



Diets enriched in red seaweed (*Pyropia columbina* and *Gracilaria chilensis*) cryo concentrates modulate the immune-relevant gene encoding the Mx antiviral protein in salmon (*Salmo salar*) white blood cells

Ivonne Lozano Muñoz¹ · Jurij Wacyk¹ · Claudio Perez¹ · Jaime Carrasco² · Marcelo Cortez-San Martin³

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Abstract

Pharmacotherapy has long been used to control viral diseases. However, its success is questionable because its use can negatively impact environmental and human health. An alternative solution is the use of functional foods and diets containing natural products, which tend to be more biodegradable than synthetic molecules and are less likely to generate resistance. Seaweed contains biologically active macronutrients and minerals that offer a natural alternative to synthetic molecules. Red seaweeds, in particular, are a rich source of anti-viral compounds. This study aimed to evaluate the effect of two edible red seaweeds, *Pyropia columbina* and *Gracilaria chilensis* cryo concentrates (RSCC), on the gene transcription levels in leukocyte proteins involved in antiviral response (INF γ , Mx, interleukin-6, cathelicidin, and lysozyme). The RSCCs were fed to fish (*Salmo salar* L.) at concentrations of 0.1, 1, or 10 g kg⁻¹ for 56 days, and blood samples were collected at 8 weeks. The transcription levels of key genes associated with the antiviral response were analyzed by qRT-PCR using leukocyte mRNA as template. The Mx transcript level was significantly decreased ($p < 0.05$) with the RSCC diets, and lysozyme transcript levels were significantly increased (1 g kg⁻¹ *P. columbina* cryo concentrate). Cathelicidin, interleukin-6, and INF γ had stable transcription levels. Importantly, RSCC modulated the immune-relevant gene that encodes the Mx antiviral protein in white blood cells.

Keywords Red seaweed concentrates · *Pyropia columbina* · *Gracilaria chilensis* · Mx antiviral protein · Lysozyme · Functional ingredient · *Salmo salar*

Introduction

The prevalence and appearance of new diseases continue to be one of the biggest obstacles to sustainable aquaculture. In

salmonids, current strategies include the use of vaccines and antibiotics, but these can create resistant pathogens, host immunosuppression, and negative effects on the environment (Asif et al. 2018). Functional diets are an alternative that considers the inclusion of natural ingredients like marine seaweed.

Marine seaweeds can absorb and concentrate minerals, trace elements, vitamins, and amino acids used to meet their metabolic needs, this makes seaweed a valuable food source containing significant amounts of protein, vitamins, taurine (Sánchez-Machado et al. 2004; Marsham et al. 2007; Garcia-Vaquero and Hayes 2016), omega-3 fatty acids, and carotenoids (Ngo et al. 2011). They have a low $\omega 6/\omega 3$ ratio (Mohamed et al. 2012) and can make an important contribution to daily mineral intake.

Most seaweeds also have health benefits due to their low Na/K ratio with values of 0.14–0.16 (Mohamed et al. 2012). Compared to land plants, seaweeds contain 10 to 20 times the amount of minerals making them rich in macrominerals and trace elements (Mohamed et al. 2012; Moredo-Piñeiro et al.

✉ Ivonne Lozano Muñoz
ivonneok@ug.uchile.cl

¹ Facultad de Ciencias Agronómicas, Producción Animal, Laboratorio de Genética y Biotecnología en Acuicultura, Universidad de Chile, Santa Rosa 11735, La Pintana, CP 8820808 Santiago, Región Metropolitana, Chile

² Salmon Division, Biomar Chile S.A., R&D, Bernardino 1994, Parque San Andrés, CP 5501619 Puerto Montt, Chile

³ Facultad de Química y Biología, Laboratorio de Virología Molecular y Control de Patógenos, Universidad de Santiago de Chile, Av. Libertador Bernardo O'higgins 3363, CP 9170022 Santiago, Región Metropolitana, Chile

2012). Seaweeds also have high ash content with values ranging from 20 to 50% on a dry basis (Pereira 2016). Red seaweeds are considered a particularly important source of many biologically active metabolites (El Gamal 2010), and are a major source of anti-viral compounds as has been shown using a variety of red seaweed extracts (Mohamed et al. 2012; Rhimou et al. 2015; Boulho et al. 2017; Castilla et al. 2017). However, few studies have been performed using whole seaweed cryo concentrates as an ingredient. We previously used two edible red seaweeds (*Pyropia columbina* and *Gracilaria chilensis*) cryo concentrates as a supplemental ingredient in salmon diet. Ex vivo assays showed that a diet supplemented with red seaweed significantly increased resistance against salmon anemia virus at the blood level and significantly increased the specific growth rate (39%) (Lozano et al. 2016).

Compared to mammals, fish immune systems have lower specificity and less robust response. They have limited immunoglobulins, low lymphocyte proliferation, and weak immunological memory, and this reduces the potential for long-term protection (Magnadóttir 2006). Thus, the innate or non-specific immunity might play a bigger role in fish than the specific or adaptive immunity (MacKenzie et al. 2004).

