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Red seaweed *Pyropia columbina* as antioxidant supplement in feed for cultured juvenile Pacú (*Piaractus mesopotamicus*)

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Abstract

The aim of this study was to evaluate the antioxidant effect of red seaweed *Pyropia columbina* on juvenile Pacú (*Piaractus mesopotamicus*). Two hundred and ten ($n = 210$) fish were fed for 90 days with a control extruded feed (CEF) or the same feed added with *P. columbina* (35 g kg^{-1}) (PcEF). No significant difference in final body weight, specific growth rate, condition factor, and liver somatic index was detected between dietary treatments ($p > 0.05$). Fish fed with PcEF showed lower lipid peroxidation (28.2 ± 2.9 vs. 39.4 ± 3.7 mmol malondialdehyde mg^{-1} protein) and superoxide dismutase (23.3 ± 2.3 vs. 37.7 ± 3.0 U mg^{-1} protein) and reduced SOD/CAT ratio (5.5 ± 0.3 vs. 8.1 ± 0.7) in intestine than those fed with CEF ($p < 0.05$). These results also were observed in liver and white muscle, indicating a systemic effect. No difference in glutathione S-transferase and catalase (CAT) activity in intestine, liver, and white muscle was detected between dietary treatments ($p > 0.05$). PcEF showed higher hemoglobin (7.5 ± 0.4 vs. 6.6 ± 0.3 g 100 mL^{-1}) and mean corpuscular hemoglobin concentration (18.4 ± 1.8 vs. 25.7 ± 2.7%) than those fed with CEF diet ($p < 0.05$). Beneficial effects on lipid metabolism were observed in fish fed with PcEF respect to control diet. *Pyropia columbina* could be used as a natural antioxidant ingredient in fish feeding contributing a better nutritional status of cultured fish.

Keywords In vivo antioxidant effect · Red seaweed · *Piaractus mesopotamicus* · Extruded fish feed

Introduction

Pacú (*Piaractus mesopotamicus*, Holmberg 1887) is a highly valued fish species for aquaculture due to its acceptance by consumers and high growth rate (Machado-Neto et al. 2018). In Argentina, Pacú is the most cultivated species, achieving a milestone by overtaking the production of the exotic rainbow trout in 2012 (Valladão et al. 2018).

Intensive fish farming involves a great challenge for the producer. Handling, biometry, vaccination, and stocking density in the intensive culture system induce changes in the physiological responses interfering with health, behavior status, adaptation, and welfare of fish, promoting situations of constant stress (Machado-Neto et al. 2018). In this regard, oxidative stress arises when the organism has elevated levels of reactive oxygen species (ROS). High levels of ROS can produce lipid peroxidation of cell membranes and may damage proteins, DNA, and other biological molecules. To neutralize the harmful effects of ROS, fish have developed a complex antioxidant system that includes enzymes, minerals, and vitamins. In addition, fish may obtain antioxidant compounds they cannot synthesize through their food (Biller-Takahashi et al. 2015), such as phenolic compounds, ascorbic acid, selenium, and β-carotene.

The recognition of seaweed as a natural source of functional ingredients has grown rapidly in last years (Kumar et al. 2008; Thirunavukkarasu et al. 2013; Magnoni et al. 2017). In fact, seaweeds have been associated frequently with health benefits due to the radical scavenging and singlet O_2 quenching activity present in dry, raw and cooked preparations (Sachindra et al. 2010; Kumar and Brown 2013). Antioxidant compounds in seaweed have been suggested as an endogenous defense mechanism protecting against oxidative stress due to extreme environmental conditions (Aguilera et al. 2002). Red seaweeds such as *Pyropia columbina* are characterized by their pigments, including phycobiliproteins, halogenated compounds, mycosporine-like amino acids, sulphated polysaccharides, and polyphenols with antioxidant

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activity (Yuan et al. 2005; Duan et al. 2006; Yuan and Walsh 2006; Duan et al. 2006; Yuan and Walsh 2006; Cabrita et al. 2010; Cian et al. 2013), which make this group of seaweed promising supplements in aquatic feeds (Holdt and Kraan 2011). Studies in white shrimp *Litopenaeus vannamei* fed with a diet supplemented with *Gracilaria vermiculophylla* suggested a modulatory effect on antioxidant capacity when animals were subjected to biotic and abiotic stressors (Sirirustananun et al. 2011; Chen et al. 2012). Moreover, Magnoni et al. (2017) evaluated the effects of dietary supplementation with heat-treated seaweeds (5% *G. vermiculophylla* or 5% *Ulva lactuca*) on stress bioindicators in sea bream subjected to a hypoxic challenge. They found that the hepatic antioxidant enzyme activities were differently modulated by changes in environmental O_2 level, particularly in sea bream fed with the red seaweed diet, suggesting that the antioxidant properties of heat-treated seaweed may have a protective role against oxidative stress.

Currently, no research is available on in vivo antioxidant effects of extruded fish feed with red seaweed *P. columbina* added. Even less is known about the in vivo effect of this red seaweed on antioxidant status of temperate water fish like Pacú. Therefore, the aim of this work was to evaluate the effects of red seaweed *P. columbina* incorporated in extruded fish feed as antioxidant supplement using a juvenile Pacú as model.

