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Administration of Red Macroalgae (*Galaxaura oblongata*) in the Diet of the Rainbow Trout (*Oncorhynchus mykiss*) Improved Immunity and Hepatic Gene Expression

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Abstract: This study is designed to evaluate the effects of dietary red macroalgae (*Galaxaura oblongata*) on growth performance, serum, and skin mucus immunological and antioxidant responses in rainbow trout (*Oncorhynchus mykiss*). For this, rainbow trout were fed diets containing different levels of *G. oblongata* (0 (ctrl), 0.5 (G1), and 1 (G2) %) for 8 weeks. Following the feeding trial, there were no significant differences in growth performance between the experimental treatments ($p > 0.05$). Total immunoglobulin (Ig) content and lysozyme (LYZ) activity in serum were increased in fish fed *G. oblongata* ($p < 0.05$), with the highest value at (0.5%). Regardless of the inclusion level, mucus total Ig levels were significantly increased in the *G. oblongata* groups ($p < 0.05$), and mucus LYZ activity was not changed ($p > 0.05$). All groups fed *G. oblongata* showed higher serum catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities than the control group ($p < 0.05$). However, skin mucus SOD activity increased more in the group fed 1% of *G. oblongata* than the other groups ($p < 0.05$). Additionally, the skin mucus GPx activity showed higher values in the group fed 0.5 and 1% *G. oblongata* than in the control ($p < 0.05$). No significant differences were recognized between the experimental treatments in terms of CAT activity and malondialdehyde (MDA) concentration ($p > 0.05$). *G. oblongata* up-regulated *gpx* gene expression with the maximum value at the group fed 1% *G. oblongata* ($p < 0.05$). Additionally, interleukin 6 (*il-6*) and tumor necrosis factor-alpha (*tnf-α*) gene expressions were significantly up-regulated in fish fed 1% compared with the control and 0.5% groups. Based on the results, 0.5–1% *G. oblongata* can be used in the fish diet and enhance immunity without causing impairment in growth.

Keywords: seaweed; immune system; antioxidant enzyme; cytokine; fish liver

Key Contribution: The positive effects of *Galaxaura oblongata* on immunity and antioxidant activity in rainbow trout have shown that it has significant potential in aquaculture as a functional feed additive.

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1. Introduction

Intensive aquaculture is accompanied by numerous stressors that cause stressful and undesirable conditions for fish and may cause infectious diseases and significant financial losses [1,2]. The administration of antibiotics to prevent and treat aquatic animal disease has been restricted or forbidden because of its adverse consequences on target and non-target animals and natural habitats [3–5]. Recently, there has been growing attention paid toward safer alternatives to antibiotics, such as natural active compounds, to control and treat diseases in aquaculture [6–8].

Among marine organisms, photosynthetic microalgae and some macroalgae (seaweeds) can be considered the foundation of the aquatic food web [9,10]. Macroalgae can generate a broad spectrum of primary and secondary metabolites [11,12] and thus is known as one of the most valuable sources of natural antioxidants and antimicrobial agents [13]. Seaweeds have recently received attention as dietary supplements in aquaculture because of their biologically active substances and chemical composition [14,15]. The dietary administration of suitable macroalgae and its extracts have been proven to enhance the growth performance and health status of different aquatic species with no adverse effects [16–20]. For example, Vazirzadeh et al. [18] stated that adding red macroalgae to rainbow trout feeds improved immunity, antioxidant status, and immune-related genes in a time- and dose-dependent manner. Moreover, Hoseinifar et al. [19] reported that supplementing the diet with 0.5% *Halopithys incurva* increased immunity and antioxidant defense in zebrafish (*Danio rerio*). In another recent study involving red macroalgae, it has been reported that *Laurencia caspica* extract enhances resistance to bacterial diseases and promotes immune system improvement in Nile tilapia (*Oreochromis niloticus*) [20]. Accordingly, red algae (Rhodophyta), as one category of macroalgae, are an excellent source of bioactive compounds, generally essential fatty acids (polyunsaturated fatty acids and sterols), terpenes, mycosporine-like amino acids, proteins, pigments, phenolic compounds, polysaccharides, vitamins, and minerals [11]. Red seaweeds have been well recognized for their antioxidant, antitumor, antimicrobial, anti-inflammatory, anticancer, antidiabetic, and anti-amyloidogenic properties [11,19,21].

Most Rhodophyta belong to the *Florideophyceae*, which are chiefly multicellular [22]. *Galaxaura oblongata* is a prevalent benthic and macroscopic marine algae belonging to phylum Rhodophyta, class *Florideophyceae*, with expansive distribution in shallow tropical and subtropical marine environments [23]. However, there is still a gap in understanding its structure, chemical composition, and bioactive compounds.

It has been proposed that *G. oblongata* has been proposed as a natural pharmaceutical compound with possible therapeutic functions due to its potent anti-inflammatory, anti-edema, antitumor, anticancer, and antioxidant properties [24–26]. With such promising attributes, *G. oblongata* may promote fish health and welfare. The nutritional and anti-nutritional contents of macroalgae commonly differ between genera, according to the region where the algae are collected and the season. Therefore, not all macroalgae have the same beneficial effects on all aquatic species [10]. In this sense, most of the research evaluating macroalgae's effects in aquafeeds has focused on some genera such as *Ulva*, *Sargassum*, *Gracilaria*, and *Porphyra* species [12]. To the best of our knowledge, no data is available regarding the effects of *G. oblongata* on fish. Rainbow trout, *Oncorhynchus mykiss*, is a leading fish cultivated globally. Its production reached 959,000 tons in 2020, accounting for 1.67% of the total finfish production (FAO, 2022). Hence, the current study aimed to investigate the possible impact of *G. oblongata* on growth performance, serum, mucosal antioxidant status, and antioxidant-related gene expression of *O. mykiss*.

2. Materials and Methods

2.1. Algal Collection and Preparation

During the summer season *G. oblongata* was collected at depths of 0 and 20 m near Iskenderun Bay, Türkiye, in the Mediterranean. Identification of *G. oblongata* was performed using SZX16 stereo zoom and BX51 binocular light microscopes (STEMI 2000, Carl Zeiss, Jena, Germany). It was carried out by immersion in 1000 mL of distilled water for 72 h to remove the water-soluble components on the macroalgae. The filtered *G. oblongata*, described before [27], were ground four times in a rotary mill operating at 300 rpm for 10 min and then vacuum dried at 50 °C for 24 h. Algal powders to be added to the diet were sieved with a 0.2 mm sieve and stored at 4 °C until used.

2.2. Experimental Diets

In the present study, a commercial diet (Coppens Co., Helmond, The Netherland; size 2 mm, 48% crude protein, 22% crude fat, 8.8% ash, 0.9% fiber; 20.7 MJ Kg^{-1} digestible energy) was the basal diet (control diet (C)). Two other experimental diets were made by the addition of *G. oblongata* powder at 0.5% (diet G1) and 1% (diet G2) to the basal diet of rainbow trout using the method described previously [19]. Briefly, the basal diet (which served as the control) was milled, supplemented with selected levels of *G. oblongata* powder at the expense of cellulose, and repelleted after mixing and adding water. The experimental diets were held in a sealed bag at 4 °C until use.

2.3. Experimental Design

Rainbow trout (*O. mykiss*) fingerlings were provided from a local farm and were adapted to indoor conditions for two weeks. Fish were fed a control diet three times daily (2% of body weight). A total number of 135 healthy fish (4.29 ± 0.37 g) were placed randomly into nine fiberglass tanks (100 L) at a density of 15 fish per tank as three experimental treatments in three replicates. During the 8-week feeding experiment, one dietary treatment received the control diet (C), and two other treatments received the basal diet supplemented with different levels of *G. oblongata* powder (G1 and G2). Feeding was performed three times a day at 8:00 a.m., 12:00 p.m., and 4:00 p.m. at a ratio of 2% body weight, and one hour later, the uneaten feed was collected, dried, and weighed to assess the feed conversion ratio (FCR). During the feeding trial, each tank was aerated constantly using an air pump, and water was exchanged daily (70%) with clean, fresh water. Water physicochemical variables such as temperature, pH, and dissolved oxygen were measured daily throughout the experimental period and kept at 14.30 ± 1.20 °C, 7.85 ± 0.56, 7.15 ± 0.10 mg/L, respectively.

2.4. Growth Performance

After feeding for 8-week, the weight of all remaining fish per tank was recorded individually to measure growth performance according to the formula below [28]:

$$\text{Weight gain (WG, \%)} = 100 \times [(\text{final weight} - \text{initial weight})/\text{initial weight}]$$

$$\text{Feed conversation ratio (FCR)} = \text{Feed intake (g)}/(\text{final weight} - \text{initial weight})$$

$$\text{Specific growth rate (SGR, \%/d)} = 100 \times [(\text{Ln final weight} - \text{Ln initial weight})/\text{days}]$$

2.5. Sample Collection

Before sampling, fish were fasted for 24 h. Three individuals were chosen randomly per replicate and anesthetized with clove powder (250 mg L^{-1}). A serum sample was provided using blood drained from the caudal vein of each fish with a 2 mL syringe that was instantly placed in 1.5 mL non-heparinized Eppendorf tubes. Following blood collection, the tubes were left for one hour at room temperature and four hours in a refrigerator (4 °C). After the incubation period, the clotted blood was centrifuged at 1500× *g* for five minutes at 4 °C, and the obtained supernatant was pipetted out and kept at −80 °C until needed [29]. For skin mucus collection, three fish from each tank were anesthetized and placed in an individually sealed bag containing 10 mL NaCl (50 mM). The samples were softly rubbed for around two minutes. The obtained mucus was transferred into 15 mL sterile tubes and centrifuged at 1500× *g* for 10 min at 4 °C. The supernatant was stored at −80 °C until used [30]. Thereafter, to evaluate the gene expression of antioxidant and immune parameters, the liver from each fish was removed and frozen with liquid nitrogen, then subsequently preserved at −80 °C for further gene expression analysis.

2.6. Serum and Mucus Non-Specific Immune Parameters

The activity of serum and skin mucus lysozyme (LYZ) was assayed according to the degradation of *Micrococcus luteus* (Sigma, Welwyn Garden City, UK), lysozyme-sensitive

bacteria using the turbidimetric method [31]. The quantity of sample that caused a decline in absorbance of 0.001 min^{-1} was evaluated as one unit of LYZ activity and pointed to as U mL^{-1}.

The serum and mucus total immunoglobulin (Ig) amount were achieved according to Siwicki et al. [32] and estimating the difference among total protein quantity of samples pre and post the addition of 12% polyethylene glycol (Sigma, UK) and shown as g dL^{-1}.

2.7. Serum and Mucus Antioxidant Parameters

The serum and skin mucus superoxide dismutase (SOD) and catalase (CAT) activity were determined utilizing a commercially available kit (ZellBio GmbH, Lonsee, Germany) based on the manufacturer's instructions as described elsewhere [33,34]. The SOD and CAT activity unit was marked as the quantity of the sample which would catalyze disintegration of 1 µmole of O_2– to H_2O_2 and O_2 per min^{-1} as well as the amount of the sample which will catalyze disintegration of 1 µmole of H_2O_2 to H_2O and O_2 per min^{-1}, respectively. Glutathione peroxidase (GPX) activity was assessed using a commercial kit (ZellBio GmbH, Lonsee, Germany), considering the reduction of the glutathione to glutathione disulfide. Determination of mucus malondialdehyde (MDA) concentration was also assayed using a kit (ZellBio GmbH, Lonsee, Germany) according to a reaction with thiobarbituric acid at 95 °C.

2.8. RNA Isolation, cDNA Synthesis, and Real-Time PCR

The total RNA of the liver tissues was isolated by a Sinaclon extraction kit (Sinaclon, Tehran, Iran), as stated in the kit's instructions. A Nanodrop Spectrophotometer (Wilmington, DE, USA) and electrophoresis on agarose gel (1.5%) were applied to confirm the quantity and quality of isolated RNA.DNase I (Sinaclon, Tehran, Iran) was subsequently used to avoid DNA impurity. Next, cDNA was constructed utilizing a commercial synthesis kit (Fermentas, Vilnius, Lithuania). Expression of superoxide dismutase (*sod*), glutathione peroxidase (*gpx*), tumor necrosis factor-alpha (*tnf-α*), and interleukin-6 (*il-6*) were estimated by quantitative real-time PCR (qPCR) method in triplicates using the SYBR green (SYBR biopars, GUASNR, Gorgan, Iran) method as described previously [35,36]. The β-actin gene was selected as the reference gene. The distinct primers for the selected genes were designed via Oligo 7 software based on GeneBank (Table 1). Serial dilutions previously evaluated the efficiency of primers for each gene to ensure that it was close to 100%. The relative expression of selected genes was calculated using the IQ5 program (Bio-Rad, Hercules, CA, USA) and ΔΔCt procedure [37].

Table 1. Sequences of the primers used in the study.

Gene Name	Sequences of Primers	Accession No	Efficiency
ß-actin	Forward: GACATCAGGGTGTCATGGTTGGT Reverse: CTCAAACATGATCTGTGTCAT	M24113.1	97%
il-6	Forward: ACTCCCCTCTGTCACACACC Reverse: GGCAGACAGGTCCTCCACTA	DQ866150	98%
tnf-α	Forward: GGTGATGGTGTCGAGGAGGAA Reverse: TGGAAAGACACCTGGCTGTA	AJ311800.1	97%
sod	Forward: GTAGTCGTGGCTCAATGGTAAG Reverse: GCTTTATATTCTGCGGGTCATT	AJ311800.1	97%
gpx	Forward: AAATTGCCATTCCCCTCCGA Reverse: TCCATCAGGACTGACCAGGA	AF281338	97%

2.9. Statistical Analysis

The present research was conducted in the form of a completely randomized design. Firstly, the normality and homogeneity of data were surveyed by Kolmogorov–Smirnov's and Levene's tests, respectively. Afterward, a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test were used to assess statistical differences among dietary treatments. The level of significance used was $p < 0.05$. All statistical analyses

were computed utilizing SPSS software (ver. 22 IBM, Armonk, NY, USA), and the obtained values were shown as the mean ± S.E.

3. Results

3.1. Growth Performance

In accordance with Table 2, dietary *G. oblongata* imposed no remarkable variation in rainbow trout growth performance parameters and FCR following the 8-week feeding trial ($p > 0.05$). No mortality was observed in any dietary treatment.

Table 2. The growth performance of rainbow trout fed with different levels of red macroalgae (*G. oblongata*) after an 8-week feeding trial. C (Control—0% *G. oblongata*), G1 (0.5% *G. oblongata*), G2 (1% *G. oblongata*).

	C	G1	G2
IW (g)	4.50 ± 0.50 [a]	4.26 ± 0.20 [a]	4.30 ± 0.26 [a]
FW (g)	22.60 ± 2.25 [a]	23.40 ± 1.40 [a]	23.66 ± 3.88 [a]
WG (g)	18.10 ± 2.68 [a]	19.13 ± 1.18 [a]	19.36 ± 3.64 [a]
SGR (%/d)	2.88 ± 0.36 [a]	3.03 ± 0.10 [a]	3.13 ± 0.19 [a]
FCR	1.16 ± 0.14 [a]	1.08 ± 0.10 [a]	1.09 ± 0.19 [a]

IW: initial weight (g); FW: final weight (g); WG: weight gain (%); SGR: specific growth rate (%/day); FCR: feed conversion ratio. Data in a row assigned the same letters signify significant differences between treatments ($p < 0.05$). Values are presented as mean ± S.E.

3.2. Serum Immunological Indices

The serum immune parameters of fish fed diets containing *G. oblongata* for 8 weeks are presented in Table 3. Fish diets supplemented with *G. oblongata* caused a substantial increase in the serum values of total Ig and LYZ compared to that of the control group ($p < 0.05$), with a peak value achieved in the G1 group.

Table 3. The serum immune parameters of rainbow trout fed with different levels of red macroalgae (*G. oblongata*) after an 8-week feeding trial. C (Control—0% *G. oblongata*), G1 (0.5% *G. oblongata*), G2 (1% *G. oblongata*).

	C	G1	G2
Total Ig (g/dL)	1.04 ± 0.01 [c]	1.38 ± 0.04 [a]	1.21 ± 0.04 [b]
Lysozyme (U/mL)	45.69 ± 2.80 [c]	56.47 ± 3.31 [a]	49.84 ± 3.47 [b]

Data in a row assigned with different letters signify significant differences between treatments ($p < 0.05$). Values are presented as mean ± S.E ($n = 9$).

3.3. Skin Mucus Immunological Indices

The mucosal immune parameters of rainbow trout that received diets containing *G. oblongata* are displayed in Table 4. The results exhibited a notable increase in the mucus total Ig value in the group fed with *G. oblongata*-containing diets in contrast to the control group ($p < 0.05$). However, no remarkable variation was seen among the G1 and G2 groups ($p > 0.05$). In terms of the LYZ value, no remarkable variations were found among all groups ($p > 0.05$).

Table 4. The skin mucus immune parameters of rainbow trout fed with different levels of red macroalgae (*G. oblongata*) after an 8-week feeding trial. C (Control—0% *G. oblongata*), G1 (0.5% *G. oblongata*), G2 (1% *G. oblongata*).

	C	G1	G2
Total Ig (g/dL)	0.61 ± 0.03 [b]	0.96 ± 0.08 [a]	0.85 ± 0.09 [a]
Lysozyme (U/mL)	20.33 ± 1.43 [a]	21.56 ± 2.32 [a]	22.49 ± 1.91 [a]

Data in a row assigned different letters denote significant differences between treatments ($p < 0.05$). Values are presented as mean ± SE ($n = 9$).

3.4. Serum Antioxidant Indices

The results of serum-associated antioxidant variables are presented in Table 5. In contrast to the control group, the serum CAT, SOD, and GPX activities were considerably higher in the fish that received *G. oblongata* diets ($p < 0.05$). No statistical variation was noticed between the *G. oblongata*-supplemented groups regarding antioxidant parameters ($p > 0.05$).

Table 5. The serum antioxidant parameters of rainbow trout fed with different levels of red macroalgae (*G. oblongata*) after an 8-week feeding trial. C (Control—0% *G. oblongata*), G1 (0.5% *G. oblongata*), G2 (1% *G. oblongata*).

	C	G1	G2
Catalase (mL/min/n)	0.22 ± 0.04^{b}	0.73 ± 0.10^{a}	0.63 ± 0.06^{a}
SOD (U/mL)	656.26 ± 7.13^{b}	780.52 ± 24.28^{a}	719.61 ± 18.56^{a}
GPx (nmol/min/mL)	131.50 ± 0.52^{b}	170.90 ± 1.84^{a}	166.87 ± 1.18^{a}

Data in a row assigned different letters denote significant differences between treatments ($p < 0.05$). Values are presented as mean ± SE ($n = 9$). SOD (superoxide dismutase), GPx (glutathione peroxidase).

3.5. Skin Mucus Antioxidant Indices

The skin mucosal antioxidant parameters of fish fed with *G. oblongata*-containing diets are displayed in Table 6. *G. oblongata*-supplemented diets increased SOD activity only at the highest dietary inclusion level (G2) ($p < 0.05$). Moreover, dietary *G. oblongata* showed a markedly higher GPx value ($p < 0.05$). However, no remarkable variations were found among *G. oblongata*-supplemented groups ($p > 0.05$). Additionally, the results revealed no significant variation in the impact of *G. oblongata* on CAT and MDA levels ($p > 0.05$).

Table 6. The skin mucus antioxidant parameters of rainbow trout fed with different levels of red macroalgae (*G. oblongata*) after an 8-week feeding trial. C (Control—0% *G. oblongata*), G1 (0.5% *G. oblongata*), G2 (1% *G. oblongata*).

	C	G1	G2
Catalase (mL/min/n)	0.25 ± 0.01^{a}	0.26 ± 0.01^{a}	0.25 ± 0.01^{a}
SOD (U/mL)	502.83 ± 1.29^{b}	499.61 ± 0.98^{b}	509.12 ± 5.33^{a}
GPx (nmol/min/mL)	136.78 ± 0.96^{b}	141.78 ± 0.92^{a}	142.02 ± 1.11^{a}
MDA (nmol/mL)	417.13 ± 3.32^{a}	416.97 ± 2.34^{a}	414.93 ± 1.25^{a}

Data in a row assigned different letters denote significant differences between treatments ($p < 0.05$). Values are presented as mean ± SE ($n = 9$). SOD (superoxide dismutase), GPx (glutathione peroxidase), MDA (Malondialdehyde).

3.6. Antioxidant and Immune-Related Gene Expression

The results related to the effects of *G. oblongata* on the expression of antioxidant-related genes and immune-related gene expressions in the liver fish are shown in Figure 1A,B and Figure 2A,B. *G. oblongata*-supplemented diets up-regulated *gpx* gene expression (Figure 1A), with the highest value in the G2 group ($p < 0.05$). A dose-dependent increase was observed in *gpx* expression. However, the group that received a diet enriched with *G. oblongata* did not remarkably influence *sod* gene expression when compared with the control diet ($p > 0.05$) (Figure 1B). The results of immune-related genes also demonstrated that the G2 group noticeably up-regulated the expression of interleukin 6 (*il-6*) (Figure 2A) and tumor necrosis factor-alpha (*tnf-α*) genes compared with the control and G1 groups ($p < 0.05$) (Figure 2B).

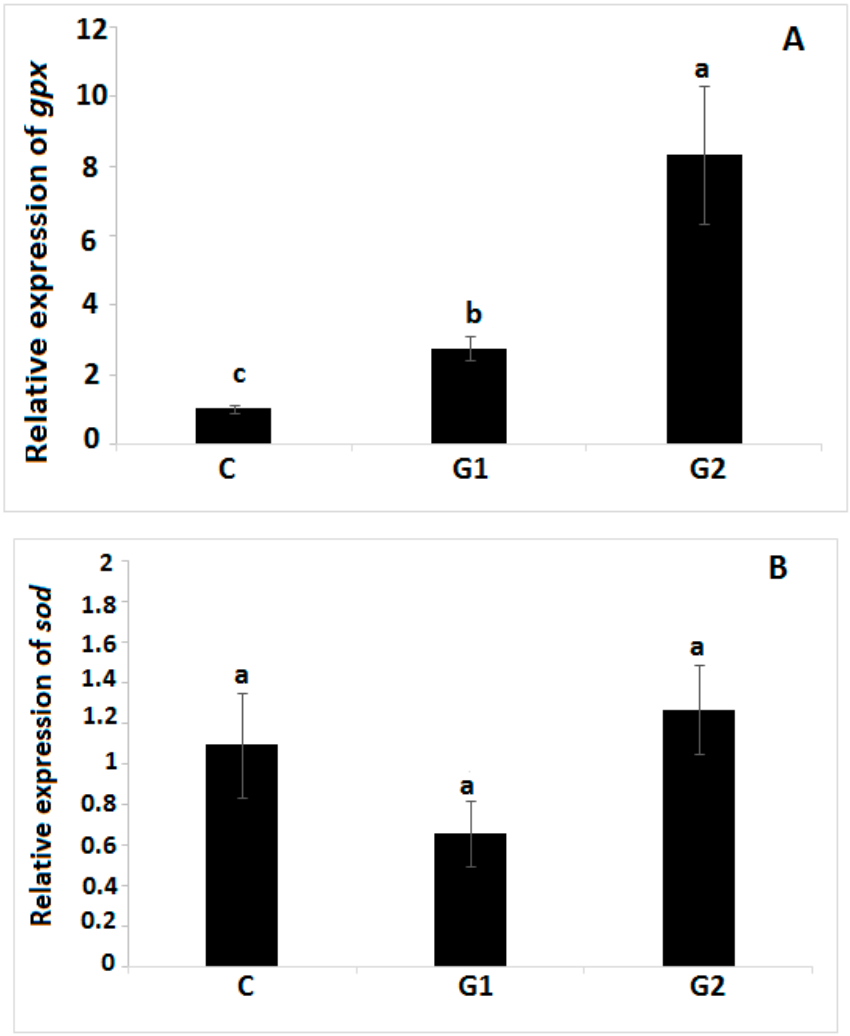

Figure 1. Changes in the relative expression of antioxidant related genes in the liver of rainbow trout fed with different levels of red macroalgae (*G. oblongata*). (**A**) *gpx* and (**B**) *sod* gene expression. C (control—0% *G. oblongata*), G1 (0.5% *G. oblongata*), G2 (1% *G. oblongata*). Bars labeled with identical superscripts indicate no significant differences ($p > 0.05$), while different letters above the bars signify a significant difference between treatments ($p < 0.05$); values are presented as the mean ± SE ($n = 9$).

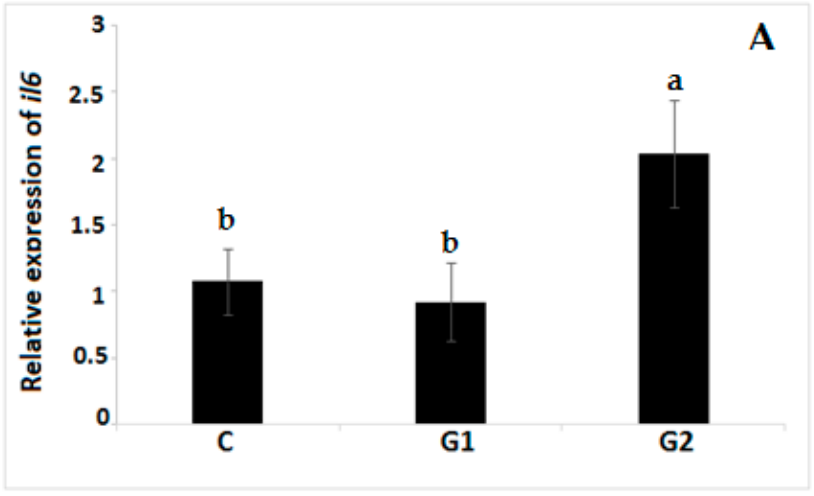

Figure 2. *Cont.*

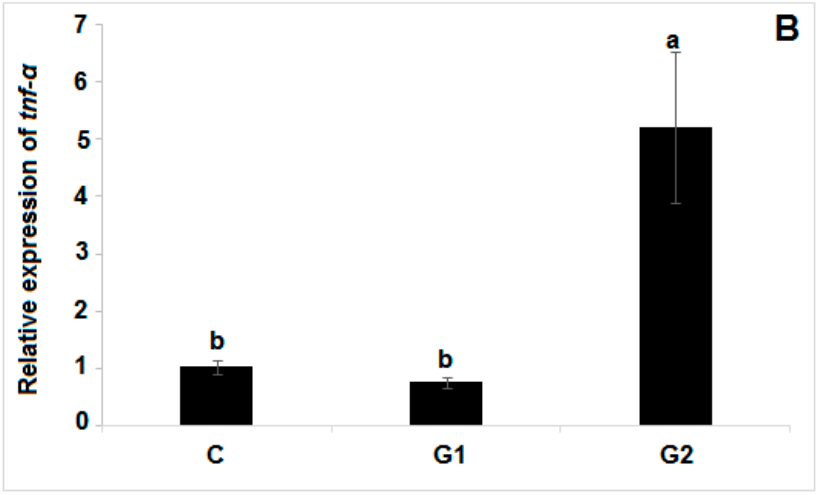

Figure 2. Changes in the relative expression of immune related genes in the rainbow trout liver fed with different levels of red macroalgae (*G. oblongata*). (**A**) *tnf-α* and (**B**) *il-6* gene expression. C (control—0% *G. oblongata*), G1 (0.5% *G. oblongata*), G2 (1% *G. oblongata*). Different letters above the bars signify a significant difference between treatments ($p < 0.05$); values are presented as the mean ± SE ($n = 9$).

4. Discussion

Previous works with red and brown seaweeds reported an excellent nutritional composition, which may be a functional and cost-effective replacement for proteins, minerals, and vitamins [13,38]. In this regard, several publications have addressed the potency of different red and brown seaweed species to ameliorate the growth performance and feed revenue of several aquatic animal models [39–46]. In addition, based on evidence, red algae contain higher PUFAs (n−3 (eicosapentaenoic acid) and n−6 (arachidonic acid)) than green algae and have a higher nutritional value than brown algae [13], which in turn may have growth promotion effects on aquatic animals. Nevertheless, in this study, the fish that received dietary *G. oblongata* exhibited no significant improvement in growth parameters (WG, SGR, and FCR). These findings are consistent with those obtained by Vazirzadeh et al. [10], who claimed that the long-term (83-day) administration of 5% and 10% red algae (*Gracilariopsis persica* and *Hypnea flagelliformis*) and brown algae (*Sargassum boveanum*) had no remarkable effect on the growth performance and FCR of rainbow trout. Similarly, the growth performance in zebrafish supplemented with *Gracilaria gracilis* (0.25, 0.5, and 1%) [47] and Persian sturgeon (*Acipenser persicus*) supplemented with *G. persica* (2.5, 5, and 10 g kg^{-1} diet) [48] was not significantly different from fish fed non-supplemented feeds. These discrepancies in the results reveal the theory that dietary macroalgae may exert species-specific effects [48] and seaweeds' nutritional values may change notably depending on the species, genera, dose, duration, and other factors [47].

The beneficial role of phytobiotics, seaweeds, and their products in the health of aquatic animals and their providing of resistance against viral and bacterial infections have been widely reported [19,20,49–51]. For this purpose, serum and mucus non-specific immune parameters can be considered helpful tools for monitoring the influence of medicinal herbs on fish health status [52]. Indeed, skin mucus is a significant part of the fish defense system. It consists of numerous antimicrobial components, such as complement systems, lectins, LYZ, immunoglobulins, antimicrobial peptides, and antibodies, which provide a robust physiological barrier against invasive pathogens [53]. Ig and LYZ are common humoral components of host mucosal and systemic immunity and are usually admitted as biomarkers of immune responses with feed supplements in aquatic species [54,55]. Our results highlighted the immunostimulatory effects of *G. oblongata* in rainbow trout, characterized by a higher serum (LYZ and total Ig) and mucosal (total Ig) immune response compared to the control. Consistent with our results, a remarkable increase in aquatic

animal immunological indices was also reported following the dietary administration of different red macroalgae species [16,39,56,57].

Additionally, *G. persica* powder boosted serum and mucus total Ig and LYZ values in Persian sturgeon compared to the non-supplemented group [48]. Chen and Zhang [42] found that grass carp (*Ctenopharyngodon idella*) that received *Porphyra yezoensis* polysaccharide-containing diets (3 and 5 g kg^{-1}) showed substantially higher serum LYZ activity. Furthermore, adding *G. gracilis* (1%) to the fish diet caused a remarkable increase in the mucosal total Ig levels of zebrafish [47]. In another study, dietary Spirulina platensis increased serum Ig and LYZ activity in coral trout (*Plectropomus leopardus*) [58]. In addition, increased serum LYZ activity was determined in juvenile black sea bream (*Acanthopagrus schlegelii*) after feeding brown algae (*Sargassum hornei*) [41]. However, concerning serum, the maximum immune responses (LYZ and Total Ig) were observed in rainbow trout fed with a lower concentration of *G. oblongata* (G1). This study's findings agree with a previous study on barramundi (*Lates calcarifer*), where a significant decline was observed in LYZ and Ig in response to increased dietary levels of *Gracilaria pulvinata* [56]. In line with our results, Ghafarifarsani et al. (2022) reported that adding savory essential oil to roach diets increased Ig and lysozyme activity in mucus and serum. However, *G. oblongata* employed in the current study at different doses exhibited no significant differences in the mucus total Ig value. A similar result has been reported for Ig value in the research carried out on zebrafish evaluating the influence of red macroalgae (*Halopithy sincurva*) [19]. Although the exact mechanism responsible for the immunostimulatory effect of seaweeds is still unclear, it is well known that they are rich sources of polysaccharides and polyphenolic compounds with high antibacterial features and a promising influence on the welfare of fish [12]. However, including dietary *G. oblongata* had no marked effects on mucus LYZ activity in this research. Contrary to our finding, a study on the impacts of *G. persica* (5 and 10 g kg^{-1}) in Persian sturgeon showed a promoting effect on skin mucus LYZ activity [48]. It may, therefore, be concluded from the contradictory results that the immunostimulatory effects of dietary seaweeds on fish may differ from one species to another, and various parameters, e.g., fish and algae species, administration dose and duration, as well as experimental conditions may alter the outputs [19,56].

It has been proved that dietary exogenous antioxidant compounds may cooperate with endogenous antioxidants to diminish the overgeneration of reactive oxygen species (ROS) [19,59]. Correspondingly, in vitro studies have well exhibited the antioxidant effects of dietary macroalgae on several cultured organisms because of the existence of carotenoids, specific polysaccharides, prebiotics, and phenolic compounds in their chemical profile, scavenging free radicals [60]. SOD, CAT, and GPx are generally considered appropriate biomarkers of oxidative stress in fish, with an increase in concentration being translated into an increase in antioxidant performance [61,62]. Accordingly, in the current study, the potential of *G. oblongata* to improve antioxidant capacity is also exhibited through increased serum SOD, CAT, and GPx activity compared to the unsupplemented group. Parallel to our results, Chen, L. and Y. Zhang [42] reported improved serum SOD, CAT, and GPx activity in grass carp fed with dietary polysaccharides extracted from *P. yezoensis* (1, 3, and 5 g kg^{-1}). MDA is a biomarker of lipid peroxidation and cell and tissue disorders. When the generation of ROS is above its elimination, the MDA levels subsequently increase [63]. In this study, the observed increase in mucus GPx and SOD levels following macroalgae supplementation, coupled with the absence of elevation in MDA levels, suggests that *G. oblongata* could be employed to mitigate oxidative stress in *O. mykiss* [14]. These findings are concurrent with Hoseinifar et al. [19], who assayed the effects of dietary red macroalgae (*H. incurva*) on zebrafish and claimed that this supplement boosted serum and mucus SOD, CAT, GPx, and decreased MDA levels.

It has also been determined that GPx and CAT may play the same role since both are antioxidant enzymes responsible for detoxifying H_2O_2 to H_2O and O_2 [64]. The existence of the GPx enzyme may have a limiting effect on the CAT enzyme, as featured in the present research just for skin mucus. Similar results were also obtained in the case of serum

GPx and CAT activity in rainbow trout fed with red algae (*Laurencia caspica*) extract [65]. Additionally, the results of the present study support the hypothesis that antioxidant enzyme activity may possess contradictory patterns in different tissues [66].

Many studies aim to confirm the antioxidant and immunostimulatory capabilities of different macroalgae at the molecular level (21, 48, 66). However, the current trial is the first attempt to investigate the molecular-level effectiveness of *G. oblongata* on the antioxidant response and immunity of rainbow trout, demonstrating a positive role in specific genes. The up-regulation of hepatic *gpx* was shown in the group fed by *G. oblongata*-supplemented diet. Based on the documents, it can be suggested that herbal remedies could notably up-regulate hepatic antioxidant gene expressions of aquatic species [67,68]. However, in the present trial, no significant variation was found between fish fed *G. oblongata* supplemented and no-supplemented diets on *sod* gene expression.

Similarly, Hoseinifar et al. [47] reported that *G. gracilis* enriched diet (0.25, 0.5, and 1%) resulted in up-regulation of *cat* gene expression. At the same time, no significant difference in *sod* value was detected in the whole body of zebrafish. Also, the oral administration of dietary red macroalgae (*H. incurva*) (0.25 and 0.5%) in zebrafish [19] and ulvan in labeorohita (25, 50, and 100 mg kg^{-1}) [69] increased *gpx* gene expression. However, the up-regulation of an enzyme mRNA would not always lead to increased enzyme synthesis due to numerous causes [70]. Therefore, the higher *gpx* gene expression in the fish liver may suggest promising impacts of *G. oblongata* on hepatic antioxidant activity. Nevertheless, further studies are required to support this hypothesis with different genes in subsequent research.

An inflammatory response usually accompanies extra pro-oxidant production [69,71]. Additionally, the inflammatory and immune responses of fish are moderated by cytokines [72]. In this regard, *tnf-α* and *il-6* are two important pro-inflammatory cytokines that could trigger inflammatory reactions and play an essential task in modifying the immune strength of the host [73,74]. Accordingly, our findings demonstrated that feeding the highest level of *G. oblongata* could significantly up-regulate the liver's *tnf-α* and *il-6* gene expression. The up-regulation of *tnf-α* and *il-6* as the mediator of inflammation through macroalgae treatments could stimulate antibodies and numerous immune cell production [75,76]. In line with our results, dietary glycoprotein extracted from hizikia fusiformis (10 g kg^{-1}) [39] and *P. yezoensis* (20 g kg−1) [77] increased hepatic *il-6* gene expression of olive flounder only at the highest dietary inclusion level. In addition, a significant elevation of the hepatic *tnf-α* gene expression value was exhibited in zebrafish following feeding by all dietary inclusion levels of *H. incurva* (0.25, 0.5, and 1%) [19]. In an 8-week feeding trial carried out to assess the impacts of an *L. caspica* containing diet on rainbow trout, marked increases in the kidney *tnf-α* and interleukin-1β (*il-1β*) cytokine gene expression were reported after bacterial challenge in fish fed 1.5% of this supplement [65]. Additionally, an elevation in kidney *tnf-α* and *il-6* gene expression was reported in rainbow trout after feeding with dietary *Aloe vera* (10 and 15 g kg^{-1}) [78]. Finally, in juvenile black sea bream supplemented with *S. hornei* (3, 6, and 9%), the liveril-*1β*, interleukin-8 (*il-8*), and *tnf-α* genes expression increased significantly [41]. Such positive changes in these pro-inflammatory cytokine genes may explain why dietary macroalgae can be considered immunostimulants, which can incite the expression of significant immune genes.

5. Conclusions

Dietary red macroalgae (*G. oblongata*) did not affect the growth performance of rainbow trout but increased total immunoglobulin content and lysozyme activity in the serum and skin mucus. Feeding with red macroalgae enhanced the activities of catalase, superoxide dismutase, and glutathione peroxidase in the serum, as well as increased antioxidant and immune-related gene expression in the liver. Taken together, our results showed that supplementation of red macroalgae in the diet is a potential strategy to improve the health of rainbow trout by increasing immune and antioxidative responses.

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