J Appl Phycol
DOI 10.1007/s10811-017-1259-3

Low level of red seaweed *Pyropia columbina* added to extruded maize products promotes colonic and systemic antioxidant environment in growing Wistar rats

Raúl E. Cian[1] · **Emilce Llopart**[1] · **María E. López-Oliva Muñoz**[2] · **Adriana R. Weisstaub**[3] · **Ángela Zuleta**[3] · **Silvina R. Drago**[1]

Received: 11 February 2017 / Revised and accepted: 29 August 2017

Abstract The aim of this work was to evaluate the effect of consumption of extruded maize product added with a low level of the red seaweed *Pyropia columbina* on colonic and systemic oxidative status using a growing Wistar rat model. Twenty-four (*n = 24*) male Wistar rats were fed for 60 days with control (C), extruded maize product group (M), or extruded maize product added with red seaweed *P. columbina* (MP) diets. Rats fed whit MP showed higher catalase (CAT) and glutathione reductase (GR) colon expression than those fed with M or C. Beneficial effects on intestinal mucosal barrier function were observed, which was manifested in decrease of cecal pH (10%) and mucinase activity (67%) and increase of sIgA content (53%). Rats fed with MP diet showed lower cyclooxygenase-2 (COX-2; 43%), inducible nitric oxide synthase (iNOS; 49%), and NF-κβ transcription factor (27%) expression in distal colon than those fed with M or C diets. Also, MP diet exerted a significant antioxidant effect on the serum and liver, increasing hepatic redox index, CAT, and GR activity. Apparent calcium absorption, total skeleton bone mineral content, and bone mineral density of total body were the same among groups. The type of dietary fiber and phenolic compounds from *P. columbina* could promote antioxidant environment in growing Wistar rats.

✉ Raúl E. Cian
rec_704@yahoo.com.ar

[1] Instituto de Tecnología de Alimentos, CONICET, FIQ – UNL, 1° de Mayo 3250, (3000), Santa Fe, Argentina

[2] Sección Departamental de Fisiología, Facultad de Farmacia, Universidad Complutense de Madrid, 28040 Madrid, Spain

[3] Departamento de Bromatología y Nutrición, Facultad de Farmacia y Bioquímica, (UBA), Buenos Aires, Argentina

Keywords In vivo antioxidant effect · Cecum environment · Colon mucosa · Rhodophyta · Expanded products · Bio-functional foods

Introduction

Seaweeds represent a considerable part of the ocean biomass (mainly located on the coastline), and their use as food dates back to 2700 BC in China. This practice remains widespread currently in Eastern countries such as China, Japan, and Korea (Gressler et al. 2010). In Japanese and Korean cuisine, red algae include "nori" (or "kim") and "laver" from *Pyropia* species. In China, *Sargassum fusiforme* (*Hizikia fusiformis)* ("hijiki"), *Undaria pinnatifida* ("wakame"), *Saccharina japonica* ("makonbu"), and other species of the brown algae *Saccharina*/*Laminaria* are also consumed. Furthermore, green algae from *Ulva* species are consumed as part of traditional Hawaiian cuisine known as "limu palahalaha" (Fitzgerald et al. 2011).

From a nutritional point of view, edible seaweeds are a low-calorie food, with high concentration of minerals, vitamins, and proteins, and low lipid content. Also contain large amounts of polysaccharides (agar, carrageenan, etc.), which cannot be digested in human gastrointestinal tract, and therefore may be regarded as a good source of dietary fiber and a potential source of prebiotics (Fitzgerald et al. 2011). In this regard, seaweed fiber has been proven to be health-related and beneficial, showing antitumor, anticoagulant, antiviral, and antihypercholesterolemic properties in humans (Hamed et al. 2015). Other putative beneficial health effects have been identified, such as decreased blood pressure and sugar and anti-inflammatory, immunomodulatory, and neuroprotective effects, among others (Figueiredo et al. 2016). A mechanistic link has been proposed due to the presence in seaweed of

Published online: 07 September 2017

different bioactive compounds, including sulfated polysaccharides, polyphenols, carotenoids, amino acids, proteins/peptides, and lipids (Holdt and Kraan 2011; Hamed et al. 2015). In this regard, specific in vivo and in vitro functional properties have been attributed to red seaweeds proteins/peptides and polysaccharides because of their unique composition (Cian et al. 2015). According to this, red seaweeds are a very interesting natural source of compounds with biological activity that may be used as functional ingredients.

Aside from the scientific interest in the use of algae functional ingredients, there are various challenges ahead that have to be overcome to use them in new functional foods. Foods should have good sensorial characteristics in order to be accepted by the consumers since very few of them are willing to compromise taste for healthiness in food (Honkanen 2009). In this regard, Cian et al. (2014a) developed an extruded maize products added with red seaweed *Pyropia columbina* in different levels (1.7, 3.5, and 5.2 g (100 g)$^{-1}$). Sensory analysis of the extrudates revealed the most suitable addition rate was 3.5 g (100 g)$^{-1}$, since it allows good sensory characteristics in terms of color, flavor, and mouth texture. In this work, the presence and resistance to in vitro gastrointestinal digestion of bioactive compounds with angiotensin-converting enzyme inhibition and antioxidant properties of selected product was evaluated resulting that in vitro bio-accessibility of bioactive compounds was enhanced in the snacks added with 3.5 g red seaweed (100 g)$^{-1}$. Currently, no research is available on antioxidant in vivo effects of extruded products added with seaweeds. Even less is known about the in vivo effect of red seaweed *P. columbina*. Therefore, the aim of this work was to evaluate the effect of consumption of extruded maize product added with a low level of red seaweed *P. columbina* on colonic and systemic oxidative status using a growing Wistar rat model.

Materials and methods

Reagents

All reagents were obtained from Sigma Chemical Co. (USA).

Raw materials and ingredients

One kilogram of different specimens of *Pyropia columbina* was handpicked in Punta Maqueda (Comodoro Rivadavia, Argentina). The seaweed was processed according to Cian et al. (2014b). *Pyropia columbina* composition in dry base was (as g (100 g)$^{-1}$): protein, 24.61; fat, 0.25; ash, 6.46; total dietary fiber, 48.09; and moisture, 12.79. The most abundant phenolic compounds of *P. columbina* were *epicatechin* and *gallic acid*. In this regard, Cian et al. (2012) reported that epicatechin and gallic acid content was 5.20 ± 0.02 and 2.47 ± 0.02 mg kg^{-1}, respectively. Other phenolic compounds such as cathechin, coumaric acid, quercetin, and kaempferol accounted for 7.5% of the total content. Additional components of *P. columbina* such as saturated and unsaturated fatty acids and minerals were reported by Cian et al. (2014c), being the main saturated and unsaturated fatty acids C16:0 and C20:5 (n-3), respectively. Commercial maize grits corresponded to hull and degermined corn. Its composition in dry base was as follows: (as g (100 g)$^{-1}$): protein, 8.92; fat, 0.27; ash, 0.28; total dietary fiber, 6.96; carbohydrates, 76.17; and moisture, 7.39.

Preparation of extruded maize products added with red seaweed *P. columbina*

Commercial maize grits and red seaweed *P. columbina* were blended in the following ratios: 100:0 and 96.5:3.5 corresponding to maize (M) and maize plus *P. columbina* (MP) samples, respectively. Extrusion was carried out with a 20 DN Brabender single screw extruder using 4:1 compression ratio screw, 150 rpm, 16.5 g (100 g)$^{-1}$ grits moisture, 175 °C barrel temperature, and 160 °C die temperature. The feeding rate of the extruder was at full capacity. Experimental samples were taken after stationary state was established. Extruded M and MP products were ground to obtain flours with a particle size lower than 1.5 mm using a hammer mill (Retsch, Haan, Germany). The composition of extruded M and MP flours has been reported by Cian et al. (2014a).

