



# *Spirulina platensis* in rainbow trout (*Oncorhynchus mykiss*) feed: effects on growth, fillet composition, and tissue antioxidant mechanisms

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## Abstract

An experiment was conducted to investigate the effects of *Spirulina platensis* (SP) on growth, fillet composition, and intestinal, skin, and gill mucosal antioxidants of rainbow trout, *Oncorhynchus mykiss* (initial weight  $17.18 \pm 0.59$  g). One-hundred and thirty-five fish were randomly distributed among nine cement tanks (1.8 m × 0.22 m × 0.35 m) with 15 fish per tank. Three isonitrogenous (37.8% crude protein) feeds containing either 0, 2.5, or 5% SP were prepared, randomly assigned to triplicate tanks of fish, and given at 2% body weight per day for 7 weeks. At the end of the trial, fish in each tank were assessed for growth (final weight, thermal growth coefficient), condition factor, fillet composition, mucosal antioxidant activity, and gene expression analysis related to the antioxidant enzymes catalase, glutathione peroxidase 1, and glutathione S-transferase. Neither growth nor fillet composition was influenced by inclusion of SP in feed. Total antioxidant activity in three mucosal tissues including the intestine, skin, and gill was significantly increased by 2.5% SP whereas administration of 5% SP only increased total antioxidant activity in the intestine. Feeding fish with 2.5% and 5% SP could upregulate the expression of catalase in the intestinal tissue whereas 5% SP enhanced the expression of glutathione peroxidase 1 in this tissue. Glutathione S-transferase gene expression was also increased in the intestinal and skin tissues of fish administrated with 2.5% SP while in the fish that received 5% SP-supplemented diet, an upregulation of this gene was only noted in the intestinal tissue. It was concluded that 2.5% SP had a potential to enhance some antioxidant parameters mostly in the intestine, followed by the skin and gill.

**Keywords** *Salmonidae* · Growth metrics · Diet formulation · Antioxidant enzymes

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## Introduction

Like all aerobic organisms, fish are susceptible to attack by reactive oxygen species (ROS) and reactive nitrogen metabolites (RNMs) which are capable of modifying and damaging cell and tissue components (Martínez-Álvarez et al. 2005). In aquaculture, multiple stressors can lead to oxidative damage to tissues (Livingstone 2003; Martínez-Álvarez et al. 2005; Trenzado et al. 2009; Slaninova et al. 2009). The antioxidant defense system, comprising antioxidant compounds and enzymes, is important in preventing the cascade of oxidant reactions by detoxifying ROS and RNMs.

Nutritional studies indicate that dietary supplements, such as probiotics, microalgae, and herbal extracts, can augment the antioxidant system because they contain active components such as flavonoids, alkaloids, terpenoids, tannins, saponins, or essential oils (Martínez-Álvarez et al. 2005; Chakraborty and Hancz 2011; Oliva-Teles 2012; Pohlenz and Gatlin 2014; Reverter et al. 2014). *Spirulina* is an edible multicellular and filamentous cyanobacterium with high contents of protein, vitamins, minerals, and polyunsaturated fatty acids such as  $\gamma$ -linolenic acid, essential amino acids, and antioxidant pigments such as  $\beta$ -carotenoids (Soni et al. 2017). Several studies have demonstrated beneficial effects of supplementing aqua feeds with *Spirulina platensis* (SP) (Rosas et al. 2018). For example, after feeding Nile tilapia (*Oreochromis niloticus*) with SP, serum antioxidant activities were enhanced, as shown by increased activity of superoxide dismutase, glutathione peroxidase, and catalase (Abdel-Latif and Khalil 2014). Similarly, presenting feed supplemented with SP improved antioxidant biomarkers in the liver, kidney, and gills of Nile tilapia (Abdelkhalik et al. 2015). The current study was performed with the primary aim of evaluating the effects of dietary SP supplementation on rainbow trout (*Oncorhynchus mykiss*) mucosal antioxidants in the gill, skin, and intestine.