Materials and methods

Reagents

1-chloro-2,4-dinitrobenzene (138630), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (10102946001), 4-hydroxybenzil alcohol (H20806), 4-hydroxybenzoic acid (240141), amino acid standard solution (AAS18), caffeic acid (C0625), diethyl ethoxymethylenemalonate (D94208), 2,2-diphenyl-1-picrylhydrazyl (D9132), α-aminobutyric acid (A1879), ferulic acid (128708), gallic acid (398225), malondialdehyde (63287), p-coumaric acid (C9008), sinapic acid (D7927), thiobarbituric acid (T5500), vanillic acid (W398802), and β-carotene (22040) were obtained from Sigma Chemical Co. (USA). Other reagents were of analytical grade and obtained from Chicarelli Laboratorios (San Lorenzo, Santa Fe, Argentina).

Raw materials and production of experimental diets

One kilogram of different specimens of *Pyropia columbina* was handpicked in Punta Maqueda (46° 00′ S, 67° 34′ W) in the spring of 2017. Punta Maqueda is located within the San Jorge Gulf, 30 km to the south of Comodoro Rivadavia, Argentina. The seaweed was processed according to Cian et al. (2014a). Briefly, *P. columbina* was transported to the laboratory and stored at 4 °C inside plastic bags. Seawater, sediments, organic debris, macro fauna, and epibionts were removed by scraping and subsequent washing with distilled water. Then the samples were dried at 100 ± 4 °C and ground with a particle size lower than 1 mm, using a laboratory hammer mill (Retsch, Haan, Germany). The powder obtained was passed through a 20-mesh sieve (0.85 mm) and stored at 4 °C in plastic bags until analysis or diet formulation. Chemical composition of *P. columbina* was determined using Association of Official Analytical Chemist (AOAC) (2000) methods. The protein, fat, ash, total dietary fiber, and moisture content of *P. columbina* in dry base were 24.61 g $(100\ g)^{-1}$, 0.25 g $(100\ g)^{-1}$, 6.46 g $(100\ g)^{-1}$, 48.09 g $(100\ g)^{-1}$, and 12.79 g $(100\ g)^{-1}$, respectively.

Control diet (control extruded feed, CEF) was formulated with commercial corn meal, soybean meal, bovine plasma protein concentrate, corn starch, vitamin-mineral mix, and canola oil according to Cian et al. (2018a). This formulation was made taking into account the nutrient requirement for Pacú (Bicudo et al. 2009). For *P. columbina* diet (*P. columbina* extruded feed, PcEF), the formulation was the same as CEF but 35 g kg^{-1} of commercial corn meal were replaced by *P. columbina*. The level of red algae was selected according to previous results obtained in our laboratory (Cian et al. 2018b).

Chemical composition, in vitro antioxidant capacity, amino acid profile, and phenolic acid profile of extruded feeds (CEF and PcEF)

For chemical analysis, extruded feeds were ground with a cyclone sample mill (UDY Corp, USA) using a 1-mm sieve.

Chemical composition and phosphorus of CEF and PcEF were determined using AOAC (2000) methods. Total starch was quantified according to Tovar et al. (1990). Iron, zinc and calcium contents of diets were measured by atomic absorption spectroscopy after dry mineralization using an atomic absorption spectrophotometer (Analyst 300, Perkin-Elmer, USA).

Chemical composition of diets is shown in Table 1. Chemical analysis confirmed diets were isocaloric and supplied a similar amount of macronutrients. Moreover, there were not significant differences in crude protein, crude lipid, total starch, ash, calcium, phosphorous, zinc, and iron between them ($p > 0.05$).

For amino acid analysis, samples of CEF and PcEF were hydrolyzed with 4 mL of 6 mol L^{-1} HCl. The solutions were sealed in tubes under nitrogen and incubated in an oven at 110 °C for 24 h. Amino acids were determined after derivatization with diethyl ethoxymethylenemalonate by high-performance liquid chromatography (HPLC), according to the method of Alaiz et al. (1992), using *D*,*L*-α-aminobutyric acid as internal standard. The HPLC system consisted in a Shimadzu Series LC-20AT pump, with Shimadzu SPD-M20A diode array detector, equipped with a 300 × 3.9 mm i.d. reversed-phase column (Novapack C18, 4 μm; Waters).

Table 1 Chemical composition of control extruded feed (CEF) and *P. columbina* added extruded feed (PcEF)

Components*	CEF (g kg^{-1})	PcEF (g kg^{-1})
Dry matter	895.3 ± 5.3	892.7 ± 0.9
Crude protein	276.8 ± 4.7	268.2 ± 4.3
Crude lipid	34.4 ± 2.6	31.5 ± 1.6
Total starch	444.2 ± 4.3	443.5 ± 1.2
Ash	29.5 ± 1.4	30.9 ± 1.0
Calcium	1.6 ± 0.1	1.7 ± 0.1
Phosphorous	2.2 ± 0.21	2.4 ± 0.2
Zinc	0.1 ± 0.0	0.1 ± 0.0
Iron	0.2 ± 0.0	0.2 ± 0.0

* Chemical composition expressed as mean ± SD ($n = 3$)

Amino acid content was expressed as g kg^{-1} protein. Data were processed using Shimadzu LC solution software.