Animals and diets

Twenty-four (n = 24) male Wistar rats (43.0 ± 4.5 g) were obtained from the Animal Service Laboratory, Facultad de Farmacia y Bioquímica, UBA (Argentina). Throughout the experiment, animals were allowed free access to deionized water and food and were housed in individual stainless steel cages in a temperature (21 ± 1 °C) and humidity (60 ± 10%) controlled room with a 12-h light–dark cycle. They were fed with the following diets (n = 8 per group) during a 60-day period (Table 1).

- *Control group* (*C*): rats fed with a semi-synthetic diet prepared according to the American Institute of Nutrition Diet (AIN 93) (Reeves et al. 1993), having 5 g cellulose (100 g)$^{-1}$ diet as source of fiber (Table 1).
- *Extruded maize product group* (*M*): rats fed with the AIN 93 (Reeves et al. 1993), containing 4.8 g dietary fiber (100 g)$^{-1}$ diet and 12.27 mg total phenolic compounds (100 g)$^{-1}$ diet provided by extruded maize flour.
- *Extruded maize product added with red seaweed P. columbina group* (*MP*): rats fed with the AIN 93 (Reeves et al. 1993), containing 4.89 g dietary fiber (100 g)$^{-1}$ diet and 54.25 mg total phenolic compounds

Table 1 Ingredients, proximal composition and energy of control (C), extruded maize (M), and extruded maize + *Pyropia columbina* (MP) diets

Ingredient (g kg^{-1} diet)	C	M	MP
Casein	200.0	124.6	120.9
Mineral mix (AIN-93M-MX)	35.0	34.2	34.2
Vitamin mix (AIN-93-VX)	10.0	10.0	10.0
L-Cystine	3.0	3.0	3.0
Vitamin A	1.0	1.0	1.0
Soybean oil	69.0	67.0	66.8
Choline bitartrate	71.0	71.0	71.0
Cellulose	50.0	–	–
Extruded maize flour	–	689.2	–
Extruded maize + *Pyropia columbina* flour		–	693.1
Maize dextrine	561.0	–	–
Composition (g $(100\ g)^{-1}$)	C	M	MP
Crude protein ($N \times 6.25$)	17.10 ± 0.26	16.74 ± 0.19	16.84 ± 0.18
Crude lipid	7.00 ± 0.02	6.99 ± 0.01	6.97 ± 0.02
Available carbohydrates	59.30 ± 0.28	59.70 ± 0.22	59.68 ± 0.25
Total dietary fiber	5.00 ± 0.14	4.80 ± 0.10	4.89 ± 0.08
Ash	2.20 ± 0.01	2.21 ± 0.07	2.33 ± 0.08
Moisture	9.40 ± 0.12	9.57 ± 0.13	9.32 ± 0.06
Energy (kcal kg^{-1})	3686.0	3686.7	3688.1

Data are expressed as mean ± SD ($n = 3$)

$(100\ g)^{-1}$ diet provided by extruded maize added with red seaweed *P. columbina* flour.

Chemical composition of diets was determined using the AOAC (1995) procedures and energy was calculated using Atwater factors (Maynard 1994) (Table 1). The analysis of diets confirmed that they were isocaloric and supplied a similar amount of macronutrients, calcium (0.5 g $(100\ g)^{-1}$ diet), and phosphorus (0.3 g $(100\ g)^{-1}$ diet).

This study was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Committee of Health Guide for the Care and Use of Laboratory Animals of the Facultad de Bioquímica y Farmacia, UBA (Buenos Aires, Argentina).

Sampling procedures

Body weights (BW) were recorded once a week and food intakes, every 3 days throughout the experience. Total intakes were calculated. Feed efficiency of diets was determined as the relationship between weight gained by the animal and food consumed, i.e., weight gain/food intake (Albarracín et al. 2016a).

At the end of the experience, rats were anesthetized with an intraperitoneal injection of 0.1 mg $(100\ g)^{-1}$ BW of ketamine hydrochloride, with 0.1 mg $(100\ g)^{-1}$ BW of acepromazine maleate. An abdominal incision was made; blood was withdrawn from the abdominal aorta and centrifuged at 3000–3500 rpm for 20 min at 4 °C. Serum samples were stored at −80 °C and examined within the following 3 days. The liver was excised, weighed, divided in small portions, and stored at − 80 °C for analysis. Cecal contents were weighed and their pH was measured (IQ Scientific ISFET Handheld pH/mV Meter, Cole Parmer, USA). Then, a 1/4 cecal content dilution with 0.01 mol L^{-1} PBS buffer (pH 7.3) was performed. Samples were shaken and centrifuged at 3000×*g* for 20 min and then cecal enzymes, reducing power (RP), and total phenolic compounds (TPC) from supernatant were analyzed. An aliquot of the supernatant was combined with 4 μL of protease inhibitor (Protease Inhibitor Cocktail P8340, Sigma-Aldrich, USA) in order to analyze secretory IgA (sIgA) content by ELISA. All samples were stored in freezer at − 80 °C for later analysis. The colon section was dissected. Sections (2 cm) of colon (proximal and distal) were routinely processed and paraffin embedded. Sections were cut, stained with hematoxylin and eosin (H&E), and processed for immunohistochemical staining. The right femurs were removed according to Albarracín et al. (2014).

Analytical methods

Triglycerides and total cholesterol

Pieces of 250 mg of liver were extracted with chloroform-methanol (2:1) mixture. Aliquots were evaporated and total

cholesterol and triglycerides (TG) were analyzed using enzymatic methods (commercial kits: Colestat Enzimático and TG Color GPO/PAP AA, Wiener Lab (Rosario, Argentina), respectively). Serum total cholesterol and TG were determined using the kits mentioned before.

Proteins

Protein content was determined according to Lowry et al. (1951), using bovine serum albumin (A7906, Sigma-Aldrich) as a standard.

Total phenolic compounds

Total phenolic compounds (TPC) of cecal content were determined according to Cian et al. (2014b), using Folin–Ciocalteu reagent. A standard curve with serial gallic acid solutions (0–100 mg L^{-1}) was used for calibration. Results were expressed as mg gallic acid equivalent g^{-1} cecal content.

Reducing power

The reducing power (RP) was determined according to Cian et al. (2014a). The absorbance was measured at 700 nm. A standard curve with ascorbic acid solutions (0–0.0564 mg mL^{-1}) in phosphate buffer was used. The RP of serum and cecal content was expressed as mg ascorbic acid equivalent g^{-1} protein and mg ascorbic acid equivalent g^{-1} cecal content, respectively.

Cecal enzymes (mucinase, β-glucosidase, and β-glucuronidase)

Mucinase activity of cecal content was determined according to Shiau and Chang (1983). Reducing sugar release by enzyme activity was measure by the Somogyi (1952). A standard curve with glucose solutions (0–150 mg L^{-1}), was used for calibration. The result was expressed as μmol glucose g^{-1} cecal content h^{-1}. β-glucosidase and β-glucuronidase activities of cecal content were determined according to Albarracín et al. (2016b), using a calibration curve with *p*-nitrophenol as a standard. The result was expressed as μmol *p*-nitrophenol g^{-1} cecal content h^{-1}.

Secretory immunoglobulin A

Secretory immunoglobulin A (sIgA) of cecal content was determined by ELISA (enzyme-linked immunosorbent assay) (BD Biosciences, CA, USA). The absorbance was recorded at 450 nm with a microplate reader (Biochrom Asys UVM340 Microplate Reader, UK). The result was expressed as μg sIgA g^{-1} cecal content.

Assay of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were analyzed according to Albarracín et al. (2016a) was used. The results were expressed as μmol malondialdehyde (MDA) g^{-1} protein, using MDA molar extinction coefficient (1.56×10^5 L mol^{-1} cm^{-1}). The same method was used for serum and the results were expressed as μmol MDA g^{-1} protein.