## Materials and methods

### Fish and experimental design

The experiment was carried out at a private fish farm in Firouzkooh, Iran. A total number of 135 rainbow trout ( $17.18 \pm 0.59$  g wet weight) were randomly distributed among nine indoor cement tanks ( $1.8 \text{ m} \times 0.22 \text{ m} \times 0.35 \text{ m}$ ) with 15 fish in each tank under flow-through systems. The water was maintained at  $13 \pm 1$  °C with a flow rate of  $0.5 \text{ L s}^{-1}$  and dissolved oxygen  $> 8$  ppm,  $\text{NH}_3 < 0.01 \text{ mg L}^{-1}$ ,  $\text{NO}_2 < 0.1 \text{ mg L}^{-1}$ , and pH 7.8. Fish were allowed to acclimatize for 10 days on the diet with SP 0% (Table 1) before starting the experiment.

The powdered SP was obtained from CBN Spirulina, China. The other feed ingredients were purchased from commercial suppliers. The proximate composition of SP, analyzed according to the methods described by the Association of Official Analytical Chemists (AOAC 1995) was as follows: 5.65% moisture, 60.14% crude protein, 0.75% crude fat, and 6.65% ash. Three isonitrogenous (37.8% crude protein) experimental diets were formulated containing 0, 2.5, and 5% SP by replacing with fish meal in order to attain an equal protein level (Table 1). All feed ingredients were well-mixed and pelletized by a laboratory pelletizer. After natural air-drying, pellets for each group were packed and kept in sealed bags at 4 °C until used in the feeding trial. About 60 g of each formulated diet was sampled for biochemical composition analysis according to AOAC (1995). Fish were fed twice a day at 8:00 and 17:00 for each treatment group at 2% body weight for seven continuous weeks.

**Table 1** Formulation and chemical composition of experimental diets

	SP 0%	SP 2.5%	SP 5%
Ingredients (g kg <sup>-1</sup> )			
Fish oil <sup>a</sup>	100	100	100
Soybean oil <sup>b</sup>	60	60	60
Wheat flour <sup>c</sup>	77	77	77
Fish meal <sup>d</sup>	450	425	400
Soybean meal <sup>c</sup>	200	200	200
Corn flour <sup>f</sup>	80	80	80
Lecithin	5	5	5
Cellulose <sup>g</sup>	3	3	3
Vitamin premix <sup>h</sup>	15	15	15
Mineral premix <sup>i</sup>	10	10	10
SP	0	25	50
Chemical composition (% dry matter)			
Moisture	9.75	9.69	9.63
Crude protein	37.88	37.87	37.87
Crude lipid	20.03	19.83	19.65
Ash	8.56	8.33	8.09

<sup>a,b,c,d,e,f</sup> Behparvar Company, Karaj, Iran

<sup>g</sup> Cellulose (Merck Company, Germany)

<sup>h</sup> Vitamin premix (Science Laboratories Company, Qazvin, Iran): composition per 1000 g: 160000 IU vitamin A, 400000 IU vitamin D<sub>3</sub>, 40 mg vitamin E, 2 g vitamin K<sub>3</sub>, 6 g vitamin B<sub>1</sub>, 8 g vitamin B<sub>2</sub>, 12 g pantothenate calcium, 40 g vitamin B<sub>3</sub>, 4 g vitamin B<sub>6</sub>, 2 g vitamin B<sub>9</sub>, 8 g vitamin B<sub>12</sub>, 0.24 g H<sub>2</sub>, 60 g vitamin C, 20 mg inositol, 20 mg BHT

<sup>i</sup> Mineral premix (Science Laboratories Company, Qazvin, Iran): composition per 1000 g: 26 mg Fe, 12.5 g zinc, 2 mg Se, 480 mg Co, 2.4 g Cu, 8.15 g Mn, 1 g I, 12 g chloride choline

## Growth performance

At the beginning and the end of the experiment, all fish in each tank were starved for 24 h before harvesting. After collecting and anesthetizing the fish with clove oil bath (50 µL L<sup>-1</sup>), the weight and length in each fish were determined. Survival rate and growth parameters including thermal growth coefficient (TGC) and condition factor (CF) were calculated as follow:

$$\text{TGC} = 100 \times \frac{\text{final body weight}(\text{g})^{1/3} - \text{initial body weight}(\text{g})^{1/3}}{(\text{number of days in the feeding period}) \times \text{mean daily temperature}(\text{°C})}$$

$$\text{CF} = 100 \times \frac{\text{final body weight}(\text{g})}{\text{final body length}(\text{cm})^3}$$

## The fillet composition of the examined fish

Three fish from each tank were used for fillet quality assessments according to AOAC (1995). Meat from the dorsal part of each fish was dissected (Testi et al. 2006) and autoclaved at 105 °C for 20 min, then homogenized and oven-dried at 60 °C. Total lipid was determined by a Soxhlet apparatus. Crude protein was measured by the Kjeldahl method (using Kjeltex Auto Analyser, 2300 Tecator, Sweden); and ash was determined by incineration at 600 °C for 6 h.

