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Red macroalgae *Pyropia columbina* and *Gracilaria chilensis*: sustainable feed additive in the *Salmo salar* diet and the evaluation of potential antiviral activity against infectious salmon anemia virus

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Abstract Red macroalgae (Phylum Rhodophyta) are considered to be an important source of biologically active metabolites; their use as functional feed ingredients in fish diets can increase disease resistance and animal growth. Furthermore, red macroalgae can be cultured in a sustainable and environmentally friendly manner. We evaluate the potential antiviral activity against the infectious salmon anemia (ISA) virus of Chilean red macroalgae as a sustainable feed additive in the Atlantic salmon (*Salmo salar*) diet. Lyophilized Chilean red macroalgae concentrates were elaborated and added to a commercial diet. Diets were prepared with 0.1, 1.0, and 10.0 % of *Gracilaria chilensis* and *Pyropia columbina* separately, and 0.1 and 1.0 % of a mix of both species in a 1:1 ratio. The diets were fed to *S. salar* over a period of 2 months. We collected data on production parameters and blood samples. The serum and its constituents were challenged with the ISA virus in the presence of Atlantic salmon kidney cells and tested ex vivo for antiviral capacity using a plaque reduction neutralization assay. Fish fed a diet containing 10 % *G. chilensis* showed a significantly higher specific growth rate compared with fish fed the control diet. The feed conversion ratio was not significantly affected by treatments. Sera from fish fed the algae diets showed a significant increase in antiviral activity against the ISA virus compared with sera of fish fed the control diet that did not include red macroalgae. Diets including *G. chilensis* (1.0 and 10 %) exhibited the largest increase in antiviral activity.

Keywords Red macroalgae · Antiviral activity · ISA virus · Ex vivo challenge · Atlantic salmon

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Introduction

The prevalence of infectious salmon anemia virus (ISA), detected most recently in October 2014 in southern Chile and other new diseases, is one of the biggest obstacles to Chilean aquaculture sustainability. The main strategies currently used to ameliorate the negative effects of disease outbreaks include vaccines and antibiotics. These methods carry the risk of creating resistant pathogens, causing host immunosuppression and harming the environment. One alternative is the use of diets with functional ingredients such as probiotics, prebiotics, immunostimulants, vitamins, and other bioactive components, a strategy demonstrated to potentially increase disease resistance and improve growth (Sweetman et al. 2010; Tacchi et al. 2011; Mohamed et al. 2012).

Among the alternative sources of bioactive compounds, ingredients or products derived from marine algae show great potential for use in aquaculture to enhance immune system function and disease resistance (Trichet 2010). Red, brown, and green algae have been shown to have therapeutic properties, including anticancer, antioxidant, anticoagulant, anti-inflammatory, immunomodulating, neuroprotective, antibacterial, and antiviral activities (El Gamal 2010; Mohamed et al. 2012).

Extracts from red macroalgae are a rich source of potential antiviral components for fish. Red macroalgae, for example, contain significant amounts of sulfated polysaccharides (Vo et al. 2011). These red algae components have shown to have antiviral effects on HSV-1, HSV-2, HIV-1, RSV, and the influenza virus. Some of the described mechanisms of antiviral action of these algal polysaccharides include inhibition of virus adsorption to the host cell by competing with the virus upon binding to the cell (Duarte et al. 2004), a synergistic interaction with the target cell to block viral entry (Feldman et al. 1999), and inhibition of HIV replication in vitro (Bourgougnon et al. 1996).

Red macroalgae also contain significant amounts of vitamin C, amino acids, peptides, omega-3 fatty acids, and proteins (Dawczynski et al. 2007; MacArtain et al. 2007; Matanjun et al. 2009). Among the bioactive proteins are two important groups: lectins and phycobiliproteins (Gonzalez et al. 1999). The latter are found only in cyanobacteria and red algae (Morse et al. 1984). Given the above, red macroalgae are a potential source of antiviral components for fish, hence, a good candidate to be included in fish diets.