Catalase, glutathione reductase, and glutathione peroxidase activity in the liver

Liver catalase (CAT), glutathione reductase (GR), and glutathione peroxidase activity (GPx) were analyzed according to Albarracín et al. (2016a). CAT activity was calculated using the slope of the absorbance vs. time (s) curve in a logarithmic scale (log 10). Results were expressed as μmol s^{-1} g^{-1} protein using H_2O_2 molar extinction coefficient (0.0394 L $mmol^{-1}$ cm^{-1}). GR activity was expressed as nmol NADPH min^{-1} mg^{-1} protein using the slope of the absorbance vs. time (s) curve, NADPH molar extinction coefficient (3.732 nmol mL^{-1} cm^{-1}), 0.6 cm length of multi well plate, 190 μL final reaction volume, and protein content of liver homogenate. GPx activity was calculated using the slope of absorbance vs. time (s) curve, NADPH molar extinction coefficient (4.354 μmol L^{-1} cm^{-1}), 0.7 cm length of multi well plate, 200 μL final reaction volume, and protein content of liver homogenate. Results were expressed as nmol NADPH min^{-1} mg^{-1} protein.

GSSG and GSH contents

Liver GSSG and GSH contents were determined by the fluorometric method according to Albarracín et al. (2016b). GSSG and GSH contents were expressed as μg g^{-1} of liver. Redox index was calculated as the ratio of GSH and GSSG.

Assessment of apoptosis

Apoptotic cells in colonic sections were detected using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) procedure (Gavrieli et al. 1992). In brief, colonic sections after deparaffinization and rehydration were permeabilized with proteinase K (20 μg mL^{-1}) for 15 min at 37 °C. Thereafter, the sections were quenched of endogenous peroxidase activity using 3% hydrogen peroxide for 10 min. After thorough washing with PBS, the sections were incubated with equilibration buffer for 10 min and then terminal deoxynucleotidyl transferase reaction mixture was added to the sections, except for the negative control, and incubated at 37 °C for 1 h. The reaction was stopped by immersing the sections in saline-sodium citrate buffer for 15 min. The incorporated biotinylated nucleotides were detected by

streptavidin-HRP (1:500) for 30 min at room temperature, and after repeated washings, sections were incubated with DAB until color development (5–10 min). The sections were then mounted after dehydration and counterstained with methyl green. The TUNEL labeling index (%) was calculated as the number of apoptotic cells × 100/total number of cells/crypt column height. For the quantification of the TUNEL labeling index at least 50 perpendicular well-oriented crypts were examined and counted for each animal at 400× magnification. These studies were performed using a Leica DM LB2 microscope and a digital Leica DFC 320 camera (Leica, Spain).

Inmunohistochemical staining

Before immunostaining, the sections were deparaffinized, rehydrated, and then treated in 3% hydrogen peroxide in methanol to inhibit peroxidase activity. They were then boiled in a microwave oven in 0.01 mol L^{-1} sodium citrate buffer (pH 6.0) for 20 min. To avoid background staining, blocking serum was derived from the same species in which the secondary antibody had been raised. After that, the sections were incubated with the following primary antibodies overnight at 4 °C: mouse monoclonal anti-proliferating cell nuclear antigen (anti-PCNA; PC-10) (1/50; Lab Vision Corporation, Bionova Científica SL, Madrid, Spain), rabbit polyclonal anti-COX-2 (1/200), anti-iNOS, anti-CuZnSOD (1/200), anti-GPx-1 (1/200), anti-GR (1/200), anti-CAT (1/200), anti-Nrf2 (1/50), and anti-NF-κβ (Santa Cruz Biotechnology, Quimigen, Spain). After washing with PBS, the sections were covered for 30 min at room temperature with biotinylated goat anti-mouse or goat anti-rabbit (1:400) as secondary antibodies (Santa Cruz Biotechnology). Immunochemical staining was performed for 30 min using streptavidin-biotin-conjugated horseradish peroxidase (HRP) (Sigma-Aldrich, Spain) and visualized by incubation with 3,3′-diaminobenzidine (DAB) (Sigma-Aldrich) for 10 min at room temperature. The sections were counterstained with Harris's hematoxylin, dehydrated, and mounted. Brown color indicates specific protein immunostaining and light blue color indicates nuclear hematoxylin staining. Positive and negative controls were used during the optimization of the methods.

For quantification of the PCNA labeling index % (LI), at least 20 perpendicular well-oriented crypts were examined in each animal under light microscopy at 400× magnification. LI was calculated as the number of positive nuclei × 100/total number of cells/crypt column height.

The staining intensity of epithelial COX-2, iNOS, SOD, CAT, GR, and GPx expression were evaluated according to a semi-quantitative immunohistochemical scoring system as follows: scores 0–4: 0, none; 1, equivocal; 2, low; 3, moderate; 4 and above intense. The number of nuclear Nrf2 positive cells was quantified by a percentage score with grading between 0 and 4: 0, no nuclear staining; 1, 1–10%; 2, 10–30%; 3, 30–60%; and 4, 60–100% positive cells. An additional evaluation was performed in which the stained cells were attributed either to the basal, the middle, or the luminal crypt compartments.

The crypt length was measured from H&E slices and was determined as a distance (μm) between the basal side of the lamina epithelialis at the bottom of the crypt and the apical side of the lamina epithelialis at the top of the crypt. Only crypts with an open longitudinal crypt axis were analyzed.

Total skeleton bone mineral content, bone mineral density, apparent calcium absorption, and femur calcium content

Before the end of the experiment total skeleton bone mineral content (BMC) and bone mineral density (BMD) were determined in vivo under light anesthesia (0.1 mg (100 g)$^{-1}$ body weight of ketamine hydrochloride + 0.1 mg (100 g)$^{-1}$ body weight of acepromazine maleate) with a total body scanner by dual-energy X-ray absorptiometry (DXA) provided with a specifically designed software for small animals (DPX Alpha, Small Animal Software, Lunar Radiation Corp., USA) as previously described by Albarracín et al. (2014). BMC and BMD were expressed as mg g^{-1} body weight and mg cm^{-2}, respectively.

Feces were dried under infrared light and pounded. Diets and feces were processed by wet ash with nitric acid using Parr bombs. Calcium concentration in diets, feces, and bones was determined using an atomic absorption spectrophotometer. In the case of femurs, the amount of calcium was expressed as the total content (Albarracín et al. 2014).

Food intake was determined and feces were collected and weighted during the last 3 days of the experiment and were used to calculate apparent calcium absorption (%) as [(daily calcium intake − fecal calcium excretion) / daily calcium intake] × 100.

Statistical analysis

Data were presented as the arithmetic mean ± S.E.M (Standard Error of the Mean) for each treatment group (n = 8). One-way analysis of variance (ANOVA) was performed and the statistical differences among samples were determined using the LSD test (least significant difference). Significance was accepted at $p < 0.05$.

The data obtained of the semi-quantitative immunohistochemical staining were comparatively analyzed using the Kruskal–Wallis test and multiple comparisons were made by applying Tukey's test. The level of significance was set to 5% ($p < 0.05$) on all tests. Results were expressed as means with their standard errors. All statistical analyses were performed with the Statgraphics Plus version 5.1 software (Statgraphics, USA).

