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Research Article

Dietary administration of *Sargassum angustifolium* and *Gracilaria pulvinata* extracts affect antioxidant enzyme activities and *Lactobacillus* bacterial population in intestine of rainbow trout (*Oncorhynchus mykiss*) fry

Yazdanpanah M.[1]; Sotoudeh E.[1*]; Mansouri Taee H.[2]; Habibi H.[3]

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Abstract

This study investigated the effect of *Sargassum angustifolium* and *Gracilaria pulvinata* extracts on the growth performance, body composition, antioxidant status and gut microbiota of rainbow trout (*Oncorhynchus mykiss*) fry. For this purpose, 540 fry of rainbow trout (initial weight 0.23±0.04g) were distributed in 12 fiberglass tanks. Fish were fed with experimental diets, containing 500mg kg^{-1} of *Sargassum* extract (SA), 500mg $kg^{-1}$of *Gracilaria* extract (GL) and 250mg kg^{-} *Sargassum*+250mg kg^{-1} *Gracilaria* extract (SA+GL). The control diet was a commercial diet without seaweed extract. At the end of the experiment growth and feeding performance, including final weight, specific growth rate (SGR), weight gain (WG) and feed conversion (FCR) in all treatments were not significantly different (p>0.05). Whole body protein, ash and moisture contents of fish were not significantly affected by dietary macroalgae extracts (p>0.05). However, lipid content was significantly lower in fish fed with extract og *Gracilaria* and *Sargassum* compared to control (p<0.05). The seaweed extract-fed fish utilized efficiently the body lipid and suppressed weight loss of the body during starvation. No significant difference (p>0.05) in serum total protein, creatinine alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and blood superoxide dismutase (SOD) activity was observed in all treatments. Whereas serum albumin, glutathione peroxidase (GPX) and catalase specific activity were higher in SA, GL and SA+GL treatments compared to the control group (p<0.05). ACH50 level was significantly (p<0.05) increased in GL and SA+GL treatments as compared to SA and control group. The addition of *Sargassum* and *Gracilaria* extracts to feed did not affect total bacterial population but increased *Lactobacillus* bacteria levels (p<0.05) in the intestine. These results revealed potential antioxidant enhancing and health-promoting effects of dietary *Sargassum angustifolium* and *Gracilaria pulvinata* extracts.

Keywords: Macroalgae extract, Immunostimulant, Bacterial population, Antioxidant status, Rainbow trout

1-Department of Fisheries, Faculty of Nano and Bio science and Technology, Persian Gulf University, 75169, Bushehr, Iran.
2- Lorestan Fisheries Administration, Khoramabad, Iran.
3- Department of Animal Sciences, Faculty of Agriculture and Natural Resources, Persian Gulf University, 75169, Bushehr, Iran.
*Corresponding author's Email: e.sotoudeh@pgu.ac.ir

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Introduction

Rainbow trout, *Oncorhynchus mykiss*, is a fast-growing species and one of the most important fish species that is commercially farmed in many countries (FAO, 2014). Culturing this species in both semi-intensive or intensive systems lead to a stressful condition that negatively affects fish health and immune system and increases susceptibility to infections (Trenzado *et al.,* 2007; Hoseinifar *et al.,* 2015). A practical approach for resolving the issue is by potentially stimulating the immune system of the host animal by inclusion of natural immunostimulants (Wang *et al.,* 2017). Compounds with immunostimulant effect can improve health status and disease resistance.

Seaweeds are good source of protein, lipid, pigments, minerals and vitamins (Matanjun *et al.,* 2009) and many studies focused on administration of dietary seaweeds reporing positive results on growth, feed utilization, lipid metabolism, controlling disease and disease resistance of various fish and shrimp species (Castro *et al.,* 2004; Araújo *et al.,* 2016; Peixoto *et al.,* 2016; Thanigaivel *et al.,* 2016; Sotoudeh and Jafari 2017; Morshedi *et al.,* 2018). Furthermore, seaweeds and their various derived substances are valuable sources of bioactive compounds, which can increase immune responses and be used as dietary immunostimulant in aquaculture (Jiménez-Escrig *et al.,* 2011; Araújo *et al.,* 2016; Dashtiannasb and Yeganeh, 2017). Seaweeds are identified and grouped into three different class, including brown algae (Phaeophyta), red algae (Rhodophyta) and green algae (Chlorophyta). *Gracilaria* spp. and *Sargassum* spp. are red (class: Rhodophyta) and brown algae, respectively which contain relatively high level of essential amino acids, essential fatty acids and minerals (Matanjun *et al.,* 2009). Several studies focused on effects of *Sargassum* extract on immune resistance in black tiger shrimp, *Penaeus monodon* (Immanuel *et al.,* 2012), flathead grey mullet, *Mugil cephalus* (Kanimozhi *et al.,* 2013), tilapia, *Oreochromis niloticus* (Isnansetyo *et al.,* 2016) and Asian sea bass, *Lates calcarifer* Bloch (Yangthong *et al.,* 2016). In general, these findings suggested that *Sargassum* extract can be used as an immunostimulant in fish and shrimp. There is also evidence that *Gracilaria verrucosa* enhanced some innate immune parameters in black tiger shrimp (Maftuch and Risjani, 2012) and dietary *Gracilaria* spp. supplementation in European seabass (*Dicentrarchus labrax*) led to an antioxidant capacity enhancement (Peixoto *et al.,* 2016).