In vivo challenges involve subjecting a large number of animals to pain and distress (Stokes et al. 2011). Unlike in vivo assays, ex vivo assays are performed following a feeding regimen, allowing for measurement of specific markers in tissues from fish fed experimental diets with minimum disturbance of natural conditions, revealing the effects of the diet and the response of the innate and/or adaptive immune system using in vitro assays (Kiron 2012).

Having this in mind, the goal of this study was to evaluate the use of red macroalgae as a part of the diet of Atlantic salmon to combat the effects of the ISA virus. The impacts of the diet were assessed by an ex vivo assay to measure the effect of the inclusion of red algae (0.1, 1.0, and 10.0 %) in the salmon fish diet using blood samples to evaluate a potential protection effect over ASK cells from ISA virus infection.

Materials and methods

Preparation of lyophilized red algae

The Chilean red macroalgae species selected as feed additive for the experimental diets were *Pyropia columbina*, which has high-protein content throughout the year (25.20 % crude protein in winter) and *Gracilaria chilensis*, which has the second highest protein content (24.31 % in winter) among Chile's red macroalgae (Toledo et al. 2009). Both species are accessible in Chile from natural seaweed beds (Buschmann et al. 2001) and by cultivation (Buschmann et al. 2008). The harvest and the lyophilization of algae took place during June, July, and August of 2012 at Ancud, Chiloe Island, Chile (41° 52′ S, 73° 19′ W). We collected 320 kg of *P. columbina* and 240 kg of *G. chilensis* from this area.

The algae were moved to the premises of the Granjamar Company in Calbuco on the same day of harvest. The algae were cleaned and lyophilized using a Cuddon Model FD80 General Purpose Freeze Dryer. The lyophilisate was then vacuum-packaged for storage and transfer. Subsequently, the algae were transferred to the processing plant of the University of Lagos Aquaculture Production Unit for grinding and inclusion to a balanced fish feed formulated by BioMar Chile SA.

Nutritional characterization of the lyophilized algae

Nutritional characterization of the lyophilized algae was performed. Samples of standard national fish meal, soybean meal, and rapeseed meal were also characterized and were used as a reference for comparison; ingredients were produced nationally and were provided by the Nutrition Laboratory of the Animal Production Department, University of Chile.

Proximate analysis, fatty acid profile, and amino acid profile were determined using the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC). For all samples above, the following parameters were tested:

Standard amino acid analysis: amino acid profile, AOAC Official Method 982.30.

Proximate analyses: crude protein combustion analysis, AOAC Official Method 984.13 (A–D), 2006, Kjeldahl, utilizing the calculation 6.25×nitrogen value; ash determination, AOAC Official Method 942.05; crude fat by ether extraction, AOAC Official Method 920.39 (A); crude fiber, AOAC Official Method 978.10, 2006; moisture, AOAC Official Method 934.01, 2006; total carbohydrates, crude "by difference" calculation, 100 %−%(CP + ash + crude fat + M).

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

Experimental diets

The *G. chilensis* meal was obtained with a single milling; for *P. columbina*, a second milling was necessary to obtain appropriate particle size of this macroalga. After mixing the

ingredients, the feeds were sieved, packed, and sent to BioMar Research Center in Castro, Chiloe Island, Chile.

The experimental diets were obtained by extruding 3-mm pellets from a base mixture from Bio Mar Chile feed formulation (Table 1) with 0.1, 1, and 10 % of the red algae *P. columbina* and *G. chilensis*; 0.1 and 1 % of a mixture of both algae in a 1:1 ratio; and a control diet for a total of nine experimental diets.

Experimental fish, rearing conditions, and production parameters

The fish were fed with the experimental diet for 8 weeks, from January 15 to March 15, 2013. The fish were fed to satiation twice daily. The experiment took place at the BioMar Research Center in Castro, in the Chiloe Island, Chile. Before beginning the experiment, the fish were acclimated to the experimental conditions for 2 weeks. A total of 486 *Salmo salar* fish with an initial average weight of 149.05±32 g (mean±SD) were randomly distributed into 27 tanks (18 fish per tank) with continuous 6 L min^{-1} water flow, summer photoperiod (LO 10:14), average temperature of 12 °C, and a volume of 200 L per tank. Each treatment was carried out in triplicate.