Table 2 Total intake, daily intake, body weight gain (BWG), feed efficiency, cecal content, cecal moisture, cecal pH, mucinase, β-glucosidase, and β-glucuronidase activity, secretory immunoglobulin A (sIgA), reducing power (RP), and total phenolic compounds (TPC) in rats fed with control (C), extruded maize (M), and extruded maize + *Pyropia columbina* (MP) diets

Diets	C	M	MP	*p* value
Total intake (g 60 days^{-1})	948.2 ± 9.9^{b}	978.9 ± 15.2^{b}	735.9 ± 11.6^{a}	*0.0000*
Daily intake (g day^{-1})	17.3 ± 0.5^{b}	17.7 ± 0.3^{b}	15.9 ± 0.3^{a}	*0.0006*
BWG (g 60 days^{-1})	271.3 ± 15.9^{b}	271.4 ± 11.0^{b}	217.9 ± 7.9^{a}	*0.0077*
Feed efficiency (g body weight g^{-1} diet)	0.26 ± 0.01^{a}	0.26 ± 0.01^{a}	0.26 ± 0.01^{a}	*0.3596*
Cecal content (mg g^{-1} body weight)	5.54 ± 0.11^{a}	5.23 ± 0.03^{a}	7.32 ± 0.01^{b}	*0.0004*
Cecal moisture (%)	15.33 ± 0.02^{a}	15.61 ± 0.01^{a}	18.32 ± 0.01^{b}	*0.0015*
Cecal pH	7.06 ± 0.06^{c}	6.74 ± 0.11^{b}	6.35 ± 0.13^{a}	*0.0001*
Mucinase (μmol glucose g^{-1} cecal content h^{-1})	100.87 ± 3.49^{a}	97.35 ± 2.63^{a}	33.20 ± 2.12^{b}	*0.0012*
β-D-Glucosidase (μmol *p*-nitrophenol g^{-1} cecal content h^{-1})	3.61 ± 0.04^{a}	3.00 ± 0.39^{a}	3.59 ± 0.81^{a}	*0.7005*
β-D-Glucuronidase (μmol *p*-nitrophenol g^{-1} cecal content h^{-1})	6.85 ± 0.11^{a}	7.14 ± 0.38^{a}	6.51 ± 0.24^{a}	*0.3781*
sIgA (μg g^{-1} cecal content)	11.55 ± 2.32^{a}	14.34 ± 0.84^{a}	24.71 ± 1.96^{b}	*0.0000*
RP (mg ascorbic acid g^{-1} cecal content)	121.50 ± 9.31^{a}	125.90 ± 10.3^{a}	268.97 ± 6.67^{b}	*0.0001*
TPC (mg gallic acid equivalent g^{-1} cecal content)	0.12 ± 0.01^{a}	0.18 ± 0.00^{a}	1.76 ± 0.03^{b}	*0.0020*

Data are expressed as mean ± SEM, (n = 8 per group). Different letters mean significant differences between samples analyzed by LSD test ($p < 0.05$). Italic values indicates significance by LSD test

Results

Effects on food intake and weight gain

Table 2 shows total intake, daily intake, body weight gain (BWG), and feed efficiency of rats fed with C, M, and MP diets. Animals fed with MP diet had lower total and daily intake than M and C diets, resulting in a lower BWG along 60 days. However, there were no significant differences in feed efficiency among diets.

Effect on cecum environment and colon mucosa

As shown in Table 2, rats fed with MP showed higher cecal content, sIgA, RP and TPC than those consuming M and C diets. In this regard, RP and TPC of MP diet was 114 and 1000% higher than that found for M and C diets, respectively. However, rats fed with MP showed lower cecal pH and mucinase activity than M and C diets. In spite of this, there were not significant differences in β-glucosidase and β-glucuronidase activity among diets.

Figure 1 shows the expression of copper zinc superoxide dismutase (CuZnSOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), and Nrf2 transcription factor in proximal and distal colon of rats fed with C, M, and MP diets. Rats fed whit MP showed lower CuZnSOD and higher CAT expression than M and C diets (Fig. 1a, b), both in proximal and distal colon. In addition, GR and Nrf2 transcription factor expression increased in the proximal and distal colon, whereas GPx expression did not change (Fig. 1c–e). Finally, SOD/CAT ratio of MP was lower than that found for M and C diets (Fig. 1f).

Regarding cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and NF-κβ transcription factor expression, there were not significant differences between diets in proximal colon ($p < 0.05$), but all of them were lower in Wistar rats fed with MP in distal colon (Table 3). On the other hand, no significant difference in crypt depth (cells/hemicryts), proliferating cell nuclear antigen (PCNA) and TUNEL in proximal and distal colon of rats fed with C, M, and MP were observed (Table 3).

Systemic effect

As shown in Table 4, the lower values of triglycerides (TG) and total cholesterol in serum and liver corresponded to rats fed with MP. On the other hand, MP diet showed higher serum and liver RP than M and C diets. Also, serum and liver TBARS of rats fed with MP diet were significantly lower than those found for M and C (Table 4), indicating an antioxidative environment. In line with this, rats fed with MP diet showed higher GSH than those fed with M or C diets (Fig. 2a), GSSG being lower than that found for C (Fig. 2b). According with this, redox index (GSH/GSSG) obtained for MP was significantly higher than that of M and C (Fig. 2c). Moreover, a direct relationship between RP and redox index (r^2 = 0.99) was observed. Rats fed with MP diet presented increased GR activity coupled with unmodified activity of GPx (Fig. 2d, e) and higher CAT activity than M and C diets (Fig. 2f).

As shown in Table 4, there were not significant differences in total skeleton bone mineral content (BMC),

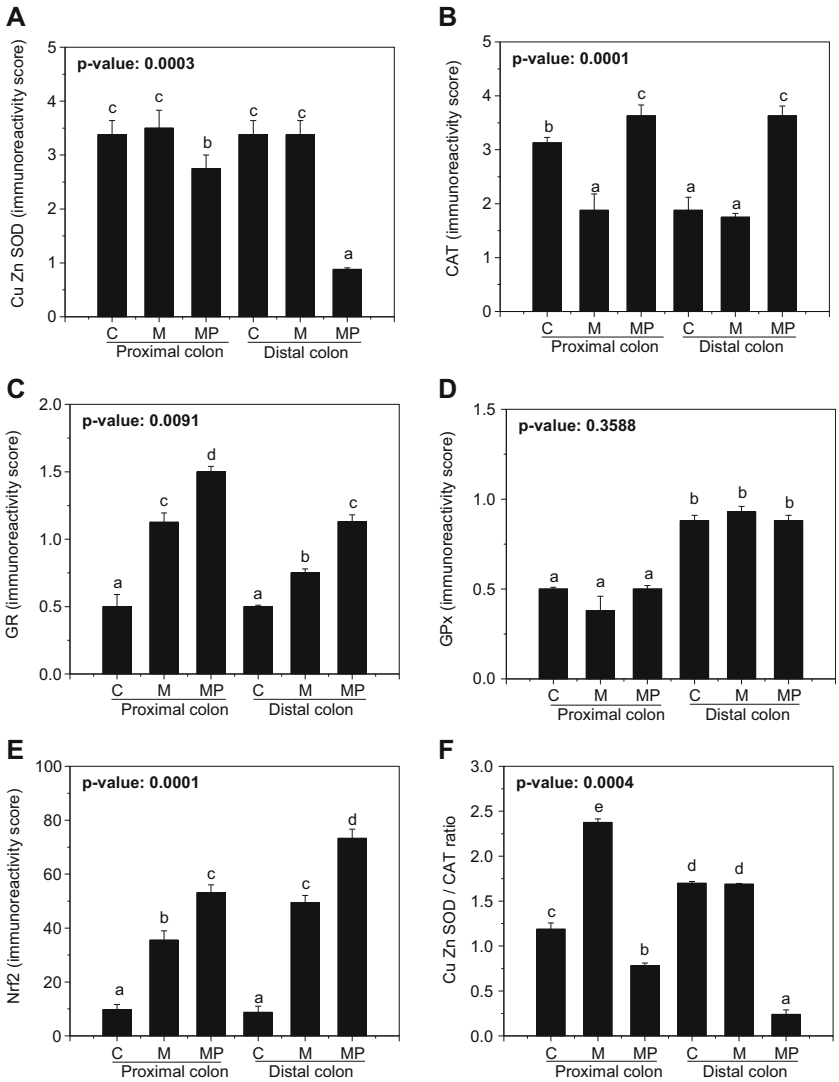

Fig. 1 Copper zinc superoxide dismutase (Cu Zn SOD) (**a**), catalase (CAT) (**b**), glutathione reductase (GR) (**c**), glutathione peroxidase (GPx) (**d**), Nrf2 transcription factor (**e**), and Cu Zn SOD/CAT ratio (**f**) in proximal and distal colon of rats fed with control (C), extruded maize (M), and extruded maize + *Pyropia columbina* (MP) diets. Data are expressed as mean ± SEM, ($n = 8$ per group). Different letters mean significant differences between samples analyzed by Tukey's test ($p < 0.05$)

bone mineral density (BMD), and apparent calcium absorption among diets. Also, there were not significant differences in femur calcium content among diets (p value: 0.5098), being 23.22 ± 0.57, 21.45 ± 2.65, and 22.26 ± 3.23 mg $(100\ g)^{-1}$ for C, M, and MP diets, respectively.