Extraction of soluble phenolic compounds from feed was performed according to Qiu et al. (2010), with some modifications. Briefly, 2 g of extruded feeds were extracted twice with 15 mL of 80% methanol for 1 h at room temperature, using a mechanical shaker. Then they were centrifuged at 3500 rpm for 5 min at room temperature (Cavour VT-3216, Argentina) and the supernatants obtained were combined. Subsequently, an acid hydrolysis for the cleavage of conjugated and condensed soluble phenolic compounds in samples was performed according to Cian et al. (2012). Phenolic acids were determined by high-performance liquid chromatography (HPLC) according to Leitao et al. (2011). The HPLC system consisted of a Shimadzu Series LC-20AT pump, with Shimadzu SPD-M20A diode array detector, equipped with a 250 mm × 4.6 mm i.d. reversed-phase column (Novapack C18, 5 μm; Gemini 110A C-18 Phenomenex column). Phenolic acid content was expressed as mg kg^{-1} dry feed. Data were processed using Shimadzu LC solution software.

In vitro antioxidant capacity of CEF and PcEF was evaluated by DPPH inhibition, β-carotene bleaching inhibition and ABTS inhibition methods according to Cian et al. (2014b). DPPH and ABTS inhibition were expressed as mg ascorbic acid equivalent g^{-1} dry feed and Trolox equivalent antioxidant capacity (TEAC) g^{-1} dry feed, respectively.

β-carotene bleaching inhibition was expressed as percent inhibition (%).

All determinations were performed in triplicate ($n = 3$).

Fish and feeding trial

Juvenile Pacú were obtained from a fish farm (Pez Campero, Paraná, Argentina). The experiment was performed in the Aquaculture Laboratory at the Instituto Nacional de Limnología (CONICET, Argentina) in a recirculating water system supplied with dechlorinated city (tap) water, and equipped with an external quartz-anthracite filter (Multiválvula Vulcano Filtro VC10). The recirculation aquatic system (RAS) was set at 15.1 L min^{-1} with artificial aeration and 12 h light/12 h dark photoperiod regime provided by artificial illumination. The replacement of 100% of the water in each tank was made every 45 min. Physico-chemical parameters of the water remained within the values recommended by Urbinati et al. (2010) for Pacú (temperature 24.0 ± 1 °C, dissolved oxygen 6.84 ± 0.62 mg L^{-1}, pH 6.18 ± 0.21, electrical conductivity 162.17 ± 8.30 μS cm^{-1}, and total ammonia nitrogen 0.26 ± 0.07 mg L^{-1}).

Prior to the feeding trial, all fish were acclimated to the indoor rearing conditions for 2 weeks. At the start of the feeding experiment, 210 juvenile Pacú (initial body weight 12.4 ± 1.9 g) were randomly stocked in six 300-L tanks with 35 fish per tank. The two test diets, CEF (control) and PcEF (*P. columbina*), were randomly assigned to triplicate tanks. Fish were fed twice a day (5% biomass weight per day), during 90 days at 24 °C. The daily ration was divided into two, and fed to the fish at 09:00 and 14:00 h. Leftover diet was collected to prevent nutrient leaching. The fish were weighed every 3 weeks and their ration adjusted accordingly.

The experiment was conducted in accordance with national and institutional guidelines (CONICET 2005) for the protection of animal welfare and approved by the Committee of Ethics and Safety in Experimental Work (Scientific-Technological Center, CONICET Santa Fe, Argentina).

Sample collection and morphometric indexes

At the end of the feeding trial, fish from each dietary treatment were anesthetized in benzocaine 0.1 g L^{-1} as described by Parma de Croux (1990). Body weight (g) and total and standard length (cm) were recorded for each individual. Growth and morphometric parameters were calculated according to Cian et al. (2017) as follows: weight gain (WG, %) = 100 × [(final body weight (g) − initial body weight (g)) × initial body $weight^{-1}$ (g)] and specific growth rate (%/day) = 100 × [(ln final weight (g) − ln initial weight (g)) / days of the trial].

Blood was collected immediately from the caudal vessel (Reichenbach-Klinke 1980). Plasma was separated from whole blood by centrifugation at 1409× *g* for 5 min, and stored at − 80 °C. Intestine, liver, white muscle, gills, and brain were dissected, quickly frozen in liquid nitrogen, and subsequently stored at − 80 °C until lipid peroxidation and enzymes involved in antioxidant system analysis. Before freezing, the wet weight of different tissues was determined. Condition factor (CF) and liver somatic index (LSI) were calculated according to Goede and Barton (1990).

Hematological parameters

Red blood cell (RBC) counts were performed with a Neubauer chamber. Hematocrit (Ht) was determined by micro-method.

Hemoglobin concentration (Hb) was measured by cyanomethemoglobin method (Houston 1990). Mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) were calculated from primary indices according to Cazenave et al. (2005).

All assays were performed in triplicate.

Analytical methods

Plasma glucose, triglyceride, cholesterol, and total protein levels were determined by spectrophotometric methods (Rossi et al. 2015). Glycogen, total protein, and lipid content were quantified in the homogenate of frozen tissues (liver and white muscle) according to Rossi et al. (2017).