Food intake was recorded daily, and the fish were weighed at 2, 4, and 8 weeks during the experimental period. Production parameters specific growth rate (SGR) and feed conversion ratio (FCR) were calculated using the following formulae:

$$\mathrm{SGR} = 100 \times (\ln W_2 - \ln W_1) \Big/ \text{feeding days},$$

where W_1 and W_2 are the initial and final weights of fish, respectively, and ln is the natural logarithm.

$$\mathrm{FCR} = \text{feed consumed} \Big/ \text{biomass increase}$$

Serum samples

After acclimatization and before the beginning of the experimental feeding period, ten fish were randomly selected to take blood samples as controls.

Serum samples were collected from fish on the experimental diets after 2 weeks of feeding, randomly selecting three fish per tank. Fish were fasted for 24 h prior to sampling. For the blood extraction, the fish was placed on ice gel-pack for approximately 30 seconds to reduce movement and a 2-mL syringe was used to extract 0.5–1 mL of blood from the caudal

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
SBC	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
CVM	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
MCP	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MVP 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
A		0.10	1.00			0.05	0.50	10.00	
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
Diets chemical composition									
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: lyophilized *Pyropia columbina*. B: lyophilized *Gracilaria chilensis*. Mix: lyophilized mixture of both algae in a 1:1 ratio

SBC soybean concentrate *CVM* chicken viscera meal, *MCP* mono calcium phosphate, *MVP mix* mineral vitamin premix, *dm* dry matter basis

vein and collected it in a 2.0-mL cryo vial without additives in order to prevent blood acidification that favors ISA virus infection (Eliassen et al. 2000). After the sample was extracted, the fish were returned to the tank for recovery, which took about 1 min.

The blood was kept in cryo vials on ice until coagulation and then centrifuged at 3000× *g* for 10 min at 4 °C. The serum was removed and placed in liquid nitrogen for transport to Santiago, Chile, where it was stored in the Genetics and Aquaculture Laboratory, Faculty of Agriculture, University of Chile, at −80 °C until ex vivo antiviral assay in the Molecular Virology Laboratory of the Center for Aquaculture Biotechnology, University of Santiago of Chile.

Cell culture and virus

The Atlantic salmon kidney (ASK) cell line, acquired from the American Type Culture Collection (ATCC) CRL-2747, was used. The cell line was cultured at 16 °C in Leibovitz's L-15 Medium with 10 % fetal bovine serum, L-glutamine (6 mM), 2-mercaptoethanol (40 mM), and gentamicin (50 μg mL^{-1}). The Chilean ISA virus 752_09 (genotype HPR7b) field strain was used (Cottet et al. 2010).

ISA virus ex vivo assay

The anti-ISA virus ex vivo assay in sera of fish fed the experimental diets was performed using the plaque reduction assay (Russell 1962; Dang et al. 2011) with modifications for use in salmonids as described below.

The ASK cell line was seeded at a density of 5×10^4 cells cm^{-2} was infected in triplicate for the formation of 10 to 40 plaque-forming units in a volume of 0.3 mL in the presence of sera samples from fish fed the experimental diets (2 %, *v*/*v*). The samples were assayed without heat inactivation to prevent changes in bioactive compounds with antiviral activity, including complement components, which in fish are completely destroyed and inactivated at 45 °C (Sakai 1992). The mixture was then incubated in 12-well plates for 4 h at 15 °C, after which the inoculum was removed and washed twice with PBS pH 7, adding 1.0 mL per well of semisolid medium composed of L-15 medium supplemented with 0.5 % fetal bovine serum and agarose (Invitrogen cat. 16520-050). The samples were incubated for 15 days at 15 °C in the presence of 6 % serum in semisolid media.

At 15 days post-infection, the samples were fixed with 37 % formalin for 1 h at room temperature. The semi-solid medium was then removed, and 2 mL of 1 % crystal violet was added, for 1 h at 25 °C. Plaque counting was performed using a light microscope. Anti-ISA virus activity was expressed as the percent reduction of the number of plaques.

