

Article

Improving Growth, Digestive and Antioxidant Enzymes and Immune Response of Juvenile Grass Carp (*Ctenopharyngodon idella*) by Using Dietary *Spirulina platensis*

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Abstract: The present study was designed to investigate the effect of *Spirulina platensis* (SP) supplemented diets on the growth performance, digestive enzymes, hepatic antioxidants and innate immunity biomarkers in juvenile grass carp (*Ctenopharyngodon idella*). Two hundred and forty grass carp juveniles (average weight 4.81 ± 0.13 g) were divided into four treatment groups in triplicates (20 fish/replicate) and fed with diets containing 0, 1, 5, and 10% *Spirulina* for 90 days. A significant increase in growth ($p < 0.05$) was observed in fish fed with diets having 1 and 5% *Spirulina*. Intestinal protease and lipase activities increased significantly ($p < 0.01$) in fish fed with a diet having 5% *Spirulina* while intestinal amylase activity increased significantly ($p < 0.01$) in fish fed with diets having 1, 5, and 10% *Spirulina*. Hepatic lipid peroxidation decreased significantly ($p < 0.05$) in fish fed with a 1 and 5% *Spirulina* supplemented diet. The activity of catalase, glutathione-S-transferase, and glutathione levels increased significantly ($p < 0.05$) in the livers of fish fed with 1% *Spirulina* supplemented diets while no significant difference ($p > 0.05$) was observed for hepatic superoxide dismutase levels when compared to the control. Significant increases in the skin mucus protease ($p < 0.05$), antiprotease ($p < 0.01$), lysozyme ($p < 0.001$), and peroxidase ($p < 0.05$) activities were observed in fish fed with 5% *Spirulina*-supplemented diets. Gene expression analysis of head kidney showed that fish fed with a 1% *Spirulina* diet had significantly ($p < 0.01$) higher expression of *tnf- α* , *il-8*, and *inf- γ* . In conclusion, the present study suggested that the inclusion of 5% *Spirulina platensis* in the diet of grass carp has positive effects on growth, digestive enzymes, antioxidants, and innate immunity.

Keywords: *Spirulina*; digestive enzymes; hepatic enzymes; gene expression; aquaculture; cyanobacteria



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1. Introduction

The world population is increasing at a tremendous rate and is expected to hit 10 billion people by 2050 [1]. Food supply, especially animal-based protein, will be crucial in the coming decades. The global demand for animal-derived protein will be doubled by 2050 which is expected to intensify pressure on the need to produce more animal-based protein [2]. Fish and shellfish are the primary sources of protein for approximately 950 million people worldwide [3]. Aquaculture is expanding faster than any other food-producing sector, and intensive aquaculture has the potential to provide animal-based protein to an exponentially growing human population [4,5]. As stocking density is high in an intensive culture system, this may lead to crowding stress. Crowding stress along with poor

water quality results in the emergence of disease outbreaks that inflict significant financial losses [6].

Infectious diseases have always been a great threat in intensive animal production systems [7–11]. Antibiotics are used to treat diseases and reduce pathogens and disease incidences [12] but antibiotic resistance has become a global issue in humans as well as poultry, livestock, and aquaculture [13–15]. Many researchers are now trying to discover sustainable alternatives to antibiotics [16–21]. The use of medicinal plants, macro and microalgae, herbs, and probiotics as oral immunostimulants has gained a great deal of interest throughout the globe [22,23]. Phenol, polyphenol, quinone, alkaloid, terpenoid, polypeptide compounds and lectin are present in algae, certain medicinal plants, and their by-products which promote growth, enhance the antioxidant status, and stimulate the immune system, thus providing a sustainable alternative to vaccines and antibiotics [24,25].

Algae and other plant-based feed additives are able to promote the growth of fish [26–29], protect against diseases [29,30], strengthen the immune system [22,26,31–33], stimulate hunger and enhance feed consumption [34,35], reduce stress [36–38] and improve digestion by increasing secretion of different digestive enzymes [39–41]. They also have antimicrobial and antiviral properties [39,42–44]. Algae and other plants are cheaper, eco-friendly, have minimum side effects, and are frequently used as substitutes to the costly antibiotics in fish health management. The World Health Organization (WHO) encourages the use of supplementary diets combined with algae and medicinal herbs or plants, which minimize the application of chemicals in fish diet [45].