Tissues homogenates for the determination of lipid peroxidation of intestine, liver, white muscle, gills, and brain were prepared from each individual fish. Briefly, tissues were homogenized using phosphate buffer (pH 7.4; 30 mmol L^{-1}). The homogenate was centrifuged at 3200 rpm (4 °C) for 30 min, and the supernatant was collected and stored at − 80 °C. Lipid peroxidation of intestine, liver, white muscle, gills, and brain were determined by measuring thiobarbituric reactive substances (TBARS), according to Yagi (1976). Protein content of each extract was determined according to Bradford (1976).

Extracts for the determination of antioxidant enzyme activities from intestine, liver, white muscle, gills, and brain were prepared from each individual (not pooled), according to Bacchetta et al. (2014). The activity of glutathione-S-transferase (GST, EC 2.5.1.18) was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, according to Habig et al. (1974). Catalase (CAT, EC 1.11.1.6) and superoxide dismutase (SOD, 1.15.1.1) activities were determined according to Beutler (1982) and Misra and Fridovich (1972), respectively. The enzyme activities were calculated in terms of sample protein content. All assays were performed in triplicate ($n = 3$).

Mineral body content

For determination of mineral body content, whole fish were ground using a Moulinex AD5661AR meat mincer in order to obtain a homogenate. Phosphorus content of homogenates was determined following AOAC (2000). Iron, zinc, and calcium content were measured by atomic absorption spectroscopy after dry mineralization. All determinations were performed in triplicate ($n = 3$).

Statistical analysis

Results were expressed as means with their standard deviation (± SD). One-way analysis of variance (ANOVA) was performed, and the statistical differences among samples were determined using LSD test (least significant difference). Significance was accepted at $p < 0.05$. All statistical analyses were performed with Statgraphics Centurion XV 15.2.06 (Statpoint Technologies, Inc., USA).

Results

Chemical composition, amino acid profile, phenolic acid profile, and in vitro antioxidant capacity of extruded feeds

Table 1 shows the chemical composition of control extruded feed (CEF) and *P. columbina* extruded feed (PcEF). Significant differences for crude protein, crude lipid, total starch, ash, and mineral content were not found ($p > 0.05$).

Table 2 shows the amino acid profile of diets. Aspartic (Asp), glutamic (Glu), and proline (Pro) contents of PcEF were higher than those found for CEF diet. However, leucine (Leu) and phenylalanine (Phe) contents were lower than those found for control diet. For all other amino acids, no significant difference between diets was observed.

Phenolic acid profile is shown in Table 3. Both diets presented a predominance of gallic acid, followed by 4-hydroxybenzoic acid, 4-hydroxybenzil alcohol, ferulic acid, p-coumaric acid, and caffeic acid. Vanillic and sinapic acid were not detected in both diets. PcEF showed significant higher content of gallic, 4-hydroxybenzoic, and p-coumaric acid than CEF.

Table 2 Amino acid profile of control extruded feed (CEF) and *P. columbina* added extruded feed (PcEF)

Amino acids	Total amino acids (g kg^{-1} protein)*		*p* value
	CEF	PcEF	
Asp + Glu	91.6 ± 3.6[a]	149.6 ± 3.1[b]	*0.0002*
Ser	71.7 ± 0.4	66.4 ± 0.1	*0.1144*
His	30.4 ± 1.1	30.0 ± 0.6	*0.7561*
Gly	46.9 ± 0.4	42.1 ± 1.3	*0.8865*
Thr	62.2 ± 0.3	62.9 ± 0.4	*0.7055*
Arg	72.3 ± 2.6	67.3 ± 2.7	*0.6582*
Ala	68.1 ± 2.5	63.1 ± 3.2	*0.1729*
Pro	38.7 ± 2.7[a]	54.8 ± 4.6[b]	*0.0031*
Tyr	56.2 ± 4.0	48.6 ± 3.7	*0.1352*
Val	52.4 ± 1.6	50.8 ± 1.2	*0.5544*
Met	53.6 ± 1.0	52.5 ± 3.2	*0.6214*
Cys	29.6 ± 3.8	22.7 ± 3.2	*0.2910*
Ile	44.0 ± 2.1	38.4 ± 3.6	*0.2633*
Leu	132.0 ± 3.9[b]	111.6 ± 2.3[a]	*0.0001*
Phe	70.0 ± 1.2[b]	63.8 ± 0.8[a]	*0.0013*
Lys	80.3 ± 3.2	75.4 ± 2.8	*0.1123*

* Total amino acid content is expressed as mean ± SD ($n = 3$). Different letters mean significant differences between samples analyzed by LSD test ($p < 0.05$). Italic values indicate significance by LSD test

Table 3 Phenolic acid profile of control extruded feed (CEF) and *P. columbina* added extruded feed (PcEF)