Gut microbiota has many functions for health of the host and is considered as an integral component of the host. Recently some researchers proposed that microbiota change in the intestine of fish may affect host immune functions (Han *et al.,* 2018). However, no study focused on administration effects of dietary seaweeds on gut microbiota in fish.

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The objective of this research was to evaluate the effects of hydroalcoholic extract of *Sargassum angustifolium* and *Gracilaria pulvinata* as dietary supplements on growth performance, blood index, chemical composition, antioxidant status and gut microbiota of rainbow trout (*Oncorhynchus mykiss*) fry.

Materials and methods

Fish and experimental diets

Five hundred and forty rainbow trout fry were obtained from Shahid Motahary Coldwater Fish Genetic and Breeding Research Center, Yasouj, Iran and transferred to fish culture experimental lab of Persian Gulf University, Bushehr Province, Iran. The fishes were acclimatized to experimental condition for two weeks. After this period, fish (0.23±0.04g) were randomly stocked into 12 square fiberglass tanks (20 litter) in triplicate and fed for 6 weeks. Following the feeding trial, the fish were kept in the tanks and starved for 10 days (Nakagawa, 2004). Body weight loss and reduction of body lipid and protein were monitored. Each replicate consisted of 45 fish in a tank with flow-through water system (3l/min). During the trial (60 days) photoperiod was 12:12h (light: dark) and water temperature, dissolved oxygen and pH were 14.7±0.5°C, 7.5±0.5mg L^{-1} and 7.2±0.2, respectively.

Diet preparation

Before preparation of the experimental diet, the brown seaweed *Sargassum angustifolium* and the red seaweed *Gracilaria pulvinata* were collected from Persian Gulf coast of Bushehr, Iran. After taxonomic identification, they were washed and dried at 50°C for 24h, then powdered and extracted as described by Thanigaivel *et al. (*2015). Then a commercial feed (Beyza, Iran) was considered as basal diet (control) and other experimental diets were prepared by spraying macroalgae solutions extract uniformly on the feed, 500mg kg^{-1} of *Sargassum* extract (SA), 500 mg kg^{-1} of *Gracilaria* extract (GL) and 250mg $kg^{-1}$of *Sargassum* + 250mg kg^{-1} *Gracilaria* extract (SA+GL, Table 1). The control group was fed by basal diet without macroalgae extract. Prepared diets were kept in plastic bags at -4°C until used. The feeding rate was 10% of body weight in equal rations at each feeding time, 08:00, 12:00, 16:00 and 20:00 (Sotoudeh *et al.*, 2016).

Table 1: Proximate composition of the basal commercial diet.

Proximate composition (dry matter basis)	
Dry matter (%)	90.00
Crude protein (%)	50.00
Crude lipid (%)	13.50
Fiber (%)	1.70
Energy (kj/g)	17.99

Growth Performance

To measure growth performance, weight and length of fish in each tank was monitored in two weeks and at the end of the experiment following a 24 hours starvation. Growth performance and survival rate of fingerlings were calculated using the following formula (Hamza *et al.*, 2008), FCR (feed

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conversion ratio)=F/(B_t–B_0), SGR (specific growth rate)=(Ln W_t -Ln W_0)×00/t, WG (weight gain)=W_t–W_0, CF or K (condition factor)=100×(Wt/TL^3), viscerosomatic index (VSI)=100×(Wv/Wt), and survival rate=100×(N_t /N_0). Where: F: relative food intake (g), B_t and B_0: final and initial fish biomass (g), W_t and W_0: final and initial body weight (g), Wv: viscera weight, t: time of rearing (days), TL: total length, N_t and N_0: final and initial fish number.

Sample collection

Five specimens were randomly selected from each replicate and anesthetized with clove solution at 30 mg/L (Velisek *et al.,* 2005) for blood sampling, proximate analysis and bacteriology. The blood samples were obtained from the caudal vein and divided into two parts.

Blood biochemistry parameters

For blood biochemistry parameters, the samples were transferred to heparinized tubes and the remainings to non-heparinized tubes for serum isolation. The samples were centrifuged (3000×g for 15 min at 4°C) and the obtained serums were stored at -80°C until use.