Statistical analysis

To test differences between dietary treatments on the SGR (specific growth rate), FCR (feed conversion ratio) and ISA virus antiviral activity, data were subjected to a one-way analysis of variance (ANOVA) using SPSS Statistics version 22 (IBM Corporation, USA). All data were subjected to check variance homogeneity prior to the ANOVA. When differences among groups were identified, multiple comparisons to the control were made using the Dunnett's post hoc test. Significant differences were considered when $p<0.05$.

Results

Nutritional characterization of lyophilized red algae

The lyophilized red alga *P. columbina* (31.12 %) showed higher protein content than *G. chilensis* (21.54 %). The crude protein values obtained from both algae are lower than the values for fish meal and similar to rapeseed meal (Table 2). Values for crude fiber and ash for the algae *G. chilensis* (5.72 and 37.71 %) and *P. columbina* (4.40 and 22.98 %) were higher than fish meal (0.22 and 17.98 %). The amino acid profile (Table 2) shows higher taurine values for the algae *P. columbina* (0.99 %) and *G. chilensis* (1.06 %) compared to fish meal (0.69 %), rapeseed meal (0.06 %), and soybean meal (0.06 %).

Crude fat content in the lyophilized was low compared to fish meal, rapeseed meal, and soybean meal. In terms of the fatty acid profile (Table 3), the essential polyunsaturated omega-3 eicosapentaenoic acid (EPA) had values for *P. columbina* (44.19 %) greater than for fish meal (12.31 %). The omega-6 fatty acid arachidonic acid (ARA) values for both algae *P. columbina* (10.88 %) and *G. chilensis* (9.63 %) were five times higher than fish meal (1.91 %). Linoleic (18:2 ω-6) and linolenic (18:3 ω-3) acid values for both algae *P. columbina* (2.09 and 0.00 %) and *G. chilensis* (1.48 and 0.00 %) were similar to fish meal (0.95 and 0.48 %), but much lower than rapeseed meal (22.14 and 8.44 %) and soybean meal (51.83 and 6.62 %). The saturated fatty acid palmitic acid (16:0) level of *G. chilensis* (51.98 %) was two times higher than fish meal (20.06 %) and *P. columbina* (22.66 %), and much higher than rapeseed meal (5.48 %) and soy bean meal (15.93 %).

Experimental fish and production parameters

All experimental diets were well accepted by the fish, no toxic or pathological signs were observed, and no mortality was observed during the experimental period.

Table 2 Amino acid profiles and proximate composition of the lyophilized algae, fish meal, rapeseed meal, and soybean meal

Units	PC *W/W*%	GC *W/W*%	FM *W/W*%	RSM *W/W*%	SBM *W/W*%
Amino acid					
Taurine	0.99	1.06	0.69	0.06	0.06
Hydroxyproline	0.07	0.01	0.58	0.41	0.10
Aspartic Acid	2.58	1.92	6.29	2.40	5.51
Threonine	1.27	0.82	2.81	1.47	1.86
Serine	1.05	0.74	2.22	1.22	2.11
Glutamic acid	2.95	1.90	8.46	5.16	8.46
Proline	0.99	0.73	2.92	2.05	2.51
Lanthionine	0.14	0.09	0.16	0.14	0.17
Glycine	1.93	0.97	4.27	1.72	2.09
Alanine	3.29	1.10	4.36	1.49	2.17
Cysteine	0.57	0.37	0.61	0.81	0.69
Valine	1.70	1.02	3.59	1.83	2.46
Methionine	0.49	0.30	1.91	0.69	0.64
Isoleucine	0.91	0.84	3.08	1.36	2.25
Leucine	1.72	1.24	5.30	2.39	3.87
Tyrosine	0.84	0.64	2.30	0.99	1.85
Phenylalanine	0.88	0.84	2.92	1.36	2.56
Hydroxylysine	0.02	0.04	0.17	0.22	0.09
Ornithine	0.08	0.18	0.10	0.02	0.04
Lysine	1.48	0.97	5.73	2.00	3.06
Histidine	0.36	0.25	2.44	0.90	1.30
Arginine	1.38	1.17	4.15	2.00	3.64
Tryptophan	0.26	0.13	0.89	0.44	0.67
Total	25.94	17.33	65.95	31.13	48.14
Composition					
Crude protein[a]	31.12	21.54	74.20	34.86	49.83
Moisture	8.76	4.22	8.17	5.88	10.05
Crude fat	0.60	0.47	8.72	12.75	2.41
Crude fiber	4.40	5.72	0.22	8.22	3.67
Ash	22.98	37.71	17.98	5.95	8.15
Silica (SiO_2)	0.70	1.34			