Innate immunity involves a large repertoire of biological mechanisms that include cellular and humoral components such as macrophages, natural killer (NK) cells, lysozymes, the complement system, cytokines such as interferon (IFN), and antimicrobial peptides including cathelicidins. All of these help regulate the receptors capable of recognizing the molecular patterns associated with foreign nucleic acids or viral surface glycoproteins. Interferon types I and II are induced as an innate antiviral immune response in vertebrates. Interferon type II, also called interferon gamma (IFN- γ), is produced by macrophages and cytotoxic cells (NK) (McBeath et al. 2007). Its most important function is the activation of the macrophages that are essential for the control of many microbial infections. It strongly stimulates the development of CD8⁺ T cell responses during an acute viral infection (Whitmire et al. 2005). In contrast, interleukin 6 (IL-6) is a vital innate immune cytokine necessary for promoting neutrophil-mediated viral clearance. IL-6 is produced by macrophages, dendritic cells, mast cells, and other innate immune cells; it is a marker of inflammation (Dienz et al. 2012).

Lysozymes are produced by leukocytes (especially monocytes and neutrophils). These enzymes contribute to the antimicrobial activity of innate immunity (Saurabh and Sahoo 2008) and are found in immune-related cells such as macrophages, neutrophils, eosinophils, as well as epithelial and intestinal cells (Fletcher and White 1976). They can lyse Gram-positive bacteria and can coordinate with complement to lyse some Gram-negative bacteria. Lysozymes also have antimicrobial properties independent of their enzymatic activity (Paulsen et al. 2003); this significantly contributes to viral inhibition (Wei et al. 2012). Lysozyme mRNA expression and enzymatic activity increase in the presence of β -glucan

and bacterial lipopolysaccharides (Paulsen et al. 2003; Ahmadi et al. 2014).

Cathelicidins are cationic peptides with antimicrobial activity that are constitutively present in granulocytes. These peptides are a part of the innate immune response in vertebrates and have been reported in different fish species (Broekman et al. 2013) including salmonids (Scocchi et al. 2009). Cathelicidins play an important role in fish innate immunity due to their antimicrobial activity and increased expression in response to pathogens (Chang et al. 2006; Broekman et al. 2013).

The Mx protein is an antiviral protein induced by interferon, the most important cytokine involved in innate immune system regulation (Haller et al. 2007). Mx is expressed in the cytoplasm and nucleus, and Mx expression has been shown to provide resistance against a broad range of viruses (Nygaard et al. 2000; Lester et al. 2012). It belongs to the dynamin superfamily of GTPases, and it might also play a role in intracellular transport, endocytosis, and in protein/vesicle transport via secretion and mitosis (Shirozu et al. 2016). The production of Mx in peripheral white blood cells has been reported as constitutive in human and other vertebrate leucocytes (Das et al. 2009). The alternative complement pathway is a powerful defense mechanism in lower vertebrates such as the teleosts (Ellis 2001; Morales et al. 2011; Kiron 2012). The complex innate defense mechanism can be constitutive or responsive in providing protection in the wild (Kiron 2012).

The aim of this study was to evaluate the effect of diets supplemented with two edible red seaweeds, *Pyropia columbina* and *Gracilaria chilensis* (Pereira 2016) cryo concentrates. The outcomes were levels of Mx, lysozyme, cathelicidin, IFN- γ , and IL-6 immune-relevant genes in uninfected *Salmo salar* blood leukocytes.

Materials and methods

Red seaweed cryo concentrates

Two edible red seaweeds *P. columbina* and *G. chilensis* were selected based on morphological description (Toledo et al. 2009) for the preparation of a cryo concentrate (CC). They were harvested from Ancud, Chile (41°52'S 73°19'W) during winter. The red seaweed cryo concentrates (RSCC) were obtained and cleaned, and the cryo concentrate was prepared using a freeze dryer. The RSCC was then vacuum packaged for subsequent grinding and inclusion in a balanced diet formulated by BioMar Chile SA (Castro, Chiloé Island, Chile).

Nutritional characterization of the RSCC

The nutritional characterization of the RSCC was performed versus reference samples of a standard national soybean meal made with ingredients produced nationally and provided by

the Nutrition Laboratory, Faculty of Agricultural Sciences, University of Chile. The nutritional characterization was performed at the University of Missouri Agricultural Experiment Station Chemical Laboratories in Columbia, MO, USA and at the Nutrition Laboratory of the Department of Animal Production in the Faculty of Agronomic Sciences at the University of Chile.

The amino acid and fatty-acid profiles as well as proximate analyses were determined using the official methods of analysis of the Association of Official Analytical Chemists (AOAC): amino acid profile, AOAC 982.30; fatty acid profile, AOAC 996.06; proximate analyses and crude protein combustion analysis, AOAC 984.13A–D (2006); Kjeldahl method multiplying the nitrogen value by (6.25); ash determination, AOAC 942.05; sodium and potassium determination, AOAC 969.23 (2005); zinc determination, AOAC 999.11 (2005); calcium determination, AOAC 991.25 (2005); crude fat by extraction, AOAC 920.39A; crude fiber, AOAC 978.10 (2006); moisture content, AOAC 934.01 (2006); and total carbohydrates, crude “by difference” method, 100% – %(CP + ash + crude fat + M).

Experimental diets

The RSCC were ground to obtain 200 µm particles and mixed with a base of the BioMar Chile feed formulation in the following proportions to produce nine experimental diets: 0.1%, 1%, and 10% *P. columbina* or *G. chilensis*; 0.1 and 1% mixtures of

both seaweeds in a 1:1 ratio; and a control diet with no supplemental seaweed (Table 1).