Discussion

Effects on food intake and weight gain

Tsuge et al. (2004) found the ingestion of diet containing 5 g sulfated galactans $(100\ g)^{-1}$ diet from *P. yezoensis* decreased

Table 3 Cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), NF-κβ transcription factor, crypt depth (cells/hemicryts), proliferating cell nuclear antigen (PCNA), and deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) in the proximal and distal colon of rats fed with control (C), extruded maize (M), and extruded maize + *Pyropia columbina* (MP) diets

	C proximal	M proximal	MP proximal	C distal	M distal	MP distal	p value
COX-2 (immunoreactivity score)	1.14 ± 0.24^{b}	0.75 ± 0.25^{b}	0.88 ± 0.30^{b}	0.88 ± 0.13^{b}	1.00 ± 0.17^{b}	0.50 ± 0.19^{a}	*0.0001*
iNOS (inmunoreactivity score)	1.00 ± 0.27^{b}	1.13 ± 0.18^{b}	1.05 ± 0.25^{b}	1.13 ± 0.30^{b}	1.07 ± 0.37^{b}	0.58 ± 0.38^{a}	*0.0025*
NF-κβ (inmunoreactivity score)	1.38 ± 0.65^{b}	1.50 ± 1.27^{b}	1.28 ± 0.63^{b}	1.31 ± 1.16^{b}	1.35 ± 0.30^{b}	0.95 ± 0.84^{a}	*0.0018*
CRYPT DEPTH (cells/hemicryts)	32.17 ± 1.56^{a}	31.93 ± 0.84^{a}	31.79 ± 1.11^{a}	30.67 ± 1.44^{a}	30.83 ± 1.65^{a}	30.29 ± 1.00^{a}	*0.3320*
PCNA (%)	58.56 ± 1.69^{b}	58.39 ± 2.99^{b}	58.60 ± 1.89^{b}	50.15 ± 2.29^{a}	48.97 ± 2.65^{a}	54.96 ± 1.75^{a}	*0.3946*
TUNEL (%)	0.66 ± 0.12^{a}	1.03 ± 0.25^{a}	0.90 ± 0.08^{a}	0.68 ± 0.17^{a}	0.79 ± 0.25^{a}	0.68 ± 0.12^{a}	*0.8056*

Data are expressed as mean ± SEM ($n = 8$ per group). Different letters mean significant differences between samples analyzed by Tukey's test ($p < 0.05$). Italic values indicates significance by LSD test

Table 4 Triglycerides (TG), total cholesterol, thiobarbituric acid reactive substances (TBARS), and reducing power (RP) in serum and liver weight; triglycerides (TG), total cholesterol, redo index (GSSG/GSH), and reducing power (RP) in liver and apparent calcium absorption; total skeleton bone mineral content g^{-1} body weight (BMC); and bone mineral density of total body (BMD) of rats fed with control (C), extruded maize (M), and extruded maize + *Pyropia columbina* (MP) diets

		Diets			*p* value
		C	M	MP	
Serum	TG (g L^{-1})	0.85 ± 0.01^{b}	0.87 ± 0.01^{b}	0.16 ± 0.01^{a}	*0.0001*
	Total cholesterol (g L^{-1})	0.46 ± 0.06^{b}	0.49 ± 0.04^{b}	0.21 ± 0.03^{a}	*0.0015*
	TBARS (nmol malondialdehyde g^{-1} protein)	57.2 ± 4.2^{b}	57.8 ± 1.9^{b}	39.4 ± 2.2^{a}	*0.0002*
	RP (mg ascorbic acid equivalent g^{-1} protein)	1.20 ± 0.1^{a}	1.21 ± 0.1^{a}	2.01 ± 0.1^{b}	*0.0022*
Liver	TG (μmol g^{-1} tissue)	30.76 ± 1.30^{c}	25.43 ± 2.16^{b}	20.10 ± 1.15^{a}	*0.0012*
	Total cholesterol (mg g^{-1} tissue)	7.78 ± 0.32^{c}	6.40 ± 0.20^{b}	5.13 ± 0.32^{a}	*0.0002*
	TBARS (nmol malondialdehyde g^{-1} protein)	225.2 ± 5.5^{b}	206.4 ± 8.5^{b}	152.5 ± 5.8^{a}	*0.0001*
	RP (mg ascorbic acid equivalent g^{-1} protein)	18.1 ± 1.2^{a}	21.3 ± 0.6^{a}	28.6 ± 1.2^{b}	*0.0010*
Calcium balance	BMC (mg g^{-1} body weight)	11.79 ± 0.46^{a}	10.28 ± 2.62^{a}	11.32 ± 3.40^{a}	*0.6558*
	BMD (mg cm^{-2})	259.75 ± 2.09^{a}	258.17 ± 4.22^{a}	259.13 ± 7.02^{a}	*0.9141*
	Apparent calcium absorption (%)	72.71 ± 6.62^{a}	71.45 ± 6.97^{a}	67.27 ± 10.19^{a}	*0.5597*

Data are expressed as mean ± SEM, ($n = 8$ per group). Different letters mean significant differences between samples analyzed by LSD test ($p < 0.05$). Italic values indicates significance by LSD test

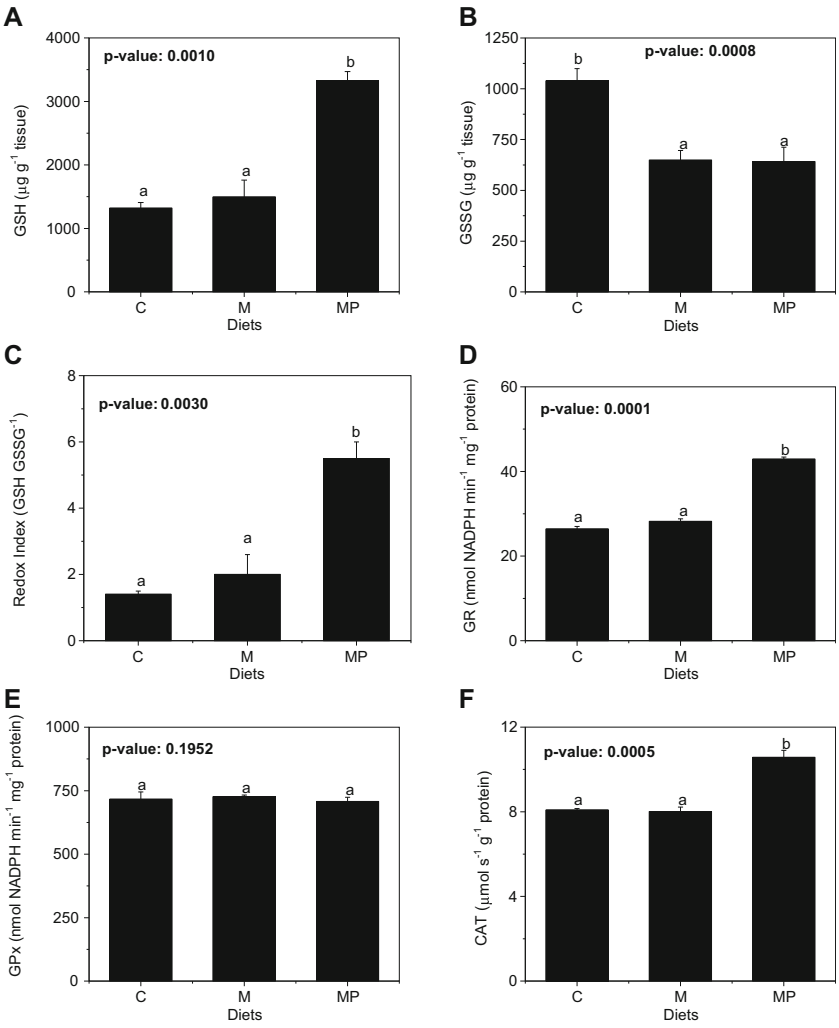

Fig. 2 Glutathione content (GSH) (**a**), glutathione disulfide content (GSSG) (**b**), redox index (GSH/GSSG) (**c**), glutathione reductase activity (GR) (**d**), glutathione peroxidase activity (GPx) (**e**), and catalase activity (CAT) (**f**) in the liver of rats fed with control (C), extruded maize (M), and extruded maize + *Pyropia co*lumbina (MP) diets. MDA: malondialdehyde. Data are expressed as mean ± SEM, ($n = 8$ per group). Different letters mean significant differences between samples analyzed by LSD test ($p < 0.05$)