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

PC Pyropia columbina, *GC Gracilaria chilensis*, *FM* fish meal, *RSM* rapeseed meal, *SBM* soybean meal

[a] Percentage $N \times 6.25$. *W/W*%=gram per 100 g of sample

Fish fed diets containing lyophilized red algae showed a significantly higher values (0.013 $p<0.05$) for SGR compared to the control diet (Table 4). As can be seen in Fig. 1, among the algae treatments, fish fed the diet with 10 % *G. chilensis* (1.51±0.12 SD) showed the largest increase in SGR relative to the control diet (0.92±0.01 SD).

The feed conversion ratio was not significantly affected by treatments in the present study (Table 4). According to Fig. 2 there is no significant difference among treatments. Moreover, 10 % *G. chilensis* (0.40± 0.03 SD) induce the lowest FCR relative to the control diet (0.88±0.08 SD).

Anti-ISA virus activity

After performing the ex vivo ISA virus challenge protocol, lysis plaques generated by the virus were obtained, counted, and recorded. In control serum samples (T0), no antiviral effect was observed, with a similar number of lysis plaques in the treated and untreated cells.

It was observed that the lysis plaques generated in the presence of serum from control fish were larger than the lysis plaques in serum-free medium. There was no discoloration of the medium in the presence of serum, whereas there was discoloration in serum-free medium. In contrast, the assay performed with the sera of fish fed with the experimental diets

Table 3 Fatty acid profile of the lyophilized algae, fish meal, rapeseed meal, and soybean meal

	PC *W/W*%	GC *W/W*%	FM *W/W*%	RSM *W/W*%	SBM *W/W*%
Crude fat	0.60	0.47	8.72	12.75	2.41
Fatty acid profile[a]					
Myristic (14:0)	0.41	7.74	5.55	0.13	0.46
Myristoleic (9c-14:1)	0.55	0.97	0.09	0.02	0.00
C15:0	0.00	0.54	0.45	0.05	0.00
Palmitic (16:0)	22.66	51.98	20.06	5.49	15.93
Palmitoleic (9c-16:1)	0.72	1.46	5.17	0.49	0.15
Margaric (17:0)	0.00	0.00	0.51	0.07	0.14
10c-17:1	0.00	0.42	1.01	0.11	0.00
Stearic (18:0)	0.52	1.37	4.91	1.53	4.38
Elaidic (9t-18:1)	0.00	0.00	0.82	0.25	0.16
Oleic (9c-18:1)	3.09	8.00	8.32	57.34	18.55
Vaccenic (11c-18:1)	1.52	3.60	4.02	0.00	0.00
Linoleic (18:2n6)	2.09	1.48	0.95	22.14	51.62
Linolenic (18:3n3)	*0.00*	*0.00*	*0.48*	*8.44*	*6.62*
Stearidonic (18:4n3)	*0.00*	*0.00*	*1.38*	*0.03*	*0.00*
Arachidic (20:0)	0.00	0.00	0.23	0.51	0.34
Gonodic (20:1n9)	3.25	0.00	0.59	1.66	0.00
Homo-a-linolenic (20:3n3)	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
Arachidonic [20:4n6]	*10.88*	*9.63*	*1.91*	*0.00*	0.00
3n-Arachidonic (20:4n3)	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
EPA (20:5n3)	*44.19*	*0.00*	*12.31*	*0.00*	*0.00*
Behenoic (22:0)	0.00	0.00	0.11	0.32	0.40
Erucic [22:1n9]	0.00	0.00	0.00	0.20	0.00
Clupanodonic (22:5n3)	*0.00*	*0.00*	*2.08*	*0.00*	*0.00*
DHA (22:6n3)	*0.00*	*0.52*	*19.36*	*0.00*	*0.00*
Lignoceric (24:0)	0.00	0.00	0.36	0.17	0.46
Nervonic (24:1n9)	0.00	0.52	0.99	0.17	0.00