Experimental animals

Atlantic salmon ($n = 486$) with an initial mean body weight of 149.05 ± 32 g were randomly distributed into 27 experimental tanks with a continuous water flow of 6 L min^{-1} , a summer photoperiod (LD 10:14), a mean temperature of $12 \text{ °C} \pm 0.52$, and a tank volume of 200 L. Each treatment was performed in triplicate at the BioMar research center in Castro on Chiloé Island, Chile. The fish were acclimated for 2 weeks prior to feeding with the experimental diets for 8 weeks. Fish were fed twice daily to apparent satiation. Food intake was recorded daily, and the fish were weighed initially and at 8 weeks after initiating the diets.

Blood samples and isolation of leukocytes

Blood was collected from the fish after 8 weeks on the experimental and control diets. Three fish per tank were randomly selected and fasted for 24 h prior to sampling. To extract the blood, a fish was placed on an ice gel pack for approximately 30 s to reduce movement, and a 2-mL syringe was used to extract 0.5 mL of blood from the caudal vein. The blood was collected in a 2.0 mL cryovial with 0.8 mL of cold sterile Hank’s BSS (Gibco) medium (Fujiwara et al. 2001) without additives to

Table 1 Formulation and chemical composition of experimental diets

	Control	0.1 A	1.0 A	0.1 B	1.0 B	0.1 Mix	1.0 Mix	10.0 A	10.0 B
Ingredients (%)									
Fish meal	28.00	28.00	28.00	28.00	28.00	28.00	28.00	28.00	28.00
Soybean concentrate	16.64	16.54	15.64	16.54	15.64	16.54	15.64	14.90	14.90
Whole wheat	14.00	14.00	14.00	14.00	14.00	14.00	14.00		
Wheat meal								7.00	7.00
Fish oil	19.7	19.7	19.7	19.7	19.7	19.7	19.7	18.4	18.4
Corn gluten	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Chicken viscera meal	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Monocalcium phosphate	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mineral vitamin premix	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38
L-Lisine	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.27	0.27
DL-Methionine	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.08	0.08
<i>Pyropia columbina</i> (A)*		0.10	1.00			0.05	0.50	10.00	
<i>Gracilaria chilensis</i> (B)*				0.10	1.00	0.05	0.50		10.00
	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Chemical composition of diets									
Lipids, %dm	20.6	23.6	26.3	25.6	25.7	26.1	20.3	25.5	28.7
Protein, %dm	45.8	43.8	42.1	42.7	43.0	42.1	48.1	43.0	37.6
Fiber, %dm	1.8	1.6	1.6	1.5	1.6	1.5	1.4	1.6	2.0
Ash, %dm	8.0	7.5	7.0	7.3	7.3	7.0	8.2	7.5	9.6
Moisture, %	6.5	6.7	6.4	6.0	5.7	4.3	8.0	5.8	10.3

A *Pyropia columbina* CC

B *Gracilaria chilensis* CC

dm dry matter basis

prevent interference in the gene expression analysis (Lortat-Jacob et al. 1996; Johnson et al. 2003). After sampling, the fish were returned to the tank and recovered in about a minute. The blood was chilled in the cryovials on ice for 5 min before centrifuging at $258\times g$ for 5 min at room temperature (RT). After centrifugation, the buffy coat and lymphocyte-rich plasma were collected in a 2.0-mL cryovial and placed in liquid nitrogen for transport. The cryovials were stored at $-80\text{ }^{\circ}\text{C}$ in the Genetics and Aquaculture Laboratory in the Faculty of Agriculture at the University of Chile until the gene expression assay.

RNA isolation and cDNA synthesis

For RNA extraction, 750 μL of TRIzol was added to the white blood cells (leukocytes estimated to be 1.6% of the blood in salmonids, or $\sim 5.8 \times 10^6$ cells) (Fujiwara et al. 2001). The sample was then centrifuged at $12000\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$. The pellet was discarded, and the supernatant was incubated for 5–10 min at RT before adding 200 μL of chloroform. The sample was mixed by inversion for 15 s, incubated for 2–3 min at RT, and centrifuged at $12,000\times g$ for 15 min at $4\text{ }^{\circ}\text{C}$ before adding 1 μL of glycogen (50 mg mL^{-1}) and 500 μL of isopropyl alcohol. The sample was then incubated for 10 min at RT and centrifuged for 10 min at $12000\times g$ at $4\text{ }^{\circ}\text{C}$, and the supernatant was then removed. The RNA pellet was washed with ethanol (75%), centrifuged at $7500\times g$ for 5 min at $4\text{ }^{\circ}\text{C}$, and allowed to dry for 10 min at RT after removal of the supernatant.

The RNA pellet was resuspended in RNase-free water and incubated in a $55\text{ }^{\circ}\text{C}$ water bath for 15 min. The quantity and purity of the extracted RNA was checked in a spectrophotometer (Biochrom WPA Biowave DNA Spectrophotometer); a 260/280 absorbance ratio of 1.8–2.1 was considered pure RNA.

Gene expression

Five genes (cathelicidin, lysozyme, IL-6, IFN γ , and Mx) associated with the antiviral innate immune response were

selected to evaluate the effects of the dietary ingredients on gene transcription levels in blood leukocytes. The elongation factor 1-alpha (EF1 α) was used as a housekeeping gene. The primers were designed using the National Center for Biotechnology Information “Pick Primers” function (ncbi.nlm.nih.gov) and purchased from Integrated DNA Technologies, Inc., Santiago, Chile (Table 2).