## Total antioxidant activity of the tissues

Total antioxidant activity of the mucosal tissues was determined for three fish from each tank as described by Benzie and Strain (1996). In each fish, the first arch of the gill, skin (about 2 cm) from the mid-body on the left side, and posterior intestine (about 1 cm) were sampled. Fish tissues were immediately frozen in liquid nitrogen and homogenized in 25 mM Tris-HCl buffer (pH 7.2). Tissue homogenates were centrifuged at 5000g for 10 min, and supernatants were collected in microtubes and stored at  $-20^{\circ}\text{C}$ . About 80  $\mu\text{L}$  aliquots were added to 2.5 mL FRAP buffer containing 10 mM TPTZ (Sigma-Aldrich, USA), 20 mM  $\text{Fe}_2\text{Cl}_3$  (Sigma-Aldrich, USA), and 300 mM sodium acetate buffer. After incubation at  $37^{\circ}\text{C}$  for 15 min, absorbance was read at 593 nm. Results were expressed as equivalent of butylated hydroxytoluene (BHT) as a potent antioxidant.

## Gene expression analysis

For similarity, the same place in the skin, gill, and intestine tissues were sampled from three other fish in each tank and were immediately frozen in liquid nitrogen. Total RNA was extracted from all sampled tissues (skin, gill, and intestine) using an AccuZol® (Bioneer, South Korea) reagent. RNA was then quantified and the purity was assessed by a Bio-Rad spectrophotometer (Bio-Rad, CA, USA). First-strand cDNA was synthesized from 1  $\mu\text{g}$  of total RNA in a 20- $\mu\text{L}$  reaction volume using a random hexamer primer (Thermo Fisher Scientific, USA), M-MuLV reverse transcriptase, and RiboLock™ RNase Inhibitor (Thermo Fisher Scientific, USA). The expression of the genes related to the antioxidant enzymes, namely, catalase, glutathione peroxidase 1, and glutathione S-transferase A (Table 2) were analyzed by real-time PCR. Reaction mixtures contained 10  $\mu\text{L}$  SYBR green supermix (TaKaRa Biotechnology Co. Ltd., China), 20 pmol of each primer, and 1  $\mu\text{L}$  of cDNA template in a final reaction volume of 20  $\mu\text{L}$ . The fold change in mRNA expressions of the genes was calculated by the  $2^{-\Delta\Delta\text{Ct}}$  method (Livak and Schmittgen 2001). In all cases, each PCR reaction was performed in triplicate to minimize the experimental error.

## Statistical analysis

Data from the present study was shown as the mean  $\pm$  standard error. After confirming the normality of data and homogeneity of variances, the one-way and two-way analysis of

**Table 2** Primers selected for the quantitative reverse transcription polymerase chain reaction analyses in rainbow trout mucosa (intestine, skin, and gill)

Target gene	Nucleotide sequence (5' → 3')	Product size (bp)	Accession no.
Catalase	TGATGTCACACAGGTGCGTA GTGGGCTCAGTGTGTTGAG	195	TC99600
Glutathione peroxidase 1	CGAGCTCCATGAACGGTACG TGCTTCCCGTTCACATCCAC	183	TC126469
Glutathione S-transferase A	CAGAGGACAGCTCCCTGCTT CTGAACCGGCTCTCCAGGTA	187	NM_001160559.1
$\beta$ -Actin	ATGGAAGATGAAATCGCCGCAC TGGCCCATCCCAACCATCAC	191	AJ438158

variance (ANOVA) were conducted using SPSS ver. 19.0 (IBM Corp. USA). The  $P$  value < 0.05 was used as the level of the statistical significance in all analyses.