Omega 3 fatty acid values are italicized. *W/W*%: grams per 100 g of sample

PC Pyropia columbina, *GC Gracilaria chilensis*, *FM* fish meal, *RSM* rapeseed meal, *SBM* soybean meal

[a] Expressed as percent of total fat

showed a decrease in plaques. Statistical analysis of the results obtained for the lysis plaques indicated a significant increase in antiviral activity against ISA virus (Table 4) for the experimental diets as compared to the control diet that did not include red algae (Fig. 3).

The range of antiviral activity found in sera treated with the experimental diets was 0.00 to 88.00 %. Diets including the 1.0 % *G. chilensis* (59.97 %±24.60 SD) and 10 % *G. chilensis* (68.02 %±18.34 SD) showed the largest increases in antiviral activity (Fig. 3) compared with the control diet (5.90 %±8.42 SD).

Discussion

The specific growth rate increased significantly with the inclusion of lyophilized *G. chilensis* (10 %). The feed conversion ratio was improved with diets containing higher levels of red seaweed (*G. chilensis* and *P. columbina* separately) versus the control. The fish performance data indicate that the inclusion of up to 10 % lyophilized red algae in the salmonid diet has no negative effect on production parameters—this confirms its potential utility as a functional ingredient.

Our study provides evidence that algae are suitable for sustainable anti-ISA virus feed additives for use in salmonids due to the presence of macro- and micronutrients such as silicon, taurine (44.9 % higher content when compared to fish meal), EPA (in the case of lyophilized *P. columbina*), ARA, and palmitic acid (lyophilized *G. chilensis*). These nutrients play important roles in the immune system of fish.

Silicon, for instance, affects lymphocyte proliferation and modulates immune function through arginine (Seaborn et al. 2002). The interaction between silicon and arginine affects immune function, and a silicon deficiency weakens

Table 4 Fish performance and antiviral activity of *Salmo salar* fed the experimental diets, values are mean±SD

	**SGR (%)[a]	**FCR[b]	Antiviral activity (%)[c]
0.0	0.92±0.01	0.88±0.08	5.90±8.42
0.1A	1.08±0.20	0.60±0.18	20.29±13.41
1.0A	1.06±0.33	0.59±0.20	42.23±36.24
0.1B	0.69±0.24	1.02±0.34	25.55±41.01
1.0B	1.05±0.29	0.63±0.19	59.97±24.60*
0.1Mix	0.98±0.08	0.64±0.09	1.23±4.67
1.0Mix	0.80±0.11	0.89±0.17	34.12±24.19
10.0A	0.76±0.27	0.69±0.30	46.54±18.32
10.0B	1.51±0.12*	0.40±0.03	68.02±18.34*

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

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

**Growth performance values are based on the mean±SD of three replicate tanks with 18 fish

[a] Specific growth rate; $n=3$

[b] Feed conversion ratio; $n=3$

[c] Antiviral activity against ISA virus was determined by plaque reduction assay and expressed as percentage reduction of plaque numbers; $n=9$