Real-time polymerase chain reactions (qRT-PCR) were performed in a two-step system using Maxima SYBR Green/ROX qPCR Master Mix (2 \times ; Thermo Scientific, Chile) and the Eco Real-Time PCR System (Illumina, USA). The first-strand cDNA was synthesized using Thermo Scientific RevertAid reverse transcriptase according to the supplier instructions.

PCR reactions were performed in duplicate in 48-well reaction plates in 20 μL reaction mixtures containing 10 μL of the 2 \times qPCR Master Mix, 0.3 μL forward primer, 0.3 μL reverse primer, 8.4 μL nuclease-free water, and 1 μL cDNA template. The wells were sealed with optical adhesive film, and the plate was centrifuged prior to amplification under the following conditions: 10 min at $95\text{ }^{\circ}\text{C}$ (initial denaturation), followed by 40 cycles of 15 s at $95\text{ }^{\circ}\text{C}$ (denaturation), and 60 s at $60\text{ }^{\circ}\text{C}$ (annealing and extension). The amplification was confirmed by a melting curve analysis to verify that a single gene product had been amplified. The C_T values provided by the real-time PCR instrumentation were imported to a Microsoft Excel spreadsheet, and the gene expression fold changes were computed using the comparative $2^{-\Delta\Delta C_T}$ method, where $\Delta\Delta C_T = (C_{T\text{ target gene}} - C_{T\text{ EF1}\alpha})_{\text{treated sample}} - (C_{T\text{ target gene}} - C_{T\text{ EF1}\alpha})_{\text{untreated control}}$ (Livak and Schmittgen 2001).

Statistical analysis

To test for differences between the dietary treatments on Mx, IL-6, IFN γ , cathelicidin, and lysozyme gene expression, the data were subjected to a one-way analysis of variance (ANOVA) using SPSS Statistics version 22 (IBM Corporation, USA). All

Table 2 Primers sequences for real-time PCR

Gene	Primer sequence 5' to 3'	Accession no.	Amplicon size (bp)
Mx	FW -GAGGACATCGACATCGGCAG RS-AAACCCCAATGCCTACTTCCA	NM_001139918.1	167
IFN γ	FW-GGTTCGACGTGTATCGGA RS-TCAGGAAGTAGTGTCTGGGT	NM_001123558.1	125
EF1 α	FW-GTGGCAGTGTGATTCGTAGT RS-GCCAATGACCACGATGTTGA	NM_001123629.	83
IL6	FW-CCTCTGTACACACCAGACC RS-AACACGCTTCCTCCTACTGG	NM_001124657.1	104
Cathelicidin	FW-CTGAGCACTCAGAAGATTCGGACA RS-ACTCCAGGACGACCTCTC	NM_001123573.1	111
Lysozyme	FW-ACCCCAAGAGCTGTGAATGC RS-TCACCACCCTCTTGCACAT	XM_014191331.1	94

data were checked for homogeneity of variance prior to the ANOVA. When differences among groups were identified, multiple comparisons to the control were made using Dunnett's post-hoc test. A difference was considered significant if p was < 0.05 .

Results

Red seaweed cryo concentrates

The nutritional characterization of the RSCC showed higher taurine values in the amino acid profile. RSCC were characterized by high amounts of ash and analyzed minerals. The RSCCs had lower values for protein content than soybean meal and a wider range of amino acids. However, the taurine levels in RSCC were 16-fold greater than that in the soybean meal. The proximate and mineral analyses showed an ash content three times higher in the RSCC than in the soybean meal; potassium, calcium, and sodium levels were twice as high in the RSCC as in the soybean meal (Table 3).

There was also a difference in the fatty acid profiles between RSCC and the soybean meal. The RSCCs had high palmitic (16:0) and arachidonic acid (20:4n6) contents, while the soybean meal had a high linoleic acid (18:2n6) content and no arachidonic acid. Myristoleic fatty acids (9c-14:1) were also only present in the RSCC. Of the omega-3 fatty acids, eicosapentaenoic acid (EPA) (20:5n3) and DHA (22:6n3), EPA was only present in *P. columbina* CC, and docosahexaenoic acid (DHA) was only present in *G. chilensis* CC. Neither of these omega-3 fatty acids were present in the soybean meal (Table 4).

Experimental animals

No mortality was observed during the experimental period, and all experimental diets were well accepted by the animals; no toxic or pathological signs were observed. No significant differences were observed in the feed intake across groups regardless of RSCC supplement. The diet supplemented with 10% *G. chilensis* resulted in a significant increase in weight gain versus control diet (Table 5).

Gene expression

After 8 weeks, the qRT-PCR results showed that the Mx transcript levels in the blood leucocytes of the animals on the diets supplemented with RSCC were significantly lower ($p < 0.05$) than those fed with an unsupplemented control diet. All of the groups fed, the RSCC-experimental diets had negative values for Mx expression in their white blood cells at the end of the experiment (Fig. 1).

The lysozyme transcript levels showed that the constitutive expression significantly increased in the animals fed with the diet supplemented with 1.0% *P. columbina* CC.