## Results

### The survival, growth performance, and proximate composition of the examined fish

During 7 weeks of the feeding trial, the survival of the examined fish was 100% in all treatment and control groups. Results showed that SP dietary supplementation had no significant effect on growth performance in any of the treatment groups as compared with the control group (Table 3). Results of the fillet composition of fish also showed that the different parameters including dry matter, total lipid, crude protein, and ash were not affected by SP feeding in any of the experimental groups (Table 4).

### Total antioxidant activity

Fish fed diet supplemented with 2.5% SP showed higher antioxidant activity in the gill and skin tissues in comparison with the control group whereas 5% SP did not change antioxidant activity compared with the other groups. Diets supplemented with 2.5% and 5% SP enhanced the antioxidant activity in the fish intestine compared with the control group (Table 5).

### Expression of antioxidant-related genes

#### Catalase

In the intestine, the expression of catalase gene was upregulated in fish fed 5% and 2.5% SP in comparison with the control group whereas in the skin and gill tissues, no significant changes were shown compared with the control group (Fig. 1).

#### Glutathione peroxidase 1

An upregulation in the expression of glutathione peroxidase 1 gene in the intestine of fish fed 5% SP-supplemented diet was seen. No significant changes were shown in other groups compared with the control group (Fig. 2).

**Table 3** Growth performance in rainbow trout (*Oncorhynchus mykiss*) after feeding different doses of *Spirulina platensis* for 7 weeks

<i>Spirulina platensis</i> in diet (%)	Final weight (g)	Final length (cm)	Thermal growth coefficient	Condition factor	Survival rate (%)
0	53.03 ± 1.56	17.02 ± 0.17	0.205 ± 0.019	1.061 ± 0.017	100
2.5	50.71 ± 1.62	16.72 ± 0.14	0.196 ± 0.012	1.077 ± 0.017	100
5	53.70 ± 2.16	17.18 ± 0.36	0.197 ± 0.031	1.059 ± 0.030	100

(Data are mean ± SEM). Those within a column superscripted by different letters are significantly different ( $P > 0.05$ )

**Table 4** Fillet composition (%) in rainbow trout (*Oncorhynchus mykiss*) after feeding different doses of *Spirulina platensis* for 7 weeks

<i>Spirulina platensis</i> in diet (%)	Dry matter	Total lipid	Crude protein	Ash
0	24.32 ± 2.53	5.55 ± 0.12	15.72 ± 0.87	1.22 ± 0.05
2.5	24.05 ± 1.31	5.80 ± 0.07	15.65 ± 1.01	1.23 ± 0.04
5	25.50 ± 2.06	5.41 ± 0.08	17.07 ± 0.93	1.28 ± 0.11

(Data are mean ± SEM). Those within a column superscripted by different letters are significantly different ( $P > 0.05$ )

### Glutathione S-transferase A

The expression of gene encoding glutathione S-transferase A in the skin and intestinal tissues was upregulated in fish that received 2.5% *S. platensis*. On the other hand, in fish fed with 5% *S. platensis*, an upregulation was noted in the skin tissue. The highest fold changes in the expression of S-transferase A gene were seen in the skin and intestinal tissues of fish that received 2.5% SP (Fig. 3).

### Discussion

The results of the present study showed that feeding fish with a diet supplemented with 2.5% SP can improve the antioxidant status of the fish even though the growth performance and fillet composition of fish were not significantly affected. In agreement with our results, Gouveia et al. (2003) showed that SP could not affect the growth performance in *Cyprinus carpio* and *Carassius auratus*. In another study, it was reported that the inclusion of SP in rainbow trout diet had no impact on fish growth performance (Teimouri et al. 2013). In contrast, a number of studies reported that fish diet enriched with SP enhanced the growth performance and fillet composition in different fish species (Promya and Chitmanat 2011; Belal and El-Hais 2012; Ibrahim et al. 2013; Abdel-Latif and Khalil 2014; Abdulrahman 2014; El-Sheekh et al. 2014).