food intake and body weight gain respect to control diet (cellulose) in Sprague-Dawley rats. Similar results were found by Dvir et al. (2000) who investigated the effects of 6.7 g dietary fiber (100 g)$^{-1}$ diet from red microalga *Porphyridium* sp. on gastrointestinal physiology and lipid metabolism in male Sprague-Dawley rats. They observed significantly lower weight gain in polysaccharide-fed rats with respect to control (cellulose), attributing this effect to the high viscosity of the intestinal contents following food consumption. Thus, the increased viscosity from gel formation with some algae-polysaccharides may promote satiety with delayed gastric emptying, increased viscosity of digest and slowed nutrient absorption in the small intestine (Kumar and Brown 2013). Taking into account that the main sulfated galactans of *P. columbina* are porphyrans (Cian et al. 2014b), the lower food intake and BWG in rats fed with MP could be due to satiating effects of these polysaccharides.

As mentioned before, there were not significant differences in feed efficiency among diets. This also was observed by Gudiel-Urbano and Goñi (2002) who studied the effect of edible seaweeds (*Undaria pinnatifida* and *Pyropia ternera*) on metabolic activity of intestinal microflora in rats fed with diets supplemented with cellulose, Nori, or Wakame.

Effect on cecum environment and colon mucosa

The increase in cecal content of rats fed with MP is due to the high amount of water held by the cecum content in comparison with M and C diets, being cecal moisture of MP higher than that found for M and C diets. Diets supplemented with red seaweed such as *Mastocarpus stellatus* (3.2 g dietary fiber (100 g)$^{-1}$ diet) and *P. tenera* (5 g dietary fiber (100 g)$^{-1}$ diet) showed considerable in vivo water retention and swelling capacity (Gudiel-Urbano and Goñi 2002; Gómez-Ordóñez et al. 2012), increasing weight and moisture of cecal content in seaweed-fed rats than control (cellulose). These hydrating properties were associated to the soluble fiber provided by red seaweeds (sulfated galactans). Thus, the higher moisture of MP cecal content could be associated to dietary fiber of *P. columbina* mainly composed by porphyrans and carrageenans as soluble fiber (Cian et al. 2014b). Other diets containing red and brown seaweeds such as *Gigartina pistillata*, *Himanthalia elongata*, and *Saccharina latissima* produced similar effects in cecal parameters of Wistar rats (Villanueva et al. 2014; Jiménez-Escrig et al. 2013). On the other hand, the reduction of cecal pH in rats fed with MP could be associated to short chain fatty acids (SCFAs) production from colonic fermentation such as acetate, propionate, and butyrate. Jiménez-Escrig et al. (2013) found SCFAs in the cecum were significantly higher in the seaweed-fed group than in the control. Also, molar proportions of both acetic and propionic were significantly higher in the seaweed-fed group than in the control (29.8 ± 5.7 and 15.8 ± 2.4 vs. 10.6 ± 1.6 and 19.9 ± 1.6%, respectively). Similar results were found for healthy Wistar rats fed with red seaweed *M. stellatus*, the molar proportion of acetate and propionate being significantly higher than for the control (Gudiel-Urbano and Goñi 2002; Gómez-Ordóñez et al. 2012). Thus, seaweed diet modulated the distribution of SCFAs through promotion of a relative increased level of acetic and propionic acids in the cecum, reducing intraluminal pH (Villanueva et al. 2014).

Differences found in the activity of cecal enzymes (mucinase, β-glucosidase, and β-glucuronidase) among diets can be due to the type of fiber provided by red seaweed. In this regard, the type and fermentation of dietary fiber can affect the composition of colonic microbiota and modify the fermentative activity of bacteria (Gómez-Ordóñez et al. 2012). In agreement with this, Gudiel-Urbano and Goñi (2002) reported a significant decrease of β-glucuronidase, azoreductase, nitroreductase, and nitrate reductase in rats fed with brown seaweed *U. pinnatifida* diet respect to the cellulose-fed group (control). However, the intake of red seaweed *P. tenera* diet did not affect β-glucosidase activity.

It is noteworthy that mucinase hydrolyses the protective mucin coat in the intestinal wall and exposes the underlying mucosa to the luminal carcinogens that are released by β-glucuronidase activity. Thus, increased gut microfloral β-glucuronidase and mucinase activity are considered precancer events associated with increased risk of colon cancer (Devasena and Menon 2003; Goñi et al. 2005). Therefore, a reduction of mucinase activity could be associated with a beneficial effect on the intestinal environment. Sulfated galactans such as porphyrans from red seaweeds are considered as potential prebiotics, since they escape digestion in the small intestine, but undergo bacterial fermentation in the bowel, thus beneficially affecting the intestinal microbiota (Kumar and Brown 2013; Macfarlane et al. 2006). The administration of whole red seaweeds (*Sarcodiotheca gaudichaudii* and *Chondrus crispus*) to chicken has been described to affect the intestinal mucosa, enhancing villus height and villus surface area, as well as the intestinal microbiota, increasing the abundance of beneficial bacteria (e.g., *Bifidobacterium longum* and *Streptococcus salivarius*) and, importantly, reducing the prevalence of *Clostridium perfringens*. These microbiota modifications were accompanied by higher SCFAs concentrations, pointing to an overall prebiotic effect of the seaweeds (Kulshreshtha et al. 2014). In line with this, MP diet increased sIgA respect to M and C diets, which could imply a beneficial effect on intestinal mucosal barrier function. Similar results were found for pigs fed with seaweeds (Katayama et al. 2011). In these works, the beneficial effect on intestinal mucosal barrier function was associated to an increase in sIgA by activation of gut immunity.

MP diet had higher contents of TPC than M and C diets (69 vs. 34 and 0 mg gallic acid equivalent (100 g)$^{-1}$ diet for MP, M, and C, respectively). Furthermore, iota, kappa, and lambda

carrageenans from red seaweeds have in vitro and in vivo antioxidant activities (Cian et al. 2014b; Gómez-Ordóñez et al. 2012; Jiménez-Escrig et al. 2012). Thus, the antioxidant capacity (RP) found in cecum could be related to phenolic content and sulfated polysaccharides from red seaweed *P. columbina*. A similar effect was found for Wistar rats fed with diets containing red and brown seaweed such as *M. stellatus* and *S. latissima*, respectively (Gómez-Ordóñez et al. 2012; Jiménez-Escrig et al. 2013). Since a high exposure to exogenous reactive oxygen species is described in the gut environment, it is likely that an increase of RP in this compartment would have a positive health influence (Jackson et al. 2002). Overall, these results suggest that the use of *P. columbina* as a source of dietary fiber with antioxidant compounds such as polyphenols would promote a beneficial reducing environment inside the cecum.