Table 3 Amino acid profile, proximate, K, Ca, Na and Zn composition of the RSCC and soybean meal

Units	W/W% <i>Pyropia columbina</i>	W/W% <i>Gracilaria chilensis</i>	W/W% Soybean meal
Taurine	0.99	1.06	0.06
Hydroxyproline	0.07	0.01	0.10
Aspartic Acid	2.58	1.92	5.51
Threonine	1.27	0.82	1.86
Serine	1.05	0.74	2.11
Glutamic Acid	2.95	1.90	8.46
Proline	0.99	0.73	2.51
Lanthionine	0.14	0.09	0.17
Glycine	1.93	0.97	2.09
Alanine	3.29	1.10	2.17
Cysteine	0.57	0.37	0.69
Valine	1.70	1.02	2.46
Methionine	0.49	0.30	0.64
Isoleucine	0.91	0.84	2.25
Leucine	1.72	1.24	3.87
Tyrosine	0.84	0.64	1.85
Phenylalanine	0.88	0.84	2.56
Hydroxylysine	0.02	0.04	0.09
Ornithine	0.08	0.18	0.04
Lysine	1.48	0.97	3.06
Histidine	0.36	0.25	1.30
Arginine	1.38	1.17	3.64
Tryptophan	0.26	0.13	0.67
Total	25.94	17.33	48.14
Crude Protein*	31.12	21.54	49.83
Moisture	8.76	4.22	10.05
Crude Fat	0.60	0.47	2.41
Crude Fiber	4.40	5.72	3.67
Ash	22.98	37.71	8.15
Potassium	3.55	5.92	1.79
Calcium	0.34	0.58	0.25
Sodium	1.59	2.66	0.04
Zinc**	2.10	3.50	2.20

*Percentage N X 6.25. W/W% gram per 100 g of sample

**mg per 100 g of sample

Results (except moisture) are presented on a dry matter basis

The lysozyme levels were increased in all animals fed with diets that included *P. columbina* CC, whether alone or mixed 1:1 with *G. chilensis* CC. In contrast, there was no effect on the lysozyme transcript levels for the animals fed with diets supplemented with only *G. chilensis* CC; their expression levels were similar to those observed in the control group (Fig. 2).

There were no significant changes in cathelicidin, IFN γ , or IL-6 gene expression in any of the groups fed with the RSCC

Table 4 Fatty acid profile of the RSCC and soybean meal

Units	W/W% <i>Pyropia columbina</i>	W/W% <i>Gracilaria chilensis</i>	W/W% Soybean meal
Crude Fat	0.60	0.47	2.41
Fatty Acid Profile (expressed as percent of total fat)			
Myristic (14:0)	0.41	7.74	0.46
Myristoleic (9c-14:1)	0.55	0.97	0.00
C15:0	0.00	0.54	0.00
Palmitic (16:0)	22.66	51.98	15.93
Palmitoleic (9c-16:1)	0.72	1.46	0.15
Margaric (17:0)	0.00	0.00	0.14
10c-17:1	0.00	0.42	0.00
Stearic (18:0)	0.52	1.37	4.38
Elaidic (9 t-18:1)	0.00	0.00	0.16
Oleic (9c-18:1)	3.09	8.00	18.55
Vaccenic (11c-18:1)	1.52	3.60	0.00
Linoleic (18:2n6)	2.09	1.48	51.62
<i>Linolenic (18:3n3)</i>	<i>0.00</i>	<i>0.00</i>	<i>6.62</i>
<i>Stearidonic (18:4n3)</i>	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>
Arachidic (20:0)	0.00	0.00	0.34
Gonodic (20:1n9)	3.25	0.00	0.00
<i>Homo-α-linolenic(20:3n3)</i>	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>
<i>Arachidonic [20:4n6]</i>	<i>10.88</i>	<i>9.63</i>	<i>0.00</i>
<i>3n-Arachidonic (20:4n3)</i>	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>
<i>EPA (20:5n3)</i>	<i>44.19</i>	<i>0.00</i>	<i>0.00</i>
Behenoic (22:0)	0.00	0.00	0.40
Erucic [22:1n9]	0.00	0.00	0.00
<i>Clupanodonic (22:5n3)</i>	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>
<i>DHA (22:6n3)</i>	<i>0.00</i>	<i>0.52</i>	<i>0.00</i>
Lignoceric (24:0)	0.00	0.00	0.46
Nervonic (24:1n9)	0.00	0.52	0.00

W/W% gram per 100 g of sample

Omega-3 fatty acid values are in italics

diets relative to the control diet. However, it is interesting that while no statistically significant changes were observed, there was a decrease in the cathelicidin and IFN γ transcript levels especially for IFN γ . The distribution of values was wide (Fig. 3a, b). In contrast, the IL-6 results suggest that its gene expression was induced. Although the values were not statistically significant, IL-6 expression increased in the animals fed with 0.1 and 1.0% mixed diets containing both *P. columbina* and *G. chilensis* CC (Fig. 3c).

Dunnnett's test showed statistically significant differences between all groups fed with the RSCC-supplemented diets and the control-diet group (Table 6). There was a significant decrease in Mx expression in all groups fed with the supplemented diets, especially those groups fed with 1% *P. columbina* CC and 1% *G. chilensis* CC. These groups had -1.25 ± 0.19 and -1.05 ± 0.37 -fold lower mRNA expression,

Table 5 Effect of edible red seaweed inclusion level on feed intake and weight-gain animals feed experimental diets

	Feed intake (g)	Weight gain (g)
Control	1403 \pm 147.5	103.06 \pm 2.69
0.1 A	1342.5 \pm 89.2	129.06 \pm 31.85
1.0 A	1251.16 \pm 157.36	127.58 \pm 51.97
0.1 B	1235 \pm 145.25	74.28 \pm 31.01
1.0 B	1311.66 \pm 48.21	126.95 \pm 51.98
0.1 Mix	1291.5 \pm 81.08	112.73 \pm 13.31
1.0 Mix	1362.5 \pm 54.08	86.94 \pm 15.64
10.0 A	1450 \pm 81.55	106.62 \pm 38.37
10.0 B	1478.16 \pm 63.81	*204.39 \pm 24.16