Phenolic acids	Phenolic content (mg kg^{-1} dry basis)*		*p* value
	CEF	PcEF	
Gallic acid	236.4 ± 2.8^{a}	439.6 ± 4.3^{b}	*0.0129*
4-hydroxybenzil alcohol	24.0 ± 6.3	38.0 ± 8.3	*0.1879*
4-hydroxybenzoic acid	78.7 ± 2.0^{a}	156.0 ± 6.0^{b}	*0.0001*
Vanillic acid	N.d.	N.d.	–
Caffeic acid	4.2 ± 0.7	4.5 ± 0.3	*0.1226*
p-coumaric acid	7.9 ± 0.1^{a}	11.6 ± 0.7^{b}	*0.0180*
Ferulic acid	13.4 ± 0.2	14.0 ± 0.8	*0.2446*
Sinapic acid	N.d.	N.d.	–

N.d. not detected

* Phenolic acid content is expressed as mean ± SD ($n = 3$). Different letters mean significant differences between samples analyzed by LSD test ($p < 0.05$). Italic values indicate significance by LSD test

As shown in Table 4, PcEF showed higher in vitro antioxidant capacity than CEF. In this regard, DPPH inhibition, β-carotene bleaching inhibition, and Trolox equivalent antioxidant capacity (TEAC) of PcEF were 46, 168, and 21% higher than those obtained for CEF, respectively.

Effects of diets on morphometric indexes, hematological parameters, mineral body content, and tissue energy reserves of juvenile Pacú

Fish promptly accepted both diets and no mortality occurred during the feeding trial. Moreover, significant differences in growth performance and condition factor were not detected between dietary treatments ($p > 0.05$) after 90 days of feeding trial (Table 5).

Except for hemoglobin and mean corpuscular hemoglobin concentration (MCHC), which were significantly higher for fish consuming PcEF ($p < 0.05$), there were no significant differences in hematological parameters between fish fed with both diets ($p > 0.05$). Hemoglobin and MCHC levels in fish fed with PcEF increased by 14 and 40% with respect to those fed with control diet, respectively. On the other hand, there were not significant differences in iron, zinc, calcium and phosphorus body content between diets (Table 5).

As shown in Table 6, fish fed with PcEF showed lower triglycerides and cholesterol in plasma than those consuming CEF diet. In spite of this, there was no significant difference in plasma glucose concentration. Moreover, no effect of PcEF diet on proteins, lipids and glycogen content from liver and white muscle was observed.

Effects of diets on enzymes involved in the antioxidant system and lipid peroxidation

Table 7 shows thiobarbituric reactive substances (TBARS) and the activity of glutathione S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) in intestine, liver, white muscle, gills and brain of fish fed with CEF and PcEF. Lipid peroxidation (TBARS) in intestine, liver and white muscle of fish fed with PcEF was lower than that found for tissues from fish fed with CEF. The reduction in TBARS content was more noticeable in the muscle (37%) then in the intestine (28%) and finally in the liver (17%). In spite of this, there were no significant differences in TBARS content of gills and brain between diets. The lower lipid peroxidation was accompanied by lower SOD activity. On the other hand, no effect of PcEF diet on GST and CAT activity of different tissues was observed. However, PcEF diet induced a change in SOD/CAT ratio in intestine, liver and white muscle (Fig. 1).

Table 4 In vitro antioxidant capacity of control extruded feed (CEF) and *P. columbina* added extruded feed (PcEF)

	CEF*	PcEF*	*p* value
DPPH inhibition (mg AA equivalent g^{-1} dry feed)	0.76 ± 0.1^{a}	1.11 ± 0.0^{b}	*0.0058*
β-carotene bleaching inhibition (%)	41.8 ± 0.6^{a}	77.5 ± 0.5^{b}	*0.0001*
TEAC (μmol Trolox equivalent g^{-1} dry feed)	1.4 ± 0.0^{a}	1.7 ± 0.0^{b}	*0.0013*

TEAC Trolox equivalent antioxidant capacity, *AA* ascorbic acid

* Mean ± SD ($n = 3$). Different letters mean significant differences between samples analyzed by LSD test ($p < 0.05$). Italic values indicate significance by LSD test

Table 5 Morphometric, hematological biomarkers and mineral body content of Pacú fed with control extruded feed (CEF) and *P. columbina* added extruded feed (PcEF) during 90 days

	Diets*		*p* value
	CEF	PcEF	
Final body weight (g)	19.0 ± 3.1	19.6 ± 3.2	*0.2708*
WG (%)	52.8 ± 8.9	63.0 ± 10.5	*0.2710*
SGR (% day^{-1})	0.35 ± 0.05	0.41 ± 0.05	*0.2519*
CF	3.9 ± 0.2	3.7 ± 0.3	*0.1310*
LSI	0.5 ± 0.0	0.5 ± 0.1	*0.2344*
RBC (× 10^3 mm^{-3})	1.5 ± 0.3	1.6 ± 0.6	*0.2244*
Hematocrit (%)	32.2 ± 4.9	29.4 ± 2.5	*0.1590*
Hemoglobin (g $(100\ g)^{-1}$)	6.6 ± 0.3[a]	7.5 ± 0.4[b]	*0.0007*
MCH (pg)	49.3 ± 3.1	46.4 ± 4.4	*0.5389*
MCV (fL)	196.4 ± 39.6	190.9 ± 11.0	*0.7706*
MCHC (%)	18.4 ± 1.8[a]	25.7 ± 2.7[b]	*0.0006*
WBC (μL^{-1})	1496.3 ± 565.5	1840.9 ± 565.4	*0.2431*
Iron BC (mg kg^{-1} tissue)	39.7 ± 4.9	33.3 ± 5.9	*0.1893*
Zinc BC (mg kg^{-1} tissue)	11.1 ± 3.2	14.0 ± 1.1	*0.0846*
Calcium BC (mg kg^{-1} tissue)	2586.6 ± 257.5	2485.3 ± 361.0	*0.6801*
Phosphorus BC (mg kg^{-1} tissue)	3182.5 ± 296.7	2927.8 ± 320.2	*0.2609*