López-Oliva et al. (2013) evaluated the effect of grape antioxidant dietary fiber on oxidative environment of the distal colonic mucosa in Wistar rats. They found a reduction in the activities and expression of SOD and suggested that polyphenols from grape antioxidant dietary fiber possibly elicits a decrease in the dismutation of O_2^- to H_2O_2 in the colonic mucosa and improved the content and activity of cytosolic CAT, increased GR activity and expression and did not modified GPx activity in colonic mucosa, indicating that induction of CAT could be an important mechanism whereby grape antioxidant dietary fiber acts to reduce H_2O_2 in mucosa of treated rats. Taking into account that TPC of MP was higher than the other diets, the diminution of CuZnSOD expression coupled with the increased GR and CAT expression, and unmodified GPx expression could be due to phenolic compounds from *P. columbina*, yielding a system that can eliminate H_2O_2 faster than it is formed. In agreement with this, Nrf2 transcription factor expression in proximal and distal colon of rats fed with MP was higher than other diets. This transcription factor regulates the inducible expression of numerous detoxifying and antioxidant genes. It binds to a specific DNA sequence known as ARE (antioxidant response element) that can be activated by several electrophiles compounds of diverse chemical nature (Königsberg Fainstein 2007). These results suggest that MP diet upregulates the expression of antioxidant enzymes. In agreement, SOD/CAT ratio of MP was lower than that found for M and C diets, indicating an enhancement of the antioxidant status of rat colonic mucosa at proximal and distal colon levels.

NF-κβ increases expression of the genes for many cytokines, enzymes, and adhesion molecules, one of them is for iNOS. The expression of this enzyme is increased in colonic epithelial cells in patients with ulcerative colitis reflected by an increased amount of nitric oxide. COX-2, another inducible enzyme regulated by NF-κβ, is responsible for the increased production of prostaglandins and thromboxane in inflammatory diseases (Barnes and Karin 1997). It has been shown that red seaweed compounds such as porphyrans and proteins exert anti-inflammatory effects. Protein fractions of *P. columbina* exert induction of anti-inflammatory IL-10 secretion in isolated macrophages and T lymphocytes (Cian et al. 2012). Also, a glycoprotein isolated from *P. yezoensis* inhibits pro-inflammatory cytokine (TNF-α and IL-1β) production in LPS-stimulated RAW 264.7 mouse macrophages. This effect was accompanied by the inhibition of NO and ROS production and expression of iNOS and COX-2 (Perez-Recalde et al. 2014). Furthermore, porphyrans from *P. yezoensis* and *P. tenera* inhibit the expression of iNOS in LPS-stimulated RAW264.7 cells, indicating an anti-inflammatory activity (Jiang et al. 2012; Isaka et al. 2015). In this way, the lower cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and NF-κβ transcription factor expression in distal colon of rats fed with MP, could indicate an anti-inflammatory effect in this portion of colon exerted by porphyrans and proteins of *P. columbina*.

Finally, crypt depth (cells/hemicryts), proliferating cell nuclear antigen (PCNA) and TUNEL in proximal and distal colon were unaffected by the type of diet, indicating a balance between proliferation and epithelial cell death to maintain tissue homeostasis.

Systemic effect

Consumption of soluble fiber from seaweeds is associated with changes in lipid metabolism, including a hypocholesterolemic effect (Figueiredo et al. 2016). The results obtained in this study could indicate that soluble fiber provided by *P. columbina* produce a lipid-lowering effect. In this regard, the sulfated polysaccharides from *Ulva pertusa* reduced serum TG, total cholesterol, and low-density lipoprotein cholesterol (LDL-cholesterol) in mice. Moreover, polysaccharides from red microalga *Porphyridium* sp. had a hypocholesterolaemic effect (Dvir et al. 2000). Also, Gómez-Ordóñez et al. (2012) reported seaweed intake for 28 days reduced significantly serum and liver TG and total cholesterol in healthy rats fed with red seaweed *M. stellatus*, generating a beneficial effect on lipid metabolism respect to control (cellulose). The significant decreases of TG and total cholesterol were attributed to via propionate synthesis by colonic fermentation. Also, Villanueva et al. (2014) found the intake of red seaweed *G. pistillata* diminished the hepatic TG. Finally, the intake of extruded maize added with red seaweed *P. columbina* diet for 60 days produced a beneficial effect on lipid metabolism in healthy growing Wistar rats. Nevertheless, more research is needed in order to elucidate the lipid-lowering mechanism.

Rats fed with MP produced antioxidative environment in serum and liver. In this way, it has been suggested that polyphenols from seaweeds can reduce the in vivo formation of reactive species (Iwai 2008), modifying the enzymatic

antioxidant system (Kim et al. 2008; Figueiredo et al. 2016). This was also observed in our work, where the enzymatic activity of GR and CAT was modified, increasing redox index (GSH/GSSG). This would suggest that phenolic compounds from MP after gastrointestinal digestion could be potentially bio-accessible. Note that epicatechin and gallic acid are the most abundant phenolic compounds of *P. columbina* (Cian et al. 2012). In this regard, Cian et al. (2014a) reported that extruded maize product added with red seaweed *P. columbina* showed higher in vitro dialyzability of TPC (0.83 vs. 0.77 mg gallic acid g^{-1} dialysate) and antioxidant capacity (36.6 vs. 17.2% DPPH inhibition, 2.4 vs. 2.15 mM TEAC, 45.7 vs. 27.7 mg ascorbic acid L^{-1} reducing power and 99.4 vs. 79.8% copper-chelating activity) than extruded maize product (M diet). The highest antioxidant capacity of MP dialyzed was attributed to polyphenols and peptides provided by *P. columbina*. Therefore, as mentioned before, phenolic compounds and peptides released or generated from MP after gastrointestinal digestion would be bio-accessible and could exert a systemic antioxidant effects.

Effect on total skeleton bone mineral content, bone mineral density, apparent calcium absorption, and femur calcium content

Rats fed with MP diet showed lower cecal pH, indicating that some fermentation took place, but this was not enough to increase Ca absorption and bioavailability measured as bone mineral density. Similar results were found by Villanueva et al. (2014) in hypercholesterolemic Wistar rats fed with red seaweed *G. pistillata*. They found the mineral balance of *Gigartina*-fed rats was unaffected, and there were no significant difference in the apparent absorption and true retention of Ca and Mg. On the other hand, the femur is a representative bone tissue because it is subject to fair remodeling by the ongoing exercise stimulus (Albarracín et al. 2014). Our results showed that the intake of MP diet did not modify femur calcium content.

Conclusions

We have demonstrated a diet containing extruded maize added with a low level of red seaweed *P. columbina* (3.5 g 100 g^{-1}) exerted a significantly antioxidant effect on colon mucosa, serum, and liver in growing Wistar rats. Also, a beneficial effect on intestinal mucosal barrier function was observed, manifested as a reduction in cecal pH, mucinase activity, and COX-2, iNOS and NF-κβ transcription factor expression couple with an increase of sIgA. Interestingly, extruded maize product diet without *P. columbina* showed similar results to those found for control diet, indicating that the type of dietary fiber and phenolic compounds from *P. columbina* could promote several health benefits in growing Wistar rat model, validating the in vitro results previously observed. Further research on human trials need to be undertaken before any health claims for extruded maize product added with red seaweed *P. columbina* as functional foods.

Acknowledgements All authors read and approved the final manuscript. The authors are thankful to CAI + D 2011 PI 0292 LI of the Universidad Nacional del Litoral for the financial support.