Spirulina is a filamentous blue-green algae, which has the potential to be used in aquafeed as a growth promoter and immunostimulant [46–50]. Dried *Spirulina* powder has high protein content (up to 55–70% of dry weight). It also contains a high amount of gamma-linolenic acid (GLA), polysaccharides, phycobiliproteins, carotenoids, vitamins (especially B12), pigments such as carotenoids, and minerals. Some studies also reported the immune-stimulating effect of *Spirulina* in several fish species [5,47,51–54], but to the best of our knowledge, no study has been performed using *Ctenopharyngodon idella*, commonly known as grass carp. We hypothesized that grass carp, a herbivorous cyprinid, can show better digestive and physiological status after feeding with algal-based feed. Therefore, this study was performed to evaluate the effect of feeding *Spirulina*-based diets on the growth, physiology, and immunity of grass carp.

2. Materials and Methods

2.1. Fish Culture and Diet Preparation

A three-month feeding trial was carried out at the Animal House fish rearing facility, Department of Zoology, Government College University Lahore Campus. Fish were provided by the Himalaya Fish Hatchery, Muridke. For acclimation purposes, fish were kept in laboratory conditions for two weeks and treated with potassium permanganate to avoid any infections. During this time, fish were fed with a basal (control) diet twice a day at a rate of 3% of body weight. Temperature (20 °C), pH (7.8), electrical conductivity (618 µS/cm), and dissolved oxygen (5.7 mg/L) were measured with the help of digital meters during the experiment.

Feed was prepared by using ingredients bought from the local market. Dried *Spirulina* powder (Naturya Organic Superfoods) was purchased from a local organic store. Dried *Spirulina* was added to the fish feed and four diets were prepared with 0%, 1%, 5%, and 10% *Spirulina* supplementation. The inclusion of *Spirulina* in the diet was selected based on previously published literature [53,54]. Ingredients were ground into a fine powder and mixed together. After mixing, pellets were formed manually using a mincer. Pellets were shade dried for 48 h and were preserved in zipper bags for further use.

The diet samples were homogenized using a pestle and mortar and were analyzed by standard methods of AOAC [55]. Moisture was determined by oven-drying at 105 °C for 12 h. Crude protein ($N \times 6.25$) was estimated by micro Kjeldahl's apparatus. Ash was determined by ignition samples at 650 °C for 12 h (Eyela-TMF 3100) to constant weight.

Crude fat was determined by the petroleum ether extraction method through the Soxtec HT2 1045 system. The carbohydrate content of the feed was measured by subtracting the % values of nutrients (moisture, protein, fat, ash) from 100. The composition and proximate analysis of *Spirulina* powder and feed are given in Table 1.

Table 1. Composition of basal and experimental diet (%).

Ingredients	Control	1% <i>Spirulina</i>	5% <i>Spirulina</i>	10% <i>Spirulina</i>
Fish meal	10	10	10	10
Wheat flour	20	19.5	18	15
Soybean meal	21.5	21	19.5	18.5
Cottonseed meal	6.5	6.5	6	6
Mustard cake	21.5	21.5	21	20
Fish oil	3	3	3	3
Soya oil	4	4	4	4
Mineral premix ^a	3	3	3	3
Vitamin premix ^b	3	3	3	3
<i>Spirulina</i>	0	1	5	10
cellulose	4	4	4	4
Salt	0.5	0.5	0.5	0.5
Molasses	3	3	3	3
Crude protein (%)	28	28	28.5	29
Fat	5.5	5.6	5.5	5.7
Ash	20.37	20.34	20.45	20.39
Moisture	12.89	12.77	12.6	12.79

^a Each 1000 g of mineral premix contains copper (Cu) 0.25 g, magnesium (Mg) 25 g, calcium (Ca) 0.023 g, zinc (Zn) 2.17 g, manganese (Mn) 10 g, potassium (K) 0.5 g, selenium (Se) 0.01 g, sodium (Na) 120 g. ^b Each 1000 g of vitamin premix contains Vit A 0.8 g, Vit D₃ 0.16 g, Vit E 0.38 g, Vit B₁ 1 g, Vit B₂ 1.25 g, Vit B₁₂ 0.001 g, Vit B₃ 6.25 g, Vit B₆ 4 g, Pantothenic acid 54 g, folic acid 5 g.