WG weight gain, *SGR* specific growth rate, *CF* condition factor, *LSI* liver somatic index, *RBC* red blood cells count, *MCH* mean corpuscular hemoglobin, *MCV* mean corpuscular volume, *MCHC* mean corpuscular hemoglobin concentration, *WBC* white blood cells count, *BC* body content

* Mean ± SD ($n = 10$). Different letters in a row mean significant differences between samples analyzed by LSD test ($p < 0.05$). Italic values indicate significance by LSD test

Discussion

Composition and in vitro antioxidant capacity of extruded feeds

For most seaweed, Asp and Glu constitute a large part of the amino acid fraction, representing between 22 and 44% of the total amino acids (Fleurence 1999). Cian et al. (2014b) reported that *P. columbina* had 22.7 g Asp + Glu $(100\ g)^{-1}$ proteins, constituting the most abundant amino acid in this seaweed. Thus, the higher content of acidic amino acids of PcEF is due to the addition of *P. columbina* to the feed. It should be noted that PcEF diet has 63% more acidic amino acids than CEF.

Moreover, PcEF had higher content of phenolic acids than CEF (663.7 vs. 364.6 mg kg^{-1}, respectively), gallic and 4-hydroxybenzoic acid being the most abundant. The increase of the phenolic acid content of the diet is due to the addition of *P. columbina* to feed. It is known, seaweeds are a very rich source of phenolic compounds such as epicatechin, gallic acid, and 4-hydroxybenzoic acid (Cian et al. 2012; Sabeena

Table 6 Tissue energy reserves of Pacú fed with control extruded feed (CEF) and *P. columbina* added extruded feed (PcEF) during 90 days

		Diets*		*p* value
		CEF	PcEF	
Plasma	Glucose (g L^{-1})	1.0 ± 0.2	1.1 ± 0.2	*0.6760*
	Triglycerides (g L^{-1})	7.5 ± 1.0[b]	5.1 ± 1.3[a]	*0.0016*
	Cholesterol (g L^{-1})	3.4 ± 0.2[b]	2.1 ± 0.4[a]	*0.0014*
Liver	Proteins (mg g^{-1} tissue)	120.1 ± 5.0	127.1 ± 7.7	*0.4378*
	Lipids (μmol g^{-1} tissue)	36.5 ± 4.0	36.7 ± 8.5	*0.9701*
	Glycogen (μmol g^{-1} tissue)	1447.5 ± 331.3	1138.3 ± 351.3	*0.2173*
White muscle	Proteins (mg g^{-1} tissue)	126.7 ± 51.0	130.5 ± 43.1	*0.9003*
	Lipids (μmol g^{-1} tissue)	7.3 ± 0.4	7.2 ± 1.0	*0.7376*
	Glycogen (μmol g^{-1} tissue)	2.8 ± 0.3	2.3 ± 0.2	*0.1244*

* Mean ± SD ($n = 10$). Different letters in a row mean significant differences between samples analyzed by LSD test ($p < 0.05$). Italic values indicate significance by LSD test

Table 7 Activity of glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), and lipid peroxidation of different tissues of Pacú fed with control extruded feed (CEF) and *P. columbina* added extruded feed (PcEF) during 90 days

		Diets*		*p* value
		CEF	PcEF	
Intestine	GST (mU mg^{-1} protein)	47.5 ± 3.1	44.3 ± 2.5	*0.1540*
	SOD (U mg^{-1} protein)	37.7 ± 3.0^{b}	23.3 ± 2.3^{a}	*0.0028*
	CAT (U mg^{-1} protein)	4.6 ± 0.3	4.3 ± 0.9	*0.5245*
	TBARS (nmol MDA (100 mg protein)$^{-1}$)	39.4 ± 3.7^{b}	28.2 ± 2.9^{a}	*0.0066*
Liver	GST (mU mg^{-1} protein)	66.0 ± 8.9	69.2 ± 2.5	*0.5120*
	SOD (U mg^{-1} protein)	19.9 ± 0.5^{b}	9.4 ± 0.5^{a}	*0.0009*
	CAT (U mg^{-1} protein)	30.7 ± 0.7	31.0 ± 3.0	*0.8965*
	TBARS (nmol MDA (100 mg protein)$^{-1}$)	80.8 ± 6.2^{b}	67.4 ± 6.5^{a}	*0.0171*
White muscle	GST (mU mg^{-1} protein)	6.1 ± 0.4	5.2 ± 0.9	*0.1272*
	SOD (U mg^{-1} protein)	20.3 ± 1.9^{b}	18.7 ± 1.3^{a}	*0.0364*
	CAT (U mg^{-1} protein)	1.2 ± 0.1	1.1 ± 0.4	*0.7970*
	TBARS (nmol MDA (100 mg protein)$^{-1}$)	4.3 ± 0.5^{b}	2.7 ± 0.7^{a}	*0.0221*
Gills	GST (mU mg^{-1} protein)	25.4 ± 2.4	25.8 ± 4.4	*0.8863*
	SOD (U mg^{-1} protein)	23.3 ± 7.4	23.8 ± 6.6	*0.9180*
	CAT (U mg^{-1} protein)	1.6 ± 0.2	1.5 ± 0.1	*0.4454*
	TBARS (nmol MDA (100 mg protein)$^{-1}$)	16.6 ± 1.8	12.2 ± 2.0	*0.3987*
Brain	GST (mU mg^{-1} protein)	34.5 ± 3.5	37.9 ± 5.0	*0.2995*
	SOD (U mg^{-1} protein)	91.2 ± 8.9	90.0 ± 7.3	*0.9045*
	CAT (U mg^{-1} protein)	4.8 ± 0.4	4.8 ± 1.0	*0.9663*
	TBARS (nmol MDA (100 mg protein)$^{-1}$)	5.9 ± 1.5	5.1 ± 0.8	*0.3976*