A *Pyropia columbina*; B *Gracilaria chilensis*; and Mix mixture of both algae in a 1:1 ratio

*Significantly different from control ($p < 0.05$)

Values are based on the mean \pm S.D. of three replicate tanks of 18 fish; $n = 3$

respectively. A similar decrease was also observed for the 0.1% mixed-diet group (-1.19 ± 0.34). While this feed contained one tenth of the amount of seaweed CC, both seaweed cryo concentrates were incorporated. Finally, the lysozyme levels increased significantly (1.73 ± 0.06) in the group fed with a diet enriched with 1% *P. columbina* CC.

Discussion

The nutritional characterization showed that both RSCC materials had higher ash, taurine, potassium, calcium, sodium, as well as myristic and palmitic fatty acid contents than soybean meal. Myristoleic (9c-14:1) and arachidonic (20:4n6) fatty acids were present in both seaweed cryo concentrates but not in the soybean meal. The RSCC had ash content that was at least 3-fold higher than that in the soybean meal; the potassium and calcium contents were at least 1.5-fold higher in the marine seaweeds.

This in vivo study showed that the *G. chilensis* red seaweed concentrate increases weight gain; neither seaweed cryo concentrates had a significant effect on cathelicidin, IFN- γ , or IL-6 expression. Similar results have been reported for IFN- γ and IL-6 transcripts in the blood of pigs fed with brown seaweed *Laminaria hyperborea* and *Laminaria digitata* extracts where neither cytokine nor showed significant changes (Reilly et al. 2008). However, a significant increase in lysozyme expression was observed after 8 weeks in animals on the diet enriched with *P. columbina* (1.0%) concentrate. Similar results have been reported for enrichment diets made with free-dried powder (3%) from the red seaweed *Asparagopsis taxiformis*, garlic, and curcumin, each of these significantly increased the

Fig. 1 Effect of RSCC inclusion level on Mx expression in blood white cells ($n = 9$), expression levels are reported as fold change relative to untreated control. A *Pyropia columbina* CC, B *Gracilaria chilensis* CC, and Mix mixture of both seaweed cryo concentrates in a 1:1 ratio. *Significantly different from the control, $p < 0.05$, Dunnett’s test. Boxes represent the interquartile range (IQR) between the first and third quartiles (25th and 75th percentile), and the horizontal line defines the median

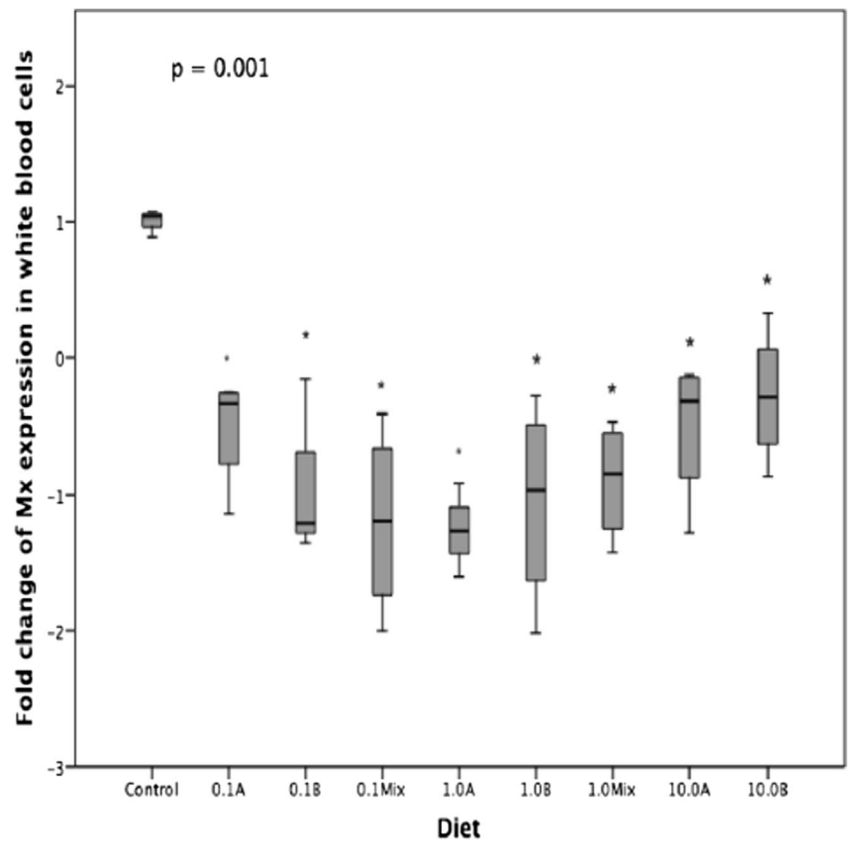
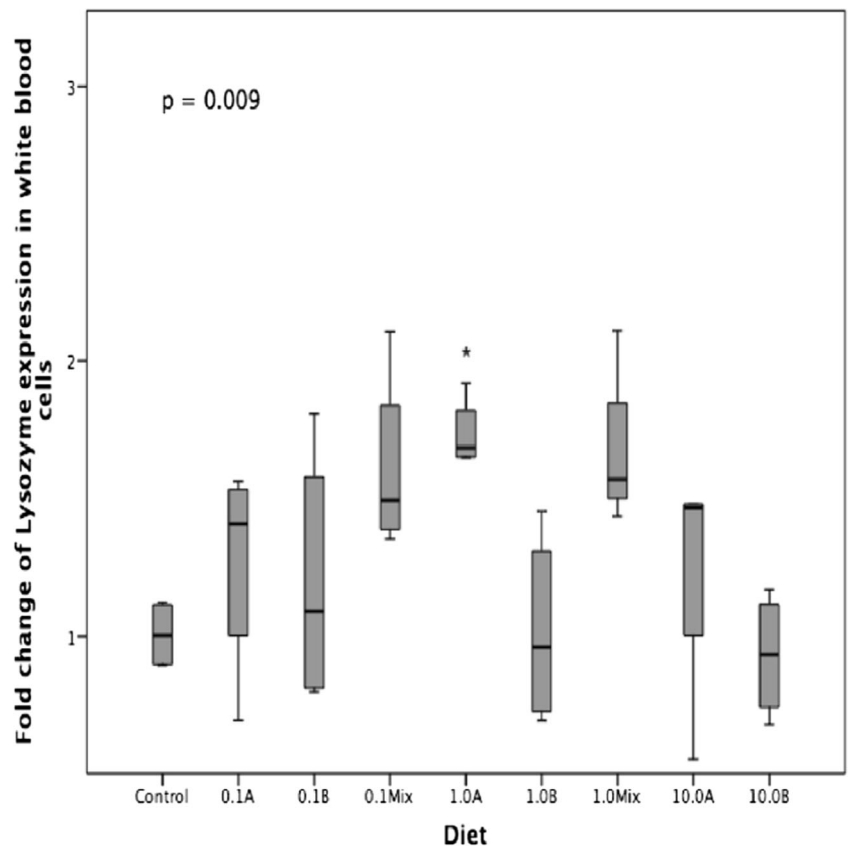