The discrepancy between the results of different studies may be originated from the differences in the source and culture condition of SP, the method by which SP was added to the diet, dose of SP, feeding regime, and fish species. For example, in an SP cultivation system, different phases including growth on culture medium, drying, and even harvesting time can directly affect the SP biomass composition and nutritional value (Soni et al. 2017). Since in the present study, a commercial brand of SP was used, no information about the culture condition of SP was available. Meanwhile, in most studies where the positive effects of SP on growth

**Table 5** Total antioxidant activity (mmol L<sup>-1</sup>) in rainbow trout (*Oncorhynchus mykiss*) mucosal tissues after feeding different doses of *Spirulina platensis* for 7 weeks

<i>Spirulina platensis</i> in diet (%)	Gill	Skin	Intestine
0	9.42 ± 1.08 <sup>a</sup>	12.78 ± 0.68 <sup>a</sup>	8.72 ± 0.39 <sup>a</sup>
2.5	12.59 ± 0.77 <sup>b</sup>	18.52 ± 1.05 <sup>b</sup>	12.99 ± 0.42 <sup>b</sup>
5	10.99 ± 1.12 <sup>ab</sup>	14.72 ± 1.01 <sup>ab</sup>	12.50 ± 1.80 <sup>b</sup>

Those within a column superscripted by different letters are significantly different ( $P > 0.05$ )

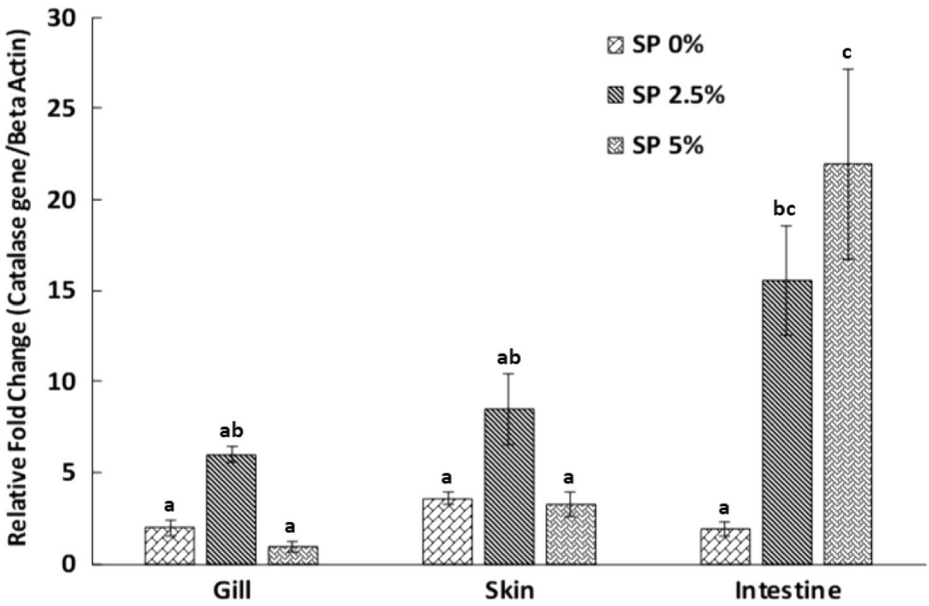


Fig. 1 Expression of gene encoding catalase in rainbow trout (*Oncorhynchus mykiss*) mucosal tissues after feeding different doses of *Spirulina platensis* for 7 weeks. Different superscript letters indicate significant differences among groups (ANOVA;  $P < 0.05$ )