Biochemical parameters, including total protein (g dl^{-1}), albumin (g dl^{-1}), triglyceride (mg dl^{-1}) and glucose (mg dl^{-1}) in serum were analyzed using a commercial kit (Pars Azmun Ltd., Iran) and a spectrophotometer (Hitachi Ltd., Tokyo, Japan) was used for measurement (Raeeszadeh and Beheshtipour, 2018).

Alanine aminotransferase (ALT, IU l^{-1}) and Aspartate aminotransferase (AST, IU l^{-1}) were measured according to Huang *et al.* (2006) method. Alkaline phosphatase (ALP, IU l^{-1}) was measured according to Bessey *et al. (*1946).

Alternative complement pathway (ACH50) activity was estimated as described by Tort *et al.* (1996) and ACH50 units were defined as the concentration of plasma giving 50% haemolysis of rabbit red blood cell, as target cells, in the presence of ethylene glycol tetraacetic acid (EGTA, Sigma) and Mg^{2+}. Briefly, fifty microlitres of plasma were used for each analysis, and samples were incubated at 37°C for 60 min. They were then centrifuged and the extent of haemolysis was estimated by measuring the optical density of the supernatant at 414nm using an ELISA reader (Epoch, BioTek, Highland Park, VT, USA). The results are presented as ACH UmL^{-1}.

Antioxidant enzymes activity

For evaluation of antioxidant enzymes activity, the liver samples were obtained from nine fish per treatment. First, the samples were homogenized in 9 volumes of 0.05M phosphate buffer pH=6.6 containing 1% Triton X-100, using a tissue homogenizer and centrifuged at 10000g at 4ºC for 30 min. The obtained supernatants were kept at -80ºC until further analysis. All enzyme preparations were carried out on ice. Dilution of the sample was done when required. Superoxide dismutase (SOD) activity was measured following

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the method described by McCord and Fridovich (1969). Catalase (CAT) activity was determined following the method described by Beers and Sizer (1951). The glutathione peroxidase (GPX) activity was measured using a GPX Assay Kit (Nanjing Jiancheng Bioengineering Institute, China) according to manufacturer's instructions.

Chemical analysis

An initial sample of five fish was taken for proximate analysis. At the end of the experiment whole body of five fish per tank were homogenized in a blender. All analyses were performed in triplicate. Analyses of crude protein, moisture and ash of whole body of fish were performed according to standard procedures (AOAC, 2000). Total lipids in the carcass of fish was analyzed according to Folch *et al. (*1957) after extraction with chloroform–methanol (2:1). Crude protein value was analyzed according to the Kjeldahl method using an Auto Kjeldahl System (Kjeltec Auto Analyzer; FOSS, Hillerød, Denmark). The moisture of samples was gravimetrically determined by drying at 110°C and ash content of fish was determined after incineration at 550°C for 6h.

Intestinal microbiota analysis

Total bacterial population and *Lactobacillus* bacteria levels were determined at the end of the experiment (n=3 fish per tank). The fish were euthanized by overdose (200mg l^{-1} water for 10 min) of tricaine methane sulphonate (MS222) and the skin was washed in a solution of 0.1% benzalkonium chloride. Intestinal tissue samples were then excised and washed with PBS and homogenized in sterile saline (0.85% (w/v) NaCl).

The enumeration of *Lactobacillus* population was performed following the procedure described by Ferguson *et al. (*2010).

Statistical analysis

Prior to statistical analyses, all data were tested for normality of distribution using Kolmogorov-Smirnov test. Then the data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. In case of all statistics analysis mean values were considered significantly different at $p<0.05$. Data were shown as means ± standard deviation. All data were analyzed using SPSS 17 for Windows software (SPSS, Chicago, IL, USA) and the figures were prepared by Microsoft Excel.

Results

Fish growth performance

The effects of dietary supplementation of *Sargassum angustifolium* and *Gracilaria pulvinata* hydroalcoholic extracts on mean body weight of fry during different experimental periods are present in Fig. 1. The average fish weight increased along the trial. Fry body weight at 28 and 42 days was not different among treatment groups ($p>0.05$). The results regarding growth performance and feed utilization of rainbow trout fry are present in Table 2. After 6 weeks trial, no significant difference ($p>0.05$) observed between

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treatments and control group in case of growth and feed utilization parameters (FCR, SGR, WG, CF).