MDA malondialdehyde

* Mean ± SD ($n = 10$). Different letters in a row mean significant differences between samples analyzed by LSD test ($p < 0.05$). Italic values indicate significance by LSD test

Farvin and Jacobsen 2013; Machu et al. 2015). Red seaweeds also contain particular phenolic compounds that differ in some respects from those of terrestrial plants, such as bromophenols (Liu et al. 2011). In this regard, it has been observed that 4-hydroxybenzoic acid is the most likely precursor of bromophenols (Whitfield et al. 1999), it being in high levels in *P. columbina* and therefore in the PcEF diet.

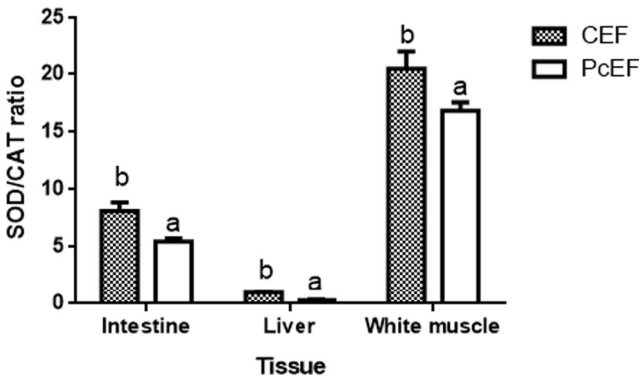

Fig. 1 SOD/CAT ratio in intestine, liver, and white muscle of Pacú fed with CEF and PcEF diets. Data are expressed as mean ± SD. Different letters mean significant differences between samples analyzed by LSD test ($p < 0.05$)

The higher in vitro antioxidant capacity of PcEF can be due to the antioxidant compounds provided by *P. columbina*. Regarding that, several studies have demonstrated the relationship between phenolics and antioxidant activity (Cian et al. 2013, 2014b). As mentioned above, PcEF had a higher content of phenolic acids which have been reported to be potent radical scavengers (Cian et al. 2013; Machu et al. 2015). In addition, red seaweeds are a rich source of iota, kappa, and lambda carrageenan, which have antioxidant activity demonstrated by in vitro and in vivo assays (Gómez-Ordóñez et al. 2012; Jiménez-Escrig et al. 2013; Cian et al. 2014a).

Finally, it has been reported that Asp and Glu are antioxidants (Cian et al. 2013) since they are hydrogen donors able to quench unpaired electrons or radicals (Quian et al. 2008). Note that PcEF had higher content of Asp + Glu than control diet.

Effects of extruded feeds on juvenile Pacú

As mentioned before, significant differences in final body weight, specific growth rate, condition factor, and liver somatic index were not detected between dietary treatments after 90 days of feeding trial. Thus, the

addition of *P. columbina* as antioxidant supplement (35 g kg^{-1} diet) did not affect the weight gain and growth performance of Pacú. This result is in agreement with that reported by Peixoto et al. (2016), who evaluated the role of dietary seaweed supplementation on growth performance, digestive capacity, and immune and stress responsiveness in European seabass (*Dicentrarchus labrax*). They found that dietary red seaweed supplementation (25 g *Gracilaria* kg^{-1} diet) had no effects on growth rate, voluntary feed intake, feed conversion ratio, and protein efficiency ratio. A similar effect was found for rainbow trout (*Oncorhynchus mykiss*) fed with diets containing 50 and 100 g of *Pyropia dioica* kg^{-1} diet (Soler-Vila et al. 2009). Moreover, these authors suggested that *P. dioica* can be included in diets for rainbow trout up to 10% without significant negative effects on weight gain and growth performance. However, at higher levels of red seaweed replacement, negative effects on the weight gain and growth performance of the fish have been reported. In this sense, Xu et al. (2011) found that dietary seaweed supplementation above 30% reduced the growth performance and feed utilization efficiency of the teleost fish *Siganus canaliculatus*.