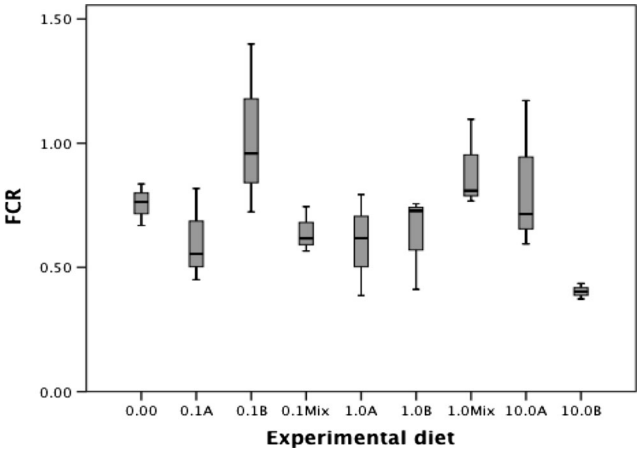

Fig. 2 Effect of macroalgae inclusion level on FCR feed conversion ratio; $n=3$ (three replicate tanks of 18 fish). A = *Pyropia columbina*, B = *Gracilaria chilensis*, and Mix = mixture of both algae in a 1:1 ratio

lymphocyte proliferation. The fish that are fed diets fortified with arginine show increased resistance to disease (Costas et al. 2011).

To date, plant-based alternatives to fish meal as protein sources have been deficient in taurine, which is involved in numerous physiological processes and other anti-inflammatory processes (Divakaran 2006). Taurine is a strong antioxidant (Zeng et al. 2010) and protects tissue against oxidative tissue damage and had been reported to promote growth and growth depression during taurine deficiency (Salze and Davis 2015). Our results show that both lyophilized algae have higher content of taurine when compared with fish meal, evidence that supports the results observed in the present study in terms of the improved SGR when fish were fed diets supplemented with these algae.

Polyunsaturated fatty acids are important components of the cell membrane in fish. The dietary requirements for these fatty acids can only be met with long-chain fatty acids docosahexaenoic acid (DHA) and EPA (Morais et al. 2012). These fatty acids are precursors of eicosanoids, prostaglandins, and leukotrienes that are involved in the immune and inflammatory responses of fish (Martinez-Rubio et al. 2013). The EPA content of lyophilized *P. columbina* (44.19 %) suggests that this alga is a promising source of this essential fatty

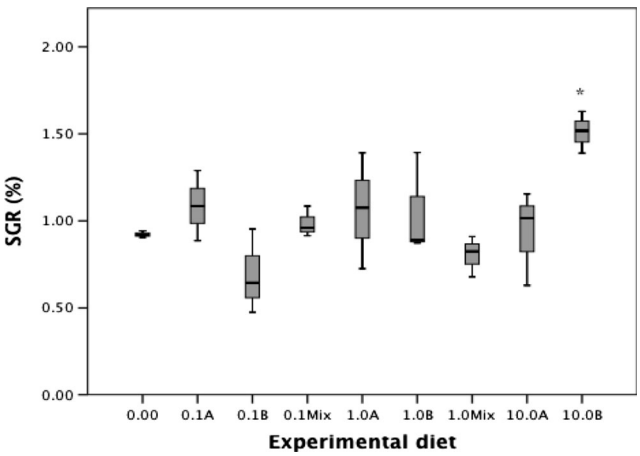

Fig. 1 Effect of macroalgae inclusion level on SGR specific growth rate (three replicate tanks of 18 fish). A = *Pyropia columbina*, B = *Gracilaria chilensis*, and Mix = mixture of both algae in a 1:1 ratio. *Significantly different from the control, $p<0.05$

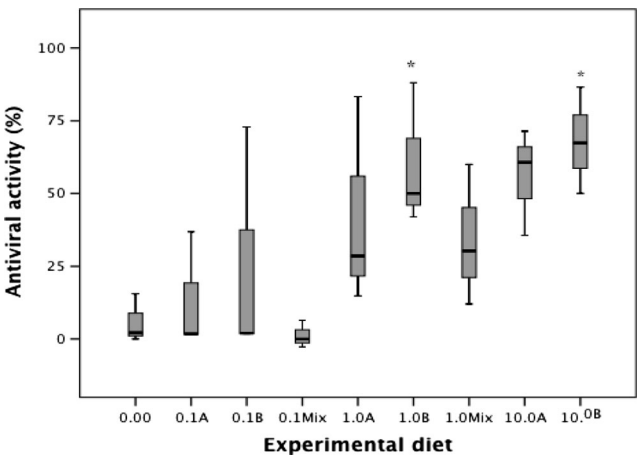

Fig. 3 Effect of macro algae inclusion level on ISA virus antiviral activity in sera from fish fed experimental diets ($n=9$). A = *Pyropia columbina*, B = *Gracilaria chilensis*, and Mix = mixture of both algae in a 1:1 ratio. *Significantly different from the control, $p<0.05$