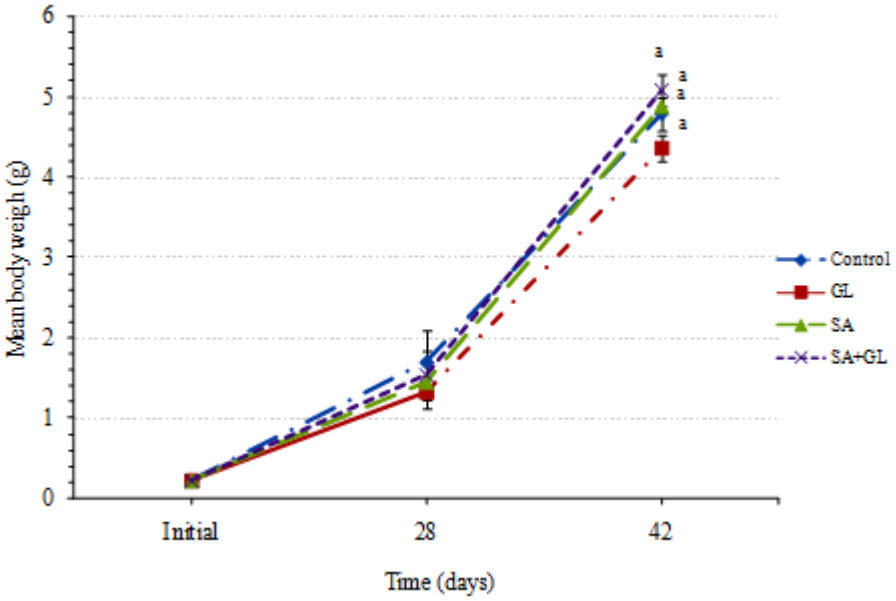

Figure 1: Mean body weight evolution for 6 weeks in fry rainbow trout fed on experimental diets (n=3). Values not sharing a common superscript were significantly different (p<0.05). Error bars show standard deviation. GL: *Gracilaria*, SA: *Sargassum*, GL+SA: *Gracilaria*+*Sargassum*.

Table 2: Growth performance, feed utilization and survival of rainbow trout fed diets supplemented with *Sargassum angustifolium* and *Gracilaria pulvinata* extracts for 6 weeks. Data are presented as mean ± standard deviation (n =3). Data assigned with different superscripts in a column differed significantly at p<0.05. FW: Final weight, FCR: feed conversion ratio, SGR: specific growth rate, WG: weight gain, CF: condition factor, VSI: viscera index, GL: *Gracilaria*, SA: *Sargassum*, GL+SA: *Gracilaria* + *Sargassum*.

Treatments	FW (g)	FCR	SGR (% day^{-1})	WG (%)	CF	VSI (%)	Survival (%)
Control	4.79±0.4	1.71±0.48	6.36±1.47	174.5±53.0	1.24±0.26	7.98±3.9	100
SA	4.36±0.6	1.06±0.14	8.11±1.17	241.2±62.9	1.35±0.05	9.20±0.45	100
GL	4.88±0.8	1.22±0.00	7.33±0.11	200.2±5.1	1.32±0.14	9.44±3.16	100
GL +SA	5.08±1.3	1.29±0.07	7.05±0.24	188.0±10.5	1.29±0.09	8.83±2.60	100

Proximate body composition

The body composition of rainbow trout fry fed with extract of *Sargassum* and *Gracilaria* macroalgae in different experimental diets are shown in Table 3. Regarding, protein, ash and moisture no significant difference was observed between treatments and control group after six weeks feeding and 10 days starvation (p>0.05). Whereas lipid was significantly lower in fish fed with extract *Gracilaria* and *Sargassum* compared to control at the end of six weeks feeding (p<0.05).

Fig. 2 shows percentage loss of body weight, whole-body protein and lipid of rainbow trout fry after 10 days of starvation. Body weight loss was

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significantly higher in control group ($P < 0.05$) in comparison to other groups. The starvation significantly depressed the lipid content in fish fed with seaweed extract ($p<0.05$). While consumption of the body protein during starvation was slightly suppressed (not significantly) by the seaweed extract supplementation.

Table 3: Proximate chemical analysis (% on dry matter basis) of whole body of rainbow trout fed diets supplemented with *Sargassum angustifolium* and *Gracilaria pulvinata* extracts for 6 weeks. Data are presented as mean±standard deviation (n=3). Data assigned with different superscripts in a column differed significantly at $p<0.05$. C: control group, GL: *Gracilaria*, SA: *Sargassum*, GL+SA: *Gracilaria+Sargassum*.