Fig. 2 Effect of RSCC inclusion level on lysozyme expression in blood white cells ($n = 9$), expression levels are reported as fold change relative to untreated control. A *Pyropia columbina* CC, B *Gracilaria chilensis* CC, and Mix mixture of both seaweed cryo concentrates in a 1:1 ratio. *Significantly different from the control, $p < 0.05$, Dunnett’s test. Boxes represent the interquartile range (IQR) between the first and third quartiles (25th and 75th percentile), and the horizontal line defines the median



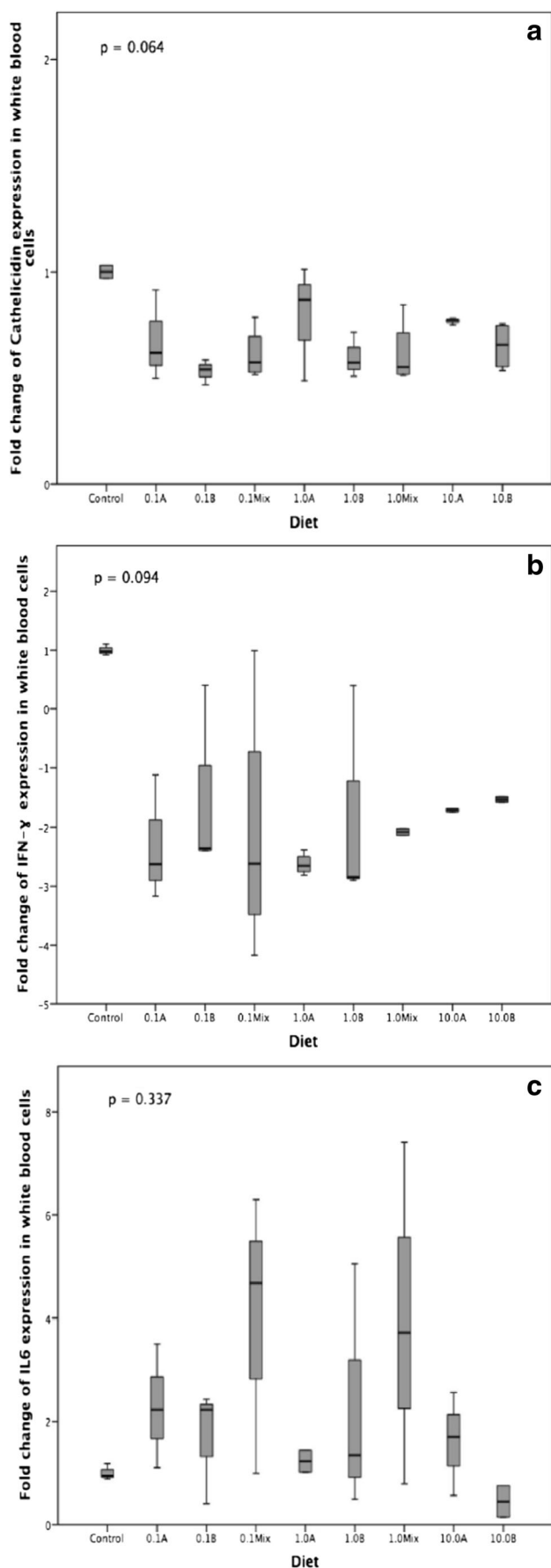


Fig. 3 Effect of RSCC inclusion level on (A) Cathelicidin, (B) INF- γ , and (C) IL6 expression in blood white cells ($n = 9$), expression levels are reported as fold change relative to untreated control. A *Pyropia columbina* CC, *B Gracilaria chilensis* CC, and *Mix* mixture of both seaweed cryo concentrates in a 1:1 ratio. Boxes represent the interquartile range (IQR) between the first and third quartiles (25th and 75th percentile), and the horizontal line defines the median

lysozyme transcript levels in the spleen of orbicular batfish (Reverter et al. 2016).