acid. It has been shown that Atlantic salmon fed vegetable-based diets have lower levels of ω-3 (Liland et al. 2013; Torstensen et al. 2005). There is a direct relationship between omega ω-3 content in aquaculture feed on the health of the fish (Midtbø et al. 2013) as well as consumer health (Seierstad et al. 2005). However, our study showed that *G. chilensis* with a low content of EPA (0.45 %) had the greatest protection against ISA virus suggesting in this case that EPA may have not had a marked influence in ISA virus antiviral activity.

The importance of ARA in fish diets has been underestimated in comparison with EPA and DHA (Bell and Sargent 2003). The presence of ARA have been linked with higher growth and survival rates (Castell et al. 1994). In Atlantic salmon during the smoltification stage, the levels of ARA and ARA/EPA ratio increased significantly (Tocher et al. 2000). Furthermore, fish with higher levels of ARA (as measured by gill phospholipid composition) are better adapted to salt water conditions and its challenges (Bell and Sargent 2003). In our study, a high content of ARA was obtained from lyophilized red algae that may have had a positive effect on SGR and antiviral activity.

Palmitic fatty acid is one of the five fatty acids involved in triglyceride biosynthesis (Arts and Kohler 2009). It has been linked with innate immunity through the induction of toll-like receptor-4/nuclear factor-kB pathway (Schaeffler et al. 2009). In fish, the antiviral protein Mx induced by interferon cytokine (Haller et al. 2007) has shown to provide resistance against a broad range of viruses including ISA virus (Lester et al. 2012), and the expression of Mx through the interferon signalling pathway has been reported nuclear factor-kB dependent (Collet et al. 2004). In the present study fish fed *G. chilensis* (10 %) showed the highest improvement in antiviral activity. Palmitic acid content in *G. chilensis* exceeded twice the content of *P. columbina* suggesting that this fatty acid could be involved in the synergistic effect of red algae to increase ISA virus antiviral activity.

Our study indicates that algae contain all of these macronutrients (EPA, ARA, palmitic fatty acid, and taurine amino acid). Previous work has indicated that vitamin C is present in other red algae (Matanjun et al. 2009) as well as phycobiliprotein, sulfated polysaccharides, and a low ω-6:ω-3 ratio (Matanjun et al. 2009). The above macro- and micronutrients affect fish health and response to pathogens. The mechanism by which these and other nutrients increase resistance to ISA virus in *S. salar* remains unknown, but it is clear that these nutrients provide a synergistic effect against the ISA virus pathogen.

The extracts and carageenans isolated from red seaweed have antiviral activity against different viruses (Schaeffer and Krylov 2000); however, our study is the first in which red algae is used as an ingredient in the diet of salmonids for the purpose of antiviral prophylaxis. Favorable results have been obtained in previous studies using lyophilized *Spirulina platensis* administered orally for a period of 3 days to carp (*Cyprinus carpio*); this study showed an increase in the resistance to the pathogen *Aeromonas hydrophila* (Watanuki et al. 2006).

In conclusion, these results show that a *S. salar* diet supplemented with lyophilized red algae *G. chilensis* increase the antiviral activity against the ISA virus. The impact of the algae was evaluated with an ex vivo assay and demonstrated that *S. salar* have increased resistance against the pathogen ISA virus at the blood level. Even more interesting is that the addition of lyophilized red algae to the salmonid diet significantly increases the specific growth rate (39 %) in Atlantic salmon. This improvement in SGR for diets with algae versus control diets suggests that those with more than 10 % red algae merit further study.

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