References

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

Albarracín M, Weisstaub A, Zuleta A, Drago S (2016a) Extruded whole grain diets based on brown, soaked and germinated rice. Effects on the lipid profile and antioxidant status of growing Wistar rats. Part II. Food Funct 7:2729–2735

Albarracín M, Weisstaub A, Zuleta A, Drago S (2016b) Extruded whole grain diets based on brown, soaked and germinated rice. Effects on cecum health, calcium absorption and bone parameters of growing Wistar rats. Part I. Food Funct 7:2722–2728

AOAC (1995) Official methods of analysis of AOAC International, 16th edn. Horowitz, Washigton DC

Barnes P, Karin M (1997) Nuclear factor-κβ—a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 336:1066–1071

Cian R, López-Posadas R, Drago S, Sánchez de Medina F, Martínez-Augustín O (2012) Immunomodulatory properties of the protein fraction from *Phorphyra columbina*. J Agric Food Chem 60: 8146–8154

Cian R, Caballero M, Sabbag N, Gonzalez R, Drago S (2014a) Bioaccessibility of bioactive compounds (ACE inhibitors and antioxidants) from extruded maize products added with a red seaweed *Porphyra columbina*. LWT-Food Sci Technol 55:51–58

Cian R, Fajardo M, Alaiz M, Vioque J, González R, Drago S (2014b) Chemical composition, nutritional and antioxidant properties of the red edible seaweed *Porphyra columbina*. Int J Food Sci Nutr 65: 299–305

Cian R, Salgado P, Drago S, González R, Mauri A (2014c) Development of naturally activated edible films with antioxidant properties prepared from red seaweed *Porphyra columbina* biopolymers. Food Chem 146:6–14

Cian R, Drago S, Sánchez de Medina F, Martínez-Augustin O (2015) Proteins and carbohydrates from red seaweeds: evidence for beneficial effects on gut function and microbiota. Mar Drugs 13:5358–5383

Devasena T, Menon V (2003) Fenugreek affects the activity of β-glucuronidase and mucinase in the colon. Phytother Res 17:1088–1091

Dvir I, Chayoth R, Sod-Moriah U, Shany S, Nyska A, Stark A, Madar Z, Arad S (2000) Soluble polysaccharide and biomass of red microalga *Porphyridium* sp. alter intestinal morphology and reduce serum cholesterol in rats. Br J Nutr 84:469–476

Figueiredo F, Encarnação T, Campos M (2016) Algae as functional foods for the elderly. Food Nutr Sci 7:1122–1148

Fitzgerald C, Gallagher E, Tasdemir D, Hayes M (2011) Heart health peptides from macroalgae and their potential use in functional foods. J Agric Food Chem 59:6829–6836

Gavrieli Y, Sherman Y, Ben-Sasso S (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493–501

Gómez-Ordóñez E, Jiménez-Escrig A, Rupérez P (2012) Effect of the red seaweed *Mastocarpus stellatus* intake on lipid metabolism and antioxidant status in healthy Wistar rats. Food Chem 135:806–811

Goñi I, Jiménez-Escrig A, Gudiel M, Saura-Calixto F (2005) Artichoke (*Cynara scolymus* L) modifies bacterial enzymatic activities and antioxidant status in rat cecum. Nutr Res 25:607–615

Gressler V, Yokoya N, Fujii M, Colepicolo P, Mancini J, Torres R, Pinto E (2010) Lipid, fatty acid, protein, amino acid and ash contents in four Brazilian red algae species. Food Chem 120:585–590

Gudiel-Urbano M, Goñi I (2002) Effect of edible seaweeds (*Undaria pinnatifida* and *Porphyra ternera*) on the metabolic activities of intestinal microflora in rats. Nutr Res 22:323–331

Hamed I, Özogul F, Özogul Y, Regenstein J (2015) Marine bioactive compounds and their health benefits: a review. Compr Rev Food Sci 14:446–465

Holdt S, Kraan S (2011) Bioactive compounds in seaweed: functional food applications and legislation. J Appl Phycol 23:543–597

Honkanen P (2009) Consumer acceptance of (marine) functional food. In: Luten J (ed) The Nordic Network on Marine Functional Food (MARIFUNC). Wageningen Academic Publishers, Oslo, pp 141–154

Isaka S, Cho K, Nakazono S, Abu R, Ueno M, Kim D, Oda T (2015) Antioxidant and anti-inflammatory activities of porphyran isolated from discolored nori (*Porphyra yezoensis*). Int J Biol Macromol 74:68–75

Iwai K (2008) Antidiabetic and antioxidant effects of polyphenols in brown alga *Ecklonia stolonifera* in genetically diabetic KK-A^y mice. Plant Foods Hum Nutr 63:163–169

Jackson C, Dini J, Lavandier C, Rupasinghe H, Faulkner H, Poysa V, Buzzell D, De Grandis S (2002) Effects of processing on the content and composition of isoflavones during manufacturing of soy beverage and tofu. Process Biochem 37:1117–1123

Jiang Z, Hama Y, Yamaguchi K, Oda T (2012) Inhibitory effect of sulphated polysaccharide porphyran on nitric oxide production in lipopolysaccharide-stimulated RAW264.7 macrophages. J Biochem 151:65–74

Jiménez-Escrig A, Gómez-Ordóñez E, Rupérez P (2012) Brown and red seaweeds as potential sources of antioxidant nutraceuticals. J Appl Phycol 24:1123–1132

Jiménez-Escrig A, Gómez-Ordóñez E, Tenorio M, Rupérez P (2013) Antioxidant and prebiotic effects of dietary fiber co-travelers from sugar Kombu in healthy rats. J Appl Phycol 25:503–512

Katayama M, Fukuda T, Okamura T, Suzuki E, Tamura K, Shimizu Y, Suda Y, Suzuki K (2011) Effect of dietary addition of seaweed and licorice on the immune performance of pigs. Anim Sci J 82:274–281

Kim M, Kim J, Choi W, Lee S (2008) Effects of seaweed supplementation on blood glucose concentration, lipid profile, and antioxidant enzyme activities in patients with type 2 diabetes mellitus. Nutr Res Pract 2:62–67

Königsberg Fainstein M (2007) Nrf2: La historia de un nuevo factor de transcripción que responde a estrés oxidativo. Revista de Educación Bioquímica 26:18–25

Kulshreshtha G, Rathgeber B, Stratton G, Thomas N, Evans F, Critchley A, Hafting J, Prithiviraj B (2014) Feed supplementation with red seaweeds, *Chondrus crispus* and *Sarcodiotheca gaudichaudii*, affects performance, egg quality, and gut microbiota of layer hens. Poult Sci 93:2991–3001

Kumar S, Brown L (2013) Seaweeds as potential therapeutic interventions for the metabolic syndrome. Rev Endocr Metab Disord 14:299–308

López-Oliva M, Pozuelo M, Rotger R, Muñoz-Martínez E, Goñi I (2013) Grape antioxidant dietary fibre prevents mitochondrial apoptotic pathways by enhancing Bcl-2 and Bcl-x L expression and minimising oxidative stress in rat distal colonic mucosa. Br J Nutr 109:4–16

Lowry O, Rosebrough N, Farr A, Randall R (1951) Proteins measurement with the folin phenol reagent. J Biol Chem 193:265–275

Macfarlane S, Macfarlane G, Cummings J (2006) Review article: prebiotics in the gastrointestinal tract. Aliment Pharmacol Ther 24:701–714

Maynard L (1994) The Atwater system of calculating the caloric value of diets. J Nutr 28:443–452

Perez-Recalde M, Matulewicz M, Pujol C, Carlucci M (2014) *In vitro* and *in vivo* immunomodulatory activity of sulfated polysaccharides from red seaweed *Nemalion helminthoides*. Int J Biol Macromol 63:38–42

Reeves P, Nielsen F, Fahey G (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. J Nutr 123:1939–1951

Shiau S, Chang G (1983) Effects of dietary fiber on fecal mucinase and beta-glucuronidase activity in rats. J Nutr 113:138–144

Somogyi M (1952) Notes on sugar determination. J Biol Chem 195:19–23

Tsuge K, Okabe M, Yoshimura T, Sumi T, Tachibana H, Yamada K (2004) Dietary effects of porphyran from *Porphyra yezoensis* on growth and lipid metabolism of Sprague-Dawley rats. Food Sci Technol Res 10:147–151

Villanueva M, Morcillo M, Tenorio M, Mateos-Aparicio I, Andrés V, Redondo-Cuenca A (2014) Health-promoting effects in the gut and influence on lipid metabolism of *Himanthalia elongata* and *Gigartina pistillata* in hypercholesterolaemic Wistar rats. Eur Food Res Technol 238:409–416