Hemoglobin and MCHC were higher in fish fed with PcEF than CEF. This means a higher iron bioaccessibility in PcEF than CEF, since the level of iron was the same in both diets (Table 1). Cian et al. (2016) studied the effect of *P. columbina* on iron bioaccessibility. They found that chelating peptides obtained from *P. columbina* protein hydrolysates had the capacity of maintaining iron in a soluble and bioaccessible form. As mentioned above, Asp and Glu were the main amino acids in PcEF. At pH 7.0, carboxyl residues of the acidic amino acids (Asp, pKa = 3.86; Glu, pKa = 4.25) are fully dissociated, being able to form complexes with iron. These complexes allow keeping the iron soluble in physiological conditions, making it more bioaccessible. Taking into account that the intestinal pH of Pacú is between 6.4 and 8.0, the higher hemoglobin and MCHC in fish fed with PcEF could be due to the higher level of these acidic amino acids in the diet, which were provided by *P. columbina*. On the other hand, a higher iron bioavailability of a diet has important benefits. In first place, higher bioavailability improves iron stores, which allow maintaining normal physiological state in situations of environmental stress associated with less feed ingestion. In second place, a diet with higher iron bioavailability means lower levels of fortifying for reach the fish requirement, reducing feed cost.

A lipid-lowering effect on juvenile Pacú consuming *P. columbina* (PcEF) was observed. In this regard, diets supplemented with 20 and 40 g kg^{-1} *Chlorella* decreased serum cholesterol levels in juvenile Japanese flounder (Kim et al. 2002). Other studies performed on various animals have recorded lowering of both plasma and liver cholesterol and lipid levels through seaweed supplementation to diets (Dvir et al. 2000; Matanjun et al. 2010; Gómez-Ordóñez et al. 2012; Villanueva et al. 2014; Cian et al. 2018b). On the other hand, the main unsaturated fatty acid of *P. columbina* is C20:5 (*n*-3) (Cian et al. 2014b), which would contribute to lowering cholesterol and triglycerides in Pacú. Ragaza et al. (2015) found that juvenile Japanese flounder (*Paralichthys olivaceus*) fed with a diet added with red seaweed *Eucheuma denticulatum* (30 g kg^{-1} diet) presented higher *n*-3 polyunsaturated fatty acid accumulation in dorsal muscle and reduced serum triglycerides and total cholesterol than those fed with a control diet**.** Since fatty acid profile of diets were not analyzed in this study, future studies that focus on this topic are recommended. Also, the fiber provided by *P. columbina* (carrageenans) could modify the intestinal flora, promoting the reduction of lipids in plasma. Thus, more research is needed in order to elucidate the lipid-lowering mechanism.

Fish fed with PcEF produced antioxidative environment in intestine of juvenile Pacú, reducing lipid peroxidation (TBARS) and superoxide dismutase (SOD) activity. A similar effect was found for liver and white muscle of fish fed with PcEF. These results suggest that *P. columbina* exerted an antioxidant effect at the systemic level. Note that SOD belongs to the antioxidant enzymatic pathways in response to oxidative stress (Ighodaro and Akinloye 2017). In this regard, 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, López-Oliva et al. 2013, Figueiredo et al. 2016, Llopart et al. 2017). Zdunczyk et al. (2002) found that flavones, catechins, anthocyanins and condensed tannins from different plant sources decreased the activity of erythrocyte SOD in rats. Estruch et al. (2011) reported that moderate consumption of red wine decreases erythrocyte superoxide dismutase activity in men. This effect was attributed to high polyphenol content of red wine. Moreover, SOD activity was reduced by hydroquinone, resorcinol, and pyrocatechol (OH-phenols) in sea bass (*Dicentrarchus labrax*) (Roche and Bogé 2000). Taking into account that gallic, 4-hydroxybenzoic and p-coumaric acid content of PcEF was higher than that of CEF diets, the diminution of SOD activity in intestine, liver and white muscle coupled with the unmodified catalase (CAT) activity could be due to phenolic compounds from *P. columbina.* In agreement, SOD/CAT ratio in intestine, liver and white muscle of Pacú fed with PcEF was lower than that found for CEF, indicating an improvement at systemic level. These results suggest that PcEF modulated the activity of SOD enzyme,

yielding a system that can promote a reducing environment. This was confirmed by the lower TBARS levels in intestine, liver, and white muscle of Pacú fed with PcEF. In this regard, You et al. (2014) reported that the dietary inclusion of *Ulva pertusa* at levels up to 10% reduced TBARS content and modified SOD and CAT activities in liver of rabbitfish *Siganus canaliculatus*.

Conclusions

We have demonstrated that extruded fish feed containing a low level of red seaweed *P. columbina* (35 g kg^{-1}) exerted antioxidant effects on intestine, liver and white muscle in juvenile Pacú. Moreover, *P. columbina* promoted higher iron bioavailability observable through an increase in hemoglobin and mean corpuscular hemoglobin concentration, and lipid-lowering effect (lower plasma triglycerides and cholesterol). *Pyropia columbina* could be used as a natural antioxidant ingredient in fish feeding contributing a better nutritional status of cultured fish.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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