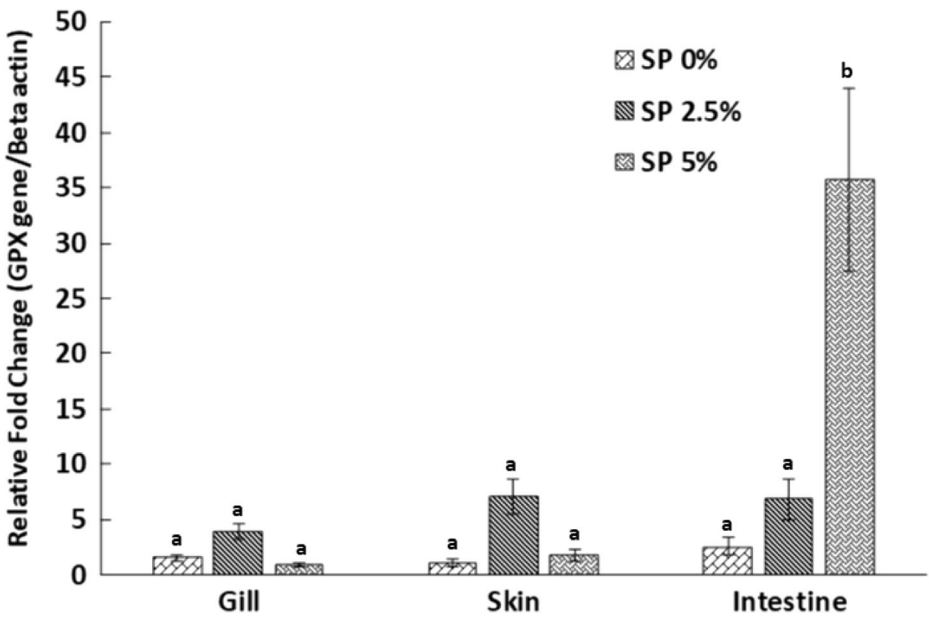
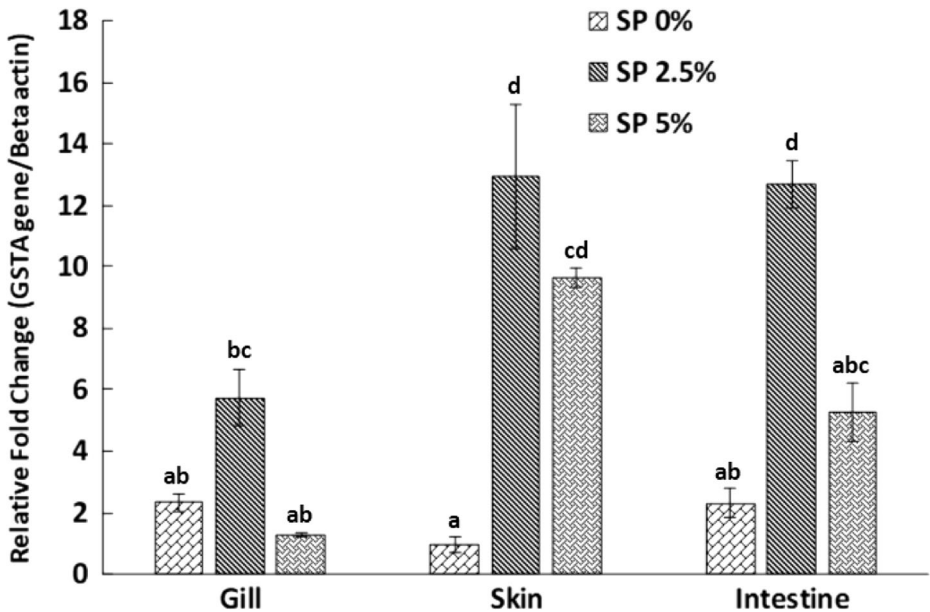


Fig. 2 Expression of gene encoding glutathione peroxidase 1 (GPX) in rainbow trout (*Oncorhynchus mykiss*) mucosal tissues after feeding different doses of *Spirulina platensis* for 7 weeks. Different superscript letters indicate significant differences among groups (ANOVA;  $P < 0.05$ )



**Fig. 3** Expression of gene encoding glutathione S-transferase A (GSTA) in rainbow trout (*Oncorhynchus mykiss*) mucosal tissues after feeding different doses of *Spirulina platensis* for 7 weeks. Different superscript letters indicate significant differences among groups (ANOVA;  $P < 0.05$ )

performance were reported, the replacement of SP was performed with the plant protein sources whereas partial replacement of SP with fish meal in most cases resulted in the similar growth performance in comparison with the control group (Rosas et al. 2018). Even in the higher amount of replacement with fish meal, diminished growth performance was also shown (Rosas et al. 2018). Besides, feeding regime can also affect the growth performance in a way that growth promotion effect is less likely to happen with fixed ration (as performed in the current study) than when fish are fed to apparent satiation.