2.2. Experimental Setup

After the acclimatization of two weeks, fish were anesthetized using clove oil and weighed individually. Fish with almost equal weight (initial weight $4.81 \text{ g} \pm 0.13$) were stocked in a glass aquarium (20 fingerlings/group) filled with 60 L of water. Experiments were performed in triplicates; therefore, 12 aquariums were set with 20 fish in each aquarium. All aquariums were supplied with air stones to maintain dissolved oxygen. The feeding trial was started 24 h post stocking in the aquarium. Fish were fed twice a day (8–9 am and 3–4 pm) at a rate of 3% of body weight. The feeding ration was adjusted fortnightly according to the weight of the fish. The control was fed with a basal diet having 0% *Spirulina* powder, while groups II, III, and IV were fed with a diet containing 1, 5, and 10% *Spirulina*, respectively.

The feeding regime was continued for 90 days. The aquaria were siphoned every other day to remove the uneaten feed particles.

2.3. Analysis of Growth

At the end of 90 days of the feeding trial, fish from each aquarium were anesthetized using clove oil [56], and weight and length were recorded again to calculate growth. Growth parameters were recorded using the following formulas:

$$\text{Weight gain (g)} = W2 - W1 \quad (1)$$

where W2 is the final weight and W1 is the initial weight;

$$\text{Specific growth rate (SGR; \%g/day)} = 100 (\ln W2(g) - \ln W1(g))/T \quad (2)$$

where W2 is the final weight (g), W1 is the initial weight (g), and T is the experimental period (day); Thermal growth coefficient = $100 (\text{final body weight}^{1/3} - \text{initial body weight}^{1/3})/T$

$\text{weight}^{1/3} / \text{experimental days} \times \text{mean daily temperature}$); Condition factor (K) = $100 \times (\text{body weight; g}) / (\text{body length; cm}^3)$; Fish survival (%) = $100 (\text{Number of fish at the end of trial} / \text{number of fish at the start of trial})$.

2.4. Analysis of Mucus Immunity

Mucus samples were collected at the end of the feeding trial for the determination of the innate immune response. To collect mucus samples, individual fish were added to a polythene bag having 10 mL of sterile saline solution. Fish were rubbed gently and all liquid was transferred to 50 mL falcon tubes and centrifuged. The supernatant was collected and used for the analysis of lysozyme, protease, antiprotease, and peroxidase activity as described in our previous work [57]. Briefly, lysozyme activity was measured using *Micrococcus luteus*. Change in turbulence was recorded using a spectrophotometer at 450 nm. Lysozyme activity was expressed as units/mL. For protease activity, mucus samples were incubated with azocasein, ammonium bicarbonate, and trichloroacetic acid. The reaction was stopped using NaOH. Optical density was recorded at 450 nm. Antiprotease activity was measured using trypsin as a substrate and optical density was recorded at 450 nm. For peroxidase activity, mucus samples were incubated with 3,3',5,5'-tetramethylbenzidine (TMB) and Hank's Balanced Salt Solution (without Ca^{2+} or Mg^{2+}). Sulphuric acid was used to stop the reaction and optical density was recorded at 450 nm.

After the collection of mucus samples, fish were dissected and the intestine, liver, and kidneys were harvested and stored in pre-labelled Eppendorf's at -80°C . The intestine was used for the estimation of digestive enzymes; liver tissue was used for antioxidant analysis, while kidney tissue was used for the expression of immune-related genes.