Marine oil-derived EPA inhibits arachidonic acid, which activates of NF- κ B, and thus many inflammatory genes (Grimm et al. 2002). Diets with moderate levels of dietary linoleic acid (0.32 to 0.63% dry weight), a precursor to EPA and DHA, have been described as significantly enhancing anti-inflammatory responses (Chen et al. 2016; Statovci et al. 2017) and non-specific immunity via lysozyme and complement activity (Chen et al. 2016). Omega-3 polyunsaturated fatty acids can also activate the anti-inflammatory transcription factor PPAR- γ and inhibit NF- κ B and production of its associated pro-inflammatory cytokine (Statovci et al. 2017). *P. columbina* concentrate was characterized by a high EPA content (44.19%), which was absent in *G. chilensis*. This suggests that the presence of the EPA in *P. columbina* is associated with the significantly increased lysozyme expression.

Interestingly, our results showed that experimental diets containing both RSCCs significantly decreased the expression of Mx transcript in peripheral white blood cells. This was observed in all doses of RSCC. Other studies have shown that Mx provides antiviral resistance for uninfected cells (Nygaard et al. 2000) and early protection for a several viral diseases (Lester et al. 2012; Purcell et al. 2012). The mechanism by which the RSCC supplementation decreases Mx expression in white blood cells is unknown. One hypothesis is that the high content of potassium, the primary intracellular cation (Halperin and Kamel 1998) provided by the RSCC, increased intracellular uptake and stimulated the sodium-potassium pump adenosine triphosphatase (Na⁺/K⁺-ATPase), a membrane-bound enzyme that imports potassium into cells by diffusion through a concentration gradient (Cantley et al. 1978; Skou and Esmann 1992; Goldstein et al. 2009).

The energy released in this reaction (Skou and Esmann 1992; Lingrel and Kuntzweiler 1994) plus the presence of the exogenous calcium ions from the RSCC could induce regulated exocytosis or, in this case, calcium-dependent exocytosis (Bi et al. 1995; Alés et al. 1999). This would decrease the Mx transcript in the white blood cells due to its participation in intracellular transport and protein export via secretion (Shirozu et al. 2016). Similar results have been reported in the liver of sea bream (*Sparus aurata*), where no Mx mRNA expression was detected in healthy animals or animals fed with a marine oil-based diet. Feeding the animals vegetable oils altered Mx transcription; this treatment markedly increased the basal Mx expression levels (Montero et al. 2008).

Table 6 Fold change in expression of target genes in white blood cells

Gene	Diet								p value
	0.1A	0.1B	0.1Mix	1.0A	1.0B	1.0Mix	10.0A	10.0B	
Mx	-0.51 ± 0.20*	-0.90 ± 0.37*	-1.19 ± 0.34*	-1.25 ± 0.19*	-1.05 ± 0.37*	-0.89 ± 0.21*	-0.51 ± 0.26*	-0.28 ± 0.24*	0.001
IL6	2.28 ± 0.69	1.69 ± 0.64	3.99 ± 1.56	1.23 ± 0.21	2.06 ± 1.01	3.97 ± 1.91	1.61 ± 0.57	0.45 ± 0.30	0.337
IFN γ	-2.30 ± 0.61	-1.67 ± 0.69	-2.10 ± 1.09	-1.77 ± 1.08	-2.08 ± 0.55	-1.70 ± 0.03	1.53 ± 0.03	-1.68 ± 0.28	0.094
Lysozyme	1.26 ± 0.19	1.19 ± 0.24	1.61 ± 0.17	1.73 ± 0.06*	1.01 ± 0.17	1.67 ± 0.14	1.24 ± 0.23	0.92 ± 0.11	0.009
Cathelicidine	0.67 ± 0.12	0.53 ± 0.33	0.61 ± 0.06	0.78 ± 0.15	0.59 ± 0.06	0.61 ± 0.07	0.76 ± 0.08	0.65 ± 0.05	0.064

Data are reported as means ± SE. The mean fold change of the target gene at each dietary treatment was calculated using $2^{-\Delta\Delta C_T}$ method, where $\Delta\Delta C_T = (C_{T \text{ target}} - C_{T \text{ EF1}\alpha})_{\text{Treated group}} - (C_{T \text{ target}} - C_{T \text{ EF1}\alpha})_{\text{untreated control}}$

*The mean difference is significant at the 0.05 level, Dunnett’s test

A *Pyropia columbina* CC, B *Gracilaria chilensis* CC, and Mix mixture of both seaweed cryo concentrates in a 1:1 ratio

Conclusions

Our in vivo study is the first to show the potential of two edible red seaweed cryo concentrates (*P. columbina* and *G. chilensis*) to modulate the expression of the immune-relevant genes encoding the Mx antiviral protein in *S. salar* white blood cells within 8 weeks of administration. In addition, diets enriched with 1% *P. columbina* cryo concentrate significantly increased white blood cell lysozyme expression over a similar period.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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