusted to 7.5 with 1 M $NaHCO_3$, and samples were incubated at 37°C at 110 oscillations/min for 2 h.

After gastrointestinal digestion, the digestive enzymes were inactivated by heat treatment for 4 min at 100°C in a polyethylenglycol bath. The samples were then cooled by immersion in an ice bath and centrifuged at $3200 \times g$ for 60 min at 4°C (CS-6R centrifuge, Beckman) to separate the soluble and non-soluble fractions. The supernatants were carefully separated and the percentages of soluble and insoluble iron were calculated from the initial iron content in diets. The supernatants were reserved and used for Caco-2 cell experiments.

2.4 Cell cultures

Caco-2 cells were purchased from the European Collection of Cell Cultures at passage 20 and used in the experiments at passages 22–30. The cells were grown in 75 cm^2 plastic

flasks containing high-glucose DMEM, with heat-inactivated fetal bovine serum (15%), $NaHCO_3$ (3.7 g/L), non-essential amino acids (1%), HEPES (15 mM), bovine insulin (0.1 UI/mL) and 1% antibiotic-antimycotic solution. The cells were maintained at 37°C in an incubator in an atmosphere of air/CO_2 (95:5) at 90% humidity, and the medium was changed every 2 days.

Trypsinisation and seeding of cells were performed as described elsewhere [28]. At 80% confluency, the cells were collected and seeded in the insert of bicameral chambers (Transwell, 24-mm diameter, 4.7-cm^2 area, 3-μm pore size, Costar) at a density of 90 000 cells/cm^2. They were maintained with 2.5 mL of medium in the well (basolateral cell side) and 1.5 mL of medium in the insert (apical cell side). The medium was changed every second day and the day before the cultures were used for the transport experiments. The integrity of the cell monolayers was monitored by determining transepithelial electrical resistance and the absorption of the phenol red marker, as described elsewhere [28, 29]. The cell monolayers used in this study exhibited adequate transepithelial electrical resistance values, ranging from 500 to 650 Ω/cm^2, and leakage rates of phenol red lower than 2%.

Twenty-one days after initial seeding, the iron transport experiments were carried out. Previously, the final concentration of glucose, HEPES and osmolarity (cryoscopic osmometer Osmomat 030-D, Berlin, Germany) of the diet digests was adjusted to 5 mM, 50 mM, and 310 mOsm/kg, respectively. Cell viability after 2 h of exposure to the supernatant digests was assessed by trypan blue exclusion, and it was never $<85\%$.

Spent culture medium was aspirated from the apical and basolateral chambers and the apical and basolateral cell surfaces of the monolayer were washed three times with Ca^{2+} and Mg^{2+}-free HBSS at 37°C. Then 2.5 mL of the transport solution (130 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L $MgSO_4$, 5 mmol/L glucose and 50 mmol/L HEPES, pH 7) was added to the basolateral chamber, and the supernatant digest of each diet (BD and WD) was added to the apical chamber (1.5 mL). The cell cultures were then incubated at 37°C in a humidified air:CO_2 atmosphere for 2 h. To calculate the iron transported across the cell monolayer, the buffer from the basolateral chamber was removed and, to ensure complete collection, the wells were washed twice with deionized water.

Iron transport in cell cultures is expressed in microgram of Fe transported to the basolateral chamber/well, and as the percentage of Fe transported/well from the experimental solution. Iron availability was expressed taking into account differences in iron solubility after *in vitro* digestion, as follows: % soluble iron × % transported iron/100.

2.5 Analytical techniques

Analyses were performed in triplicate, when possible. Before the iron analysis, aliquots of feeds, urine, faeces and serum, and solutions from diets *in vitro* digestion and cell culture experiments, were completely digested by the addition of concentrated HNO_3, $HClO_4$ and by heating at high temperatures (180–220°C) in a sand beaker (Block Digestor Selecta S-509; J.P.Selecta, Barcelona, Spain). Iron analysis, for all samples, was performed by atomic absorption spectrophotometry in a Perkin-Elmer Analyst 700 Spectrophotometer (Norwalk, CT, USA). Standard solutions were prepared from a stock Tritisol solution of iron ($FeCl_3$ in 15% HCl, 1000 mg Fe, Merck).

Pools of diet, faeces and urine were used as an internal control to assess precision. The inter-assay coefficient of variation was 7.4% in the diet, 4.8% in faeces and 2.5% in the urine. Pig kidney standard (certified reference material BCR no. 186, Community Bureau of Reference, Brussels, Belgium) was simultaneously used to quantify iron accuracy: measured value 311 ± 5 μg/g (mean ± SD of ten determinations), certified value 299 ± 10 μg/g. All glassware and polyethylene sample bottles were washed with 10 N nitric acid, and demineralized water (Milli-Q Ultrapure Water System, Millipore, Bedford, MA, USA) was used throughout.

Using the data obtained for the iron intake (I), fecal excretion (F) and urinary excretion (U), the following indices were calculated: apparent absorption ($A = I–F$), fractional absorption or digestibility ($\%A/I = A/I \times 100$), apparent retention ($R = A–U$) and utilization efficiency or bioavailability ($\%R/I = R/I \times 100$).

2.6 Statistical data analysis

SPSS for WINDOWS, version 13.0 (SPSS, 1999–2004, Chicago, IL, USA) was used for data entry and statistical analysis. The experimental data obtained after the crossover dietary treatments were analyzed by using the repeated-measures analysis of variance (ANOVA), to ascertain the consequences of the dietary treatment and to determine whether the order of presentation of the diets had an effect. There were no order effects and no treatment × order interaction for any of the dependent variables. When a significant effect between dietary treatments was found, *post-hoc* comparison of means was made using the Bonferroni's test. Differences were considered significant at $p < 0.05$. Statistical power calculation showed that a sample size of 20 give a power of 70% to find a 30% difference in iron digestibility between groups. Since 18 subjects completed the study, the power was reduced to 60%. Data from iron content in diets and from iron absorption in Caco-2 cells were statistically tested by one-way ANOVA, followed by Duncan's test to compare means that showed significant variation ($p < 0.05$). Evaluation of the relationship between the different variables was carried out by computing the relevant correlation coefficient (Pearson linear correlation) at $p < 0.05$ confidence level.

3 Results and discussion

The baseline characteristics of the subjects were weight 55.9 ± 2.9 kg and height 1.60 ± 0.03 m (mean ± SE). Weight and height increased by 2.09 ± 0.32 kg and 2.13 ± 0.36 cm during the whole experimental period (68 days), respectively, with no significant differences between groups. The BMI (21.8 ± 1.0 kg/m^2) did not change during the study.

In general, the compliance of the subjects with the diet and the urine and faeces collection was good, and they were sufficiently cooperative. The collaboration of the parents was essential, because balance studies are often problematic, especially in adolescents.

Daily intake of energy and nutrients was similar between both diets (Table 2). The intake of fat was found to be higher in the BD than in the WD ($p = 0.023$), which may be related to the preference of the adolescents for fried foods, only present in the BD and practically always consumed. The α-tocopherol intake was significantly reduced during the BD consumption ($p = 0.005$), a result that was expected as the severity of cooking procedures is known to result in a loss of tocopherols [30].

Daily iron intake did not differ significantly between the dietary treatments (Table 3). Values were similar to those found among Spanish adolescents of similar age [31], but slightly exceeded the Spanish, European or American recommendations [23] [32] [33]. In both diets, cereals contributed the majority of dietary iron (a relation was found between cereal consumption and iron intake, $r = 0.5284$, $p = 0.0005$) and meat and fish were the second-largest contributors (data not shown), which is in agreement with the dietary iron distribution found among European adolescents [34] and in the US and Italian populations [35, 36].

Table 2. Daily intakes of energy and nutrients during crossover dietary treatments with WD and BD in adolescent males aged 11–14 years

	WD	BD
Energy (kcal)	2176 ± 53	2271 ± 44
Protein (g)	84.7 ± 2.4	80.0 ± 1.9
Fat (g)	91.0 ± 2.8[a)]	97.4 ± 2.0[b)]
Carbohydrates (g)	269.7 ± 6.8	286.6 ± 6.2
Fiber (g)	20.8 ± 0.6	21.5 ± 0.6
Retinol (mg)	1.04 ± 0.05	1.05 ± 0.05
Ascorbic acid (mg)	105 ± 6	93 ± 5
α-Tocopherol (mg)	10.6 ± 0.5[a)]	8.8 ± 0.4[b)]
Calcium (mg)	1027 ± 36	1019 ± 21
Phosphorus (mg)	1360 ± 34	1371 ± 27
Magnesium (mg)	306 ± 9	305 ± 9
Iron (mg)	15.8 ± 0.42	16.67 ± 0.36
Zinc (mg)	8.6 ± 0.3	7.3 ± 0.2

Values are means ± SE, $n = 18$. The subjects consumed the WD (low in MRP) and the BD (rich in MRP) for 14-day period with a 40-day washout period.
a,b) Different letters in each row indicate significant differences (one-way ANOVA and Duncan Test, $p < 0.05$).

Table 3. Dietary iron utilization in adolescent males aged 11–14 years during crossover dietary treatments with WD and BD

	WD	BD
Intake		
(mg/d)	15.80 ± 0.42	16.67 ± 0.36
(mg kg^{-1} d^{-1})	0.28 ± 0.01	0.30 ± 0.01
Fecal excretion		
(mg/g)	0.12 ± 0.01[a)]	0.16 ± 0.01[b)]
(mg/d)	10.90 ± 0.68[a)]	14.83 ± 1.04[b)]
(mg kg^{-1} d^{-1})	0.20 ± 0.02[a)]	0.27 ± 0.02[b)]
Urinary excretion		
(μg/mL)	0.04 ± 0.00[a)]	0.05 ± 0.00[b)]
(μg/d)	55 ± 3	47 ± 4
(μg kg^{-1} d^{-1})	0.99 ± 0.07	0.84 ± 0.07
Apparent Absorption		
(mg/d)	4.91 ± 0.62[a)]	1.84 ± 0.99[b)]
(mg kg^{-1} d^{-1})	0.09 ± 0.01[a)]	0.03 ± 0.02[b)]
Apparent Retention		
(mg/d)	4.85 ± 0.62[a)]	1.79 ± 0.99[b)]
(mg kg^{-1} d^{-1})	0.08 ± 0.01[a)]	0.03 ± 0.02[b)]

Values are means ± SE, $n = 18$. The subjects consumed the WD (low in MRP) and the BD (rich in MRP) for 14-day period with a 40-day washout period. Subjects collected urine and faeces on the last 3 days of each dietary period.
a,b) Different letters in each row indicate significant differences (Repeated measures ANOVA followed by a Bonferroni test, $p < 0.05$).

Although dietary iron intake was similar in each diet, fecal excretion of iron was higher with consumption of the BD, both are expressed in milligram *per* day and when related to body weight (mg kg^{-1} d^{-1}). A positive correlation was found between iron intake and fecal excretion in the WD period ($r = 0.4458$, $p = 0.0428$), but not during consumption of the BD. Therefore, the higher level of iron excretion associated with the BD seems to be related not only to intake but also to other factors. As a consequence, the apparent absorption and digestibility of iron decreased significantly when the subjects consumed the MRP-diet (Table 3 and Fig. 1). The high values of fractional iron absorption observed during the WD consumption (31.2%) were similar to those reported in situations that occur with intense erythropoiesis, as in women with scant stores (30–35% of total dietary iron absorbed) [37] or among samples of young male adults that included blood donors (about 20% of iron fractional absorption) [38, 39]. Although some authors have reported an adverse correlation between serum ferritin and iron absorption in young women [40], unfortunately, this relationship was not analyzed in this study. On the other hand, the iron digestibility during the BD period was close to the 10% expected in standard diet patterns of the general population [41, 42] and the values

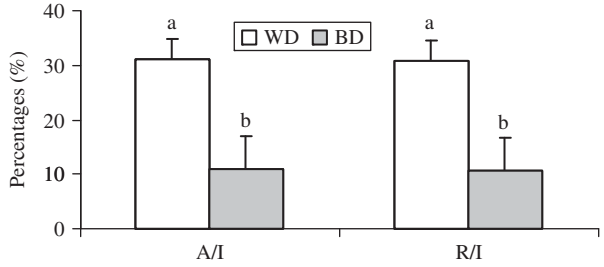

Figure 1. Iron digestibility (%*A*/*I*) and iron utilization efficiency (%*R*/*I*) in male adolescents aged 11–14 years after crossover dietary treatments with WD (low in Maillard reaction products) and BD (rich in Maillard reaction products). Values are mean ± SE, $n = 18$. Different letters indicate significant differences between diets (Repeated measures ANOVA followed by a Bonferroni test, $p < 0.05$).

used for RDA calculation [43]. The iron digestibility findings may be related to the lower iron solubility of the BD observed after the *in vitro* digestion of the diets (36.82 ± 4.72 *versus* 58.67 ± 5.10, $p < 0.05$, for BD and WD, respectively). Thus, it may be supposed that the special characteristics of the BD, or certain compounds present within it, induce the insolubilization of the mineral.

Cooking is known to cause the denaturalization of heme-iron [44], in varying proportions depending on the severity of treatment [45]. However, according to some authors, heat treatment at 120°C does not decrease the non-heme iron absorption, compared with low-temperature cooking [46]. Thus, it is likely that other factors reduced the iron absorption in the BD.

Iron is one of the minerals most affected by the presence of MRP. The ability of melanoidins to complex iron has been shown in foods such as coffee, beer and sweet wine [47–49]. MRPs from model system have also been shown to possess iron-chelating properties, especially the higher-molecular-weight fractions [50]. Glucose–lysine-heated mixtures have been analyzed for their potential iron-chelating activity [51] and it has been observed that the presence of these compounds decreases iron solubility under intestinal conditions [10]. Thus, the increased fecal iron excretion found in the BD period may be attributed to the formation of insoluble iron–MRP complexes in the intestinal lumen, which impair mineral absorption.

Results from the Caco-2 cell assay (Table 4) showed that the quantity of soluble iron transferred across the cell monolayer did not differ significantly between the WD and the BD. However, taking into account the large proportion of iron insolubilized after the *in vitro* digestion of the BD, the global process of *in vitro* digestion-absorption indicated that iron from the MRP-diet was utilized less efficiently, which is in agreement with the lower level of absorption found in the *in vivo* assay after the BD consumption. Thus, *in vitro* iron availability was significantly lower with the BD than with the WD as a consequence of the lower level of iron

Table 4. Iron transport in Caco-2 cells

Diets	Transport (μg/well)	% Transport	% Availability
WD	1.41 ± 0.01	22.01 ± 0.42	12.92 ± 0.37[a)]
BD	1.39 ± 0.02	25.87 ± 1.56	9.52 ± 0.41[b)]

Values are mean ± SE of at least three wells from a representative experiment.
a,b) Different letters indicate significant differences between diets (one-way ANOVA and Duncan Test, $p < 0.05$).

solubility from this diet, but not as a result of decreased transport of the remaining soluble iron.

Results of biological experiments of iron absorption in rats fed diets containing MRP model-system from amino acid–sugar [52–54], or from protein–sugar [55, 56], are disparate, although most of them show no variations in iron digestibility. However, when rats were fed a browning whole diet (sterilized infant formula that developed Maillard reaction), iron fractional absorption decreased [57].

Johnson *et al.* [20] found no differences in iron absorption among humans, on comparing the consumption of toasted cornflakes and corn meal, whereas a depressive effect on iron absorption has been described for chocolate, an MRP-rich food [58], although these effects have been attributed to polyphenols [59]. Bibliographic data concerning the influence of MRP intake on iron absorption in humans are scarce, and, to the best of our knowledge, this is the first study regarding the influence of whole MRP-rich diets on iron metabolism in humans.

It has been reported that the formation of certain mineral–MRP complexes could be responsible for increased trace element excretion in urine [19], especially of zinc [28]. Few studies have addressed the relationship between MRP intake and urinary iron, and most of them, performed in rats, do not find significant changes in urinary iron excretion when the diet contains MRP from amino acid–sugar model-system [18, 60]. Other experiments have reported increases in urinary iron excretion in rats when the dietary protein source was heated casein [56], or decreased values if methionine–glucose-heated mixtures were added to the diet [60]. In the present assay, the urinary iron concentration was slight but significantly higher after consumption of the BD. However, when expressed in microgram *per* day or related to body weight ($\mu g\,kg^{-1}\,d^{-1}$) iron excretion did not vary between the diets. Thus, differences in iron concentration could be related to variations in the volume of urine excreted.

Significant reductions in iron retention (Table 3) and iron bioavailability (%*R*/*I*, Fig. 1) were observed among subjects after consumption of the BD, compared with the WD. In this sense, it has been shown that iron balance is negatively affected in rats fed bottle-sterilized infant formula [57] and diets containing casein–glucose–fructose–olive oil-heated mixtures [56]. The findings of the present assay suggest that MRP intake negatively influences the iron balance as a consequence of changes at the digestive level, as the main effects occurred during digestion.

Table 5. Haematological and biochemical parameters of iron status in male adolescents aged 11–14 years after the WD and the BD

Parameters	WD	BD
Haematies (millons)	5.2±0.1	5.1±0.1
Haemoglobin (g/dL)	15.0±0.2	15.0±0.2
Haematocrit (%)	43±0	43±1
Ferritine (μg/L)	41±4	37±4
Transferrin (mg/dL)	307±7	309±8
Serum Fe (μg/dL)	140±6	147±5
Mean corpuscular volume (fL)	82.8±0.8	82.9±0.9
Mean corpuscular haemoglobin (pg)	28.3±0.6	28.9±0.3
Mean corpuscular haemoglobin concentration (g/dL)	34.9±0.2	34.7±0.2

Values are mean±SE, $n = 18$. The differences were not significant in any case (repeated measures ANOVA followed by a Bonferroni test, $p < 0.05$).

Nevertheless, we cannot discount the possibility of metabolic effects associated to with protein utilization. The consumption of a diet rich in MRP negatively affects protein absorption and digestibility in adolescents and tends to decrease nitrogen retention [16]. Given the relation between iron and nitrogen metabolism, it is feasible that the impaired protein utilization could affect iron bioavailability.

No significant differences in biochemical and haematological parameters related to iron status were found after consumption of the diets (Table 5), and all values were within the normal range, as expected in a short-term study. Therefore, although in our experimental conditions the BD consumption seemed to satisfy the needs of the adolescents, long-term studies would be required to confirm the results of the present assay. On the other hand, furthers studies are needed in girls, taking into account the differences between menstruating and non-menstruating individuals. This is a very important subject, since, in the view of the results of this first approach, women consuming MRP-rich diets could be at higher risk of developing iron-deficient anaemia than men.

4 Concluding remarks

The results obtained in the present assay show that MRP intake has a negative effect on dietary iron bioavailability in adolescents, even when consumed as part of a balanced and varied diet. This effect could be agravated with higher intakes of MRP or with unbalanced diets. In view of the present dietary habits of adolescents, and the well-established relationship between iron deficiency and learning impairment/physical performance, it seems of special interest to study the possible long-term effects of dietary MRP on iron utilization, even more in the case of young women due to the increased iron losses during menstruation.

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