2.5. Oxidative Stress and Antioxidant Defense Markers Assessment

The liver was used for the assessment of antioxidative enzymes. Tissue samples were weighed and homogenized in 0.1M phosphate buffer (pH 7.4) to make 10% homogenate. A portion of the homogenate (500 μL) was used to estimate lipid peroxidation while the remaining homogenate was centrifuged at 13,000 rpm at 4°C for 30 min. The supernatant was collected and stored in clean Eppendorf's and used for the estimation of glutathione, catalase, glutathione-S-transferase, and superoxide dismutase [56,58,59]. Protein content in the supernatant was measured using Bradford reagent.

2.6. Analysis of Digestive Enzymes

The whole intestine of ten fish from each aquarium/replicate ($n = 30$ for each treatment group) was extracted and kept in prelabeled, sterile falcon tubes and weighed. The entire procedure was performed on ice. A double amount of saline solution (0.86%) was added to each sample, homogenized, and centrifuged at a maximum speed of 4°C . The supernatant was removed and stored at -80 until analysis of digestive enzymes [41].

Protease activity was measured according to [60], and the reaction mixture of 0.2 mL of supernatant and 0.2 mL of freshly prepared azocasein was taken in an Eppendorf. The sample was incubated in a water bath for 30 min at 30°C . TCA (1.2 mL) was added and incubated for 30 min at room temperature. The reaction mixture (1.5 mL) was then centrifuged at a maximum speed for 10 min. The supernatant (1 mL) was taken in a cuvette and an equal volume of NaOH was added. Absorbance was recorded at 450 nm.

For amylase activity, 1.8 mL of 0.1M sodium phosphate buffer, 0.1 mL of 1% starch solution (substrate), and 0.1 mL of enzyme source were taken in an Eppendorf. The reaction mixture was incubated for 30 min at 37°C . The reaction was stopped by adding 2 mL of dinitro salicylic acid (DNS). The sample was then heated in a boiling water bath for 5 min and cooled. A volume of 10 mL of water was added to the sample; a brown color appeared. Absorbance was recorded at 540 nm [61].

Lipase activity was measured according to Dar et al. [61]. Briefly, the reaction mixture consists of homogenate (1 mL), phosphate buffer (0.5 mL), and olive oil emulsion (2 mL). The mixture was shaken well and incubated at 37°C for 24 h. The mixture was titrated

using NaOH (0.05N) using phenolphthalein as an indicator. The milliliter equivalent of alkali consumed was taken as a measure of the activity of lipase.

2.7. Expression Analysis of Immune-Related Genes

Head kidney (100 mg) from 5 fish per replicate ($n = 15$ per treatment group) was used to extract RNA with Trizol reagent. Quality and quantity of RNA were checked on nanodrop (Thermo Fisher Scientific). First-strand complementary DNA was synthesized using a commercially available RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific). Resultant cDNA was diluted 10 times and used to study relative gene expression. Primer sequence, accession number, and annealing temperature of genes are listed in Table 2. Primers were validated using conventional PCR. Real-time PCR was used to analyze mRNA levels of selected immune-related genes and housekeeping genes. The reaction mixture (20 μ L) contained syber green master mix (10 μ L), cDNA (1 μ L), and 1 μ L forward and reverse primers. The expression of *tumor necrosis factor- α* , *interleukin-8*, and *interferon- γ* was estimated by the $2^{-\Delta\Delta Ct}$ method [62]. *Actin- β* was used as a reference control. Each sample was analyzed in triplicate.

Table 2. Primer sequence, annealing temperature, product size, and accession number of selected genes.

Genes	Primer Sequence	Annealing Temperature ($^{\circ}$ C)	Product Size	Accession Number
<i>tnf-α</i>	GGTGCATACGACCCTGAAGT TTTGCCTCCATAGGAATCG	60	244	JQ040498.1
<i>il-8</i>	ATGAGTCTTAGAGGTCTGGGTG ACAGTGAGGGCTAGGAGGG	60	118	JN663841
<i>inf-γ</i>	TGCATGTAGGCGGATATCAA GAGGGCGCATAAGTCTGAAG	60	192	FJ695520.1
<i>actin-β</i>	ACCCACACTGTGCCATCTACG ATTTCCTCTCGGCTGTGGTGG	60	146	JQ991014.1

2.8. Statistical Analysis

All the data of growth and biochemical parameters are represented as mean $\pm$ S.E.M. ANOVA followed by Tukey's test was used to report statistical differences among groups. Statistical difference $p < 0.05$ is represented with a single asterisk (*), while double (**) and triple (***) asterisks represent statistical differences of $p < 0.01$ and $p < 0.001$, respectively. All the data were analyzed using GraphPad Prism.