Treatments	Chemical characteristics			
	Protein (%)	Lipid (%)	Ash (%)	Moisture (%)
Initial	12.3	6.1	2.7	74.8
After 6 weeks feeding				
Control	14.6±0.71	8.1±0.13^{a}	2.1±0.07	74.5±2.04
GL	15.2±1.4	6.6±0.73^{b}	2.3±0.32	72.4±1.5
SA	15.3±0.36	6.7±0.61^{b}	2.1±0.28	71.8±1.44
GL+SA	15.6±0.77	6.5±0.55^{b}	2.1±0.06	72.5±1.85

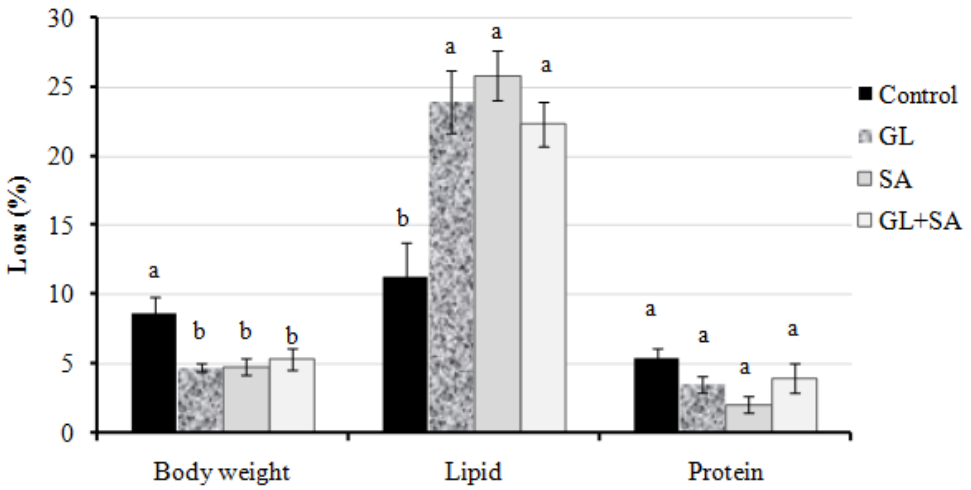

Figure 2: Mean effect of *Sargassum* and *Gracilaria* extracts supplementation to the diet on loss in body weight, body lipids (lipids) and body protein (protein) of the rainbow trout fry after 10 days starvation (n=3). Values not sharing a common superscript were significantly different ($p<0.05$). Error bars show standard deviation. GL: *Gracilaria*, SA: *Sargassum*, GL+SA: *Gracilaria+Sargassum*.

Biochemical parameters of blood

The blood biochemistry parameters of rainbow fry fed with different experimental diets are shown in Table 4. No significant difference in serum total protein, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) was observed among treatments ($p>0.05$). Fish fed on diets supplemented with *Sargassum* and *Gracilaria* extracts especially mix *Sargassum* and

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Gracilaria treatment (SA+GL) showed higher albumin contents significantly (p<0.05). The serum glucose and triglyceride levels decreased significantly in fish fed with diets containing *Sargassum* and *Gracilaria* (p<0.05). ACH50 level increased treatments as compared with SA and control groups (p<0.05).

Table 4: Blood biochemistry parameters of rainbow trout fed diets supplemented with *Sargassum angustifolium* and *Gracilaria pulvinata* extracts for 6 weeks. Values are presented as mean±standard deviation (n =3). Data assigned with different superscripts in a column differed significantly at p<0.05. ALB: albumin, GLU: glucose, ACH50: alternative complement pathway, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, GL: *Gracilaria*, SA: *Sargassum*, GL+SA: *Gracilaria+Sargassum*.

Treatment	Total Protein (g dl^{-1})	ALB (mg dl^{-1})	TG (g dl^{-1})	GLU (mg dl^{-1})	ALT (IU l^{-1})	AST (IU l^{-1})	ALK (IU l^{-1})	ACH50 (U ml^{-1})
Control	3.81±0.1	1.16±0.04^{c}	232.6±20.0^{a}	57.7±2.5^{a}	85.0±7.9	1111.6±17.5	459.0±69.9	71.1±4.1^{b}
SA	3.58±0.2	1.50±0.1^{b}	208.5±7.1^{b}	39.7±3.5^{b}	89.6±8.9	1124.3±32.0	465.6±66.1	71.1±4.2^{b}
GL	3.75±0.1	1.91±0.2^{a}	164.0±8.5^{c}	44.3±5.7^{b}	95.6±8. 5	1115.0±21.9	531.3±103.5	81.9±6.1^{a}
GL+SA	3.92±0.2	2.03±0.1^{a}	202.0±27.6^{b}	39.0±3.0^{b}	98.9±5.8	1161.5±31.4	533.0±11.1	81.3±3.2^{a}

Antioxidant enzyme activities

Effects of hydroalcoholic extract of *Sargassum* and *Gracilaria* macroalgae on antioxidant enzymes activity are shown in Fig. 3. The statistical analysis of results revealed no difference in superoxide dismutase (SOD) activity among the treatments and control group at weeks 4 and at the end of the experiment (p>0.05). However, regardless of inclusion level, feeding on *Sargassum* and *Gracilaria* macroalgae supplemented diet significantly increased glutathione peroxidase (GPX) activity compared with the control group at the end of experiment (p<0.05). Similarly these results revealed feeding on *Sargassum* and *Gracilaria* macroalgae supplemented diets increased (p<0.05) catalase (CAT) specific activity significantly among the treatments and control group at week 4 and the end of the experiment (p<0.05).

Intestinal microbiota

The effect of hydroalcoholic extract of *Sargassum* and *Gracilaria* macroalgae on total bacterial population and *Lactobacillus* bacteria levels of rainbow trout fry are shown in Fig. 4. The results revealed that feeding on *Sargassum* and *Gracilaria* macroalgae supplemented diets increased *Lactobacillus* bacteria levels significantly compared with the control group at the end of experiment (p<0.05), while total bacterial population was not affected by dietary extract of *Sargassum* and *Gracilaria* macroalgae (p>0.05).

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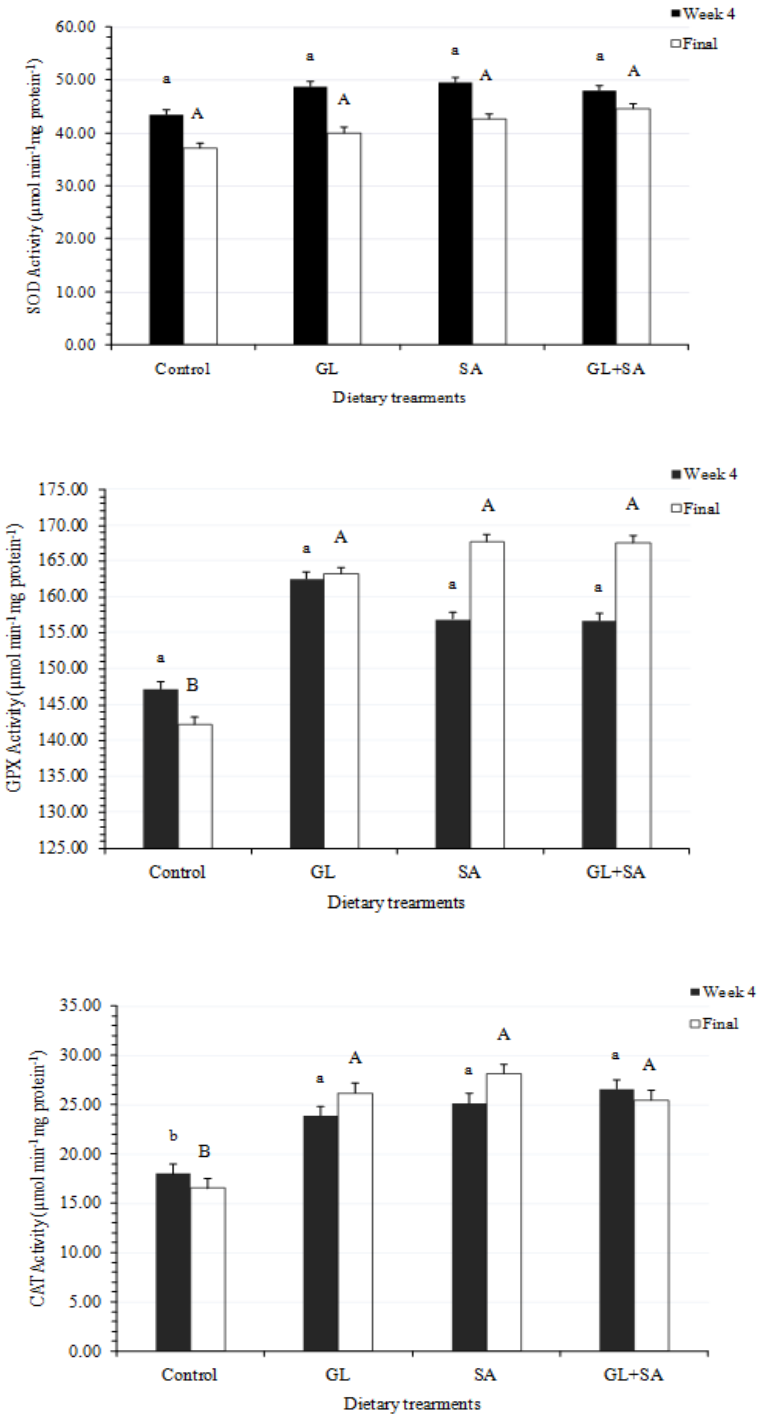

Figure 3: Mean activity of antioxidant enzymes of rainbow trout fry fed the experimental diets at fourth week and at the end of the experiment (n=3). Values not sharing a common superscript were significantly different (p<0.05). Error bars show standad deviation. GL: *Gracilaria*, SA: *Sargassum*, GL+SA: *Gracilaria*+ *Sargassum*.

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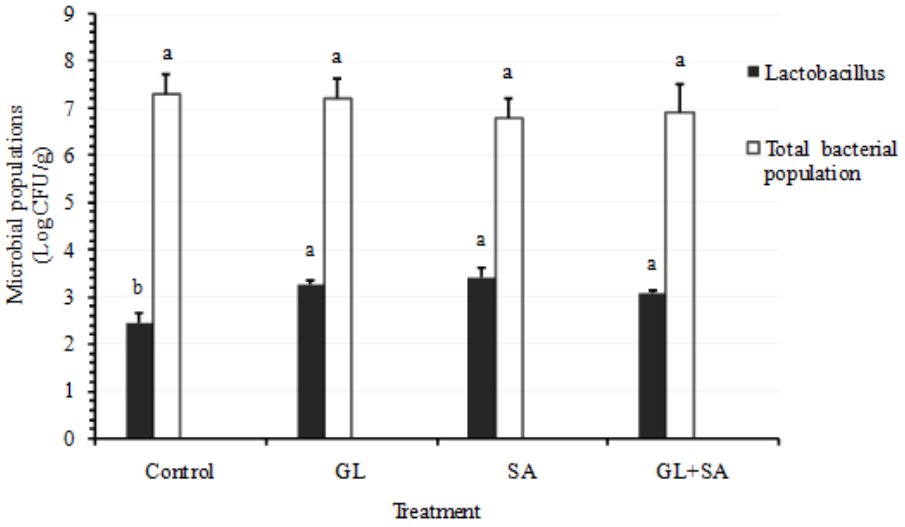

Figure 4: Mean total bacterial population and lactobacillus bacteria levels in intestine of rainbow trout fed the experimental diets at the end of the experiment (n=3). Values not sharing a common superscript are significantly different (p<0.05). Error bars show standard deviation. GL: *Gracilaria*, SA: *Sargassum*, GL+SA: *Gracilaria*+*Sargassum*.

Discussion

To our knowledge, this study is first attempt to evaluate inclusion of hydroalcoholic extract of *Sargassum angustifolium* and *Gracilaria pulvinata* macroalgae in rainbow trout fry diet. The present results revealed no significant difference in growth performance between the treatments and control group at the end of 42 days of the experiment (p>0.05). Similar results are reported in olive flounder (*Paralichthys olivaceus*) fed with *Sargassum fusiforme* (Kim *et al.*, 2014) and European seabass (*Dicentrarchus labrax*) fed with *Gracilaria* spp. (Peixoto *et al.*, 2016).

Regarding protein, ash and moisture no significant difference was observed between treatments and control group after 6 weeks feeding and 10 days starvation (p>0.05). Similar results are described by Silva *et al.* (2015) and Peixoto *et al.* (2016) for body composition of Nile tilapia (*Oreochromis niloticus*) and seabass fed with *Gracilaria vermiculophylla* and *Gracilaria* spp. respectively. Body lipid content of rainbow trout increased in fish fed with *Gracilaria* and *Sargassum* extracts compared to control. It is reported that the body lipid decreases in rainbow trout when fed with *Sargassum ilicifolium* meal (10% of basal diet). It reflects this fact that this macro algae fat are likely to play an important role in the energy of rainbow trout and are more likely to accumulate in meat. Also rainbow trout species uses unsaturated fatty acids for energy needs (Zamannejad *et al.*, 2016). In the peresent study seaweed extract-fed groups consumed efficiently the reserved lipids and suppressed body

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protein consumption during starvation. As a result, body weight loss could be minimized. The phenomenon that starvation for 10 days after the feeding diets containing *Gracilaria* and *Sargassum* extract resulted in low body weight loss was caused by preferential lipid mobilization to energy and suppression of body protein consumption. It is also showed that lipid metabolism in fish is affected by dietary supplementation of algae (Nakagawa, 1997). Feed containing *Spirulina* sp. depress triglyceride accumulation in muscle and intraperitoneal fat body of 2-year old red sea bream (*Pagrus major*) (Mustafa *et al.,* 1994). They found that dietary *Spirulina* elevate hepatic carnitine palmitoyltransferase activity and hepatic carnitine level which play important roles in P-oxidation of fatty acids. Nematipour *et al. (*1987) reported that dietary 2% Chorella-extract reduce lipid levels in muscle, liver and adipose tissue of ayu (*Plecoglossus altivelis*) by stimulating lipolytic hormones and/or influencing the adipose tissue structure. However, the effect of algae on composition of the carcasses seems to be related to their nutritional value and the level of dietary inclusion. Other influential factors include fish species, age, size and experimental protocol (Güroy *et al.,* 2007; Dantagnan *et al.,* 2009; Ergün *et al.,* 2009).

Total protein of serum is one of the components of nonspecific immune system of fish that is affected by immunostimulants (Siwicki *et al.,* 1994). In this study, fish fed with dietary *Sargassum* and *Grasilaria* showed no effect on total serum protein. Similarty, total protein in Nile tilapia were not affected by *Ulva lactuca* and *Pterocladia capillaceaas* (Khalafalla and El-Hais, 2015). Albumin is one of the main serum proteins that is associated with immune status, such as globulin, and can be used as indicator of health and immune status (Kumar *et al.,* 2005). In the present study dietary *Sargassum* and *Gracilaria* elevated albumin contents in rainbow trout.

Results of the present study showed that feeding on *Gracilaria* and *Sargassum* supplemented diet did not affect ALT, AST and ALP. The serum glucose and triglyceride level were decreased in fish fed diets containing *Sargassum* and *Gracilaria* significantly (p<0.05). Hypoglycemic plants increase insulin secretion and prevent absorption of glucose from the intestine and produce glucose from liver. With increased stimulation and glucose metabolism by the muscle, insulin reduces blood glucose concentrations. Several studies reported that seaweeds, such as *Ecklonia stolonifera* (Iwai, 2008), *Ascophyllum nodosum* and *Fucus vesiculosus* (Lordan *et al.,* 2013), and *Gracillaria arcuata* (Akbary *et al.,* 2020) have anti-diabetic effects and greatly reduce blood glucose levels. However, it is reported that triglyceride in Nile tilapia and dusky kob, *Argyrosomus japonicus*, were not affected by dietary *Ulva lactuca* and *Ulva* sp. inclusion, respectively

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(Khalafalla and El-Hais 2015; Madibana *et al.,* 2017).

The complement activity (ACH50) is an important component of non-specific immune system protecting fish from pathogens (Peixoto *et al.,* 2016). In our study ACH50 level increased in GL and SA+GL treatments as compared with SA and control groups. Similarly supplementation of *Gracilaria vermiculophylla* and *Ulva* spp. meal in diet for rainbow trout and Nile tilapia enhanced complement activity respectively (Araújo *et al.,* 2016; Valente *et al.,* 2016). Araújo *et al. (*2016) stated that algae compounds, mainly polysaccharides, such as carrageenan, alginates, fucoidan and β-glucan, stimulate immunological response and play important role in disease resistance in several species, including rainbow trout.

It is well-stablished that free radicals are produced during normal processes in body (Yang *et al.,* 2010). Antioxidant enzymes (i.e SOD, CAT and GPX) are responsible for protecting fish against oxidative stress (Zhang *et al.,* 2013). Therefore, they have vital roles in maintaining health (Santacroce *et al.,* 2012). In the present study, there was no significant difference in SOD activity compared all treatments with the control. These findings were in accordance with those of previous studies which suggest, SOD activity in liver tissue is not significantly altered in rabbitfish (*Siganus canaliculatus*) after feeding *Gracilaria lemaneiformis* (Xu *et al.,* 2011). However, activity of CAT and GPX in liver tissue homogenates showed significant increase in fish fed with *Sargassum* and *Grasilaria* extracts. In one study, supplementation of European seabass diet with different levels of *Gracilaria* spp. increased the activity of antioxidant enzymes such as GPX in liver but had no effected on CAT (Peixoto *et al.,* 2016). Red seaweeds, such as *Gracilaria* spp. are good source of antioxidants and selenium (Devi *et al.,* 2011) which functions as GPX cofactor (Rotruck *et al.,* 1973). In this case it is possible that *Gracilaria* spp. due to Selenium increment, the effect on over GPX activity (Peixoto *et al.,* 2016).

The intestinal microbiota of fish plays a key role in physiology and function of the gastrointestinal tract as well as stimulation of immune response of the host (Ringø *et al.,* 2014). It is well documented that manipulating intestinal microbiota increases diseases resistance and improves the health of fish (Ringø *et al.,* 2010a). To our knowledge there are no study to reveal the effects of macroalgae on intestinal microbiota in fish. The results also revealed a notable increase of *Lactobacillus* bacteria levels in fish fed with *Sargassum* and *Gracilaria* extracts, while total bacterial population was not affected by dietary extract of *Sargassum* and *Gracilaria* macroalgae. Studies are done to increase the level of lactic acid bacteria in intestinal microbiota following administration of dietary immunostimulant such as probiotics (Azimirad *et al.,* 2016). Lactic acid bacteria are generally known to be the beneficial component

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of fish intestinal microbiota, and producing lactic acid, bacteriocins and other antagonistic compounds affect health of the host (Ringø *et al.*, 2010b; Ringø *et al.*, 2014).

In conclusion, the present results revealed beneficial effects of dietary *Sargassum angustifolium* and *Gracilaria pulvinata* extracts on activity of antioxidant enzymes and intestinal microbiota in rainbow trout. However, additional studies are requared to evaluate effects of this algae on resistance of fish against bacterial infection and following stress conditions.

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