The present study showed that feeding fish with SP enriched diet especially at 2.5% and had a potential to augment the antioxidant system in the intestine, skin, and gill. In numerous studies in humans, veterinary medicine, and aquatic animals, the antioxidant activity of SP in the liver, brain, kidney, muscle, and blood has been investigated (Wu et al. 2005; Karadeniz et al. 2009; Ponce-Canchihuamán et al. 2010; Abdel-Daim et al. 2013; Abdel-Latif and Khalil 2014; Abdelkhalek et al. 2015; Teimouri et al. 2016), but the effects of these algae on mucosal tissue have not been evaluated. The antioxidant and protective effects of SP are related to the antioxidant active constituents such as brilliant blue polypeptide pigment phycocyanin,  $\beta$ -carotene, minerals, vitamins E and C, proteins, carbohydrates, and lipids (Soni et al. 2017).

Antioxidant enzymes such as catalase (in which NADPH protects it against inactivation by its substrate  $H_2O_2$ ), glutathione peroxidase (which reduce  $H_2O_2$  via its selenocysteine-containing active site), and glutathione-S-transferase (glutathione-related enzyme affected by ROS) are the first line of cellular defense against oxygen radical damage to aerobic cells (Mugesh and Singh 2000; Sharma et al. 2007). In parallel with the total antioxidant activity, high expression of the genes related to different antioxidant enzymes was noted in fish mucosal tissues. Similar to the results



of the current study, SP significantly increased the catalase and glutathione peroxidase 1 activities of the liver, kidney, and gill in Nile tilapia (Abdelkhalek et al. 2015). Sharma et al. (2007) also reported that feeding SP increased the activity of glutathione S-transferase enzyme A in mercuric chloride-intoxicated Swiss albino mice.

It was revealed that the expression of catalase, glutathione peroxidase 1, and glutathione S-transferase A genes was affected by SP mostly in the intestine, followed by the skin and gill. As a consequence, a higher antioxidant activity was also observed in the fish intestine than the other two mucosal tissues. In fish especially in the intestine, oxidative stress generated by food deprivation, nitrosative stress, infections, oxidants in fish feed including oxidized fish oil, and anti-nutritional factors in soybean meal could result in reduction in digestive and absorptive ability and antioxidant disturbance (Sitjà-Bobadilla et al. 2005; Peng et al. 2009; Enes et al. 2012; Zhang et al. 2013). Meanwhile, most of the chemical contaminants in the aquatic environment can be taken up from the sediment, water column, and food and enter the gastrointestinal tract of fish. Therefore, enhancement of the gut antioxidant system is of great importance. In a study by Esteban et al. (2014), the effects of bacterial probiotics, *Shewanella putrefaciens* Pdp11, and *Bacillus* spp. in combination with the extract of Tunisian date palm fruit on the expression of genes encoding antioxidant enzymes in three mucosal tissues of gilthead sea bream (*Sparus aurata*) were studied. Results showed that the expression of genes encoding antioxidant enzymes was more affected in the fish gill than the other two tissues followed by the skin and less in the gut. These findings were different from the findings of the present study.

In the current study, supplemented diet with 2.5% SP enhanced the total antioxidant activity and the expression of antioxidant-related genes in different mucosal tissues, but diet with 5% SP did not have the same effect as 2.5% SP. In this study, no side effects of administrating 5% SP were seen in the treated fish, but higher replacement of fish meal in 5% SP group might result in the lower antioxidant responses compared with the group fed 2.5% SP. It is worth mentioning that even replacing high quantities of fish meal with SP can impair the immune and antioxidant system due to the deficiencies in the amino acid composition of substituted diet (Rosas et al. 2018). Meanwhile, it was proven that phycocyanin, a pigment abundant in SP, can act as a pro-oxidant at some conditions and doses in different animals (Rosas et al. 2018). Therefore, determining the optimum dose of different additives in fish species is very important.

In general, it was concluded that feeding fish with SP especially at 2.5% enhanced the antioxidant responses in three mucosal tissues of fish including the intestine, skin, and gill. Further studies are needed to evaluate the protective role of different nutrients on mucosal surfaces following challenge by different pollutants inducing oxidative damage.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All procedures performed in this study were in accordance with the ethical standards of the Faculty of Veterinary Medicine at the University of Tabriz, under protocol number FVM.REC.1396.937.

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