3. Results

3.1. Growth Performance

A significant increase in weight gain was recorded in fish fed with 1 and 5% *Spirulina* powder. However, fish fed with a diet supplemented with 10% *Spirulina* did not differ statistically from the control. Percentage weight gain was highest in the fish fed with a diet supplemented with 5% *Spirulina* powder; the polynomial regression equation is $y = -2.8202x^2 + 32.476x + 54.179$; $R^2 = 0.9259$. The survival rate was 100% in all the treatment groups throughout the study period (Table 3). Polynomial regression analysis of dietary inclusion of *Spirulina* and specific growth rate is $y = -0.0102x^2 + 0.1117x + 0.3354$; $R^2 = 0.8693$ while the polynomial regression equation for feed conversion is $y = -0.0061x^2 + 0.0658x + 0.1781$; $R^2 = 0.8677$.

Table 3. Growth performance of grass carp fed with 0, 1, 5, and 10% *Spirulina* for 90 days.

Parameters	Control	1% <i>Spirulina</i>	5% <i>Spirulina</i>	10% <i>Spirulina</i>
Initial body weight (g)	4.9 $\pm$ 0.125	4.975 $\pm$ 0.182	4.65 $\pm$ 0.175	4.75 $\pm$ 0.153
Final body weight (g)	7.393 $\pm$ 0.4362	10.19 $\pm$ 0.7686 *	11.11 $\pm$ 0.7644 ***	7.948 $\pm$ 0.4511
%-weight gain	43.67 $\pm$ 8.310	92.43 $\pm$ 17.92	117.8 $\pm$ 16.32 **	89.09 $\pm$ 13.07
SGR	0.2874 $\pm$ 0.036	0.5035 $\pm$ 0.056	0.6154 $\pm$ 0.037	0.4401 $\pm$ 0.054
Thermal condition factor	0.1498 $\pm$ 0.020	0.2772 $\pm$ 0.034 *	0.3397 $\pm$ 0.024 ***	0.2261 $\pm$ 0.028
Survival rate (100%)	100	100	100	100

Statistical difference $p < 0.05$ is represented with a single asterisk (*), while double (**) and triple (***) asterisks represent statistical differences of $p < 0.01$ and $p < 0.001$, respectively.

3.2. Mucus Immunity

After 90 days of feeding a *Spirulina*-supplemented diet, lysozyme, and anti-protease activity increased significantly in groups fed with 5 and 10% *Spirulina*. Mucus protease and peroxidase activity increased significantly only in the group fed with 5% *Spirulina* (Table 4).

Table 4. Skin mucus enzyme activity of grass carp fed with 0, 1, 5, 10% *Spirulina* for 90 days.

Parameters	Control	1%- <i>Spirulina</i>	5%- <i>Spirulina</i>	10%- <i>Spirulina</i>
Protease (%)	51.91 ± 6.14	61.86 ± 6.480	80.53 ± 6.57 *	63.17 ± 7.26
Antiprotease (%)	66.78 ± 14.4	120.2 ± 13.84	152.2 ± 15.36 **	130.6 ± 16.77 *
Lysozyme (U/L)	2.056 ± 0.318	4.5 ± 0.606	7.00 ± 1.069 ***	5.33 ± 0.991 *
Peroxidase (U/mL)	0.2611 ± 0.030	0.2951 ± 0.028	0.4390 ± 0.057 *	0.248 ± 0.049

Statistical difference $p < 0.05$ is represented with a single asterisk (*), while double (**) and triple (***) asterisks represent statistical differences of $p < 0.01$ and $p < 0.001$, respectively.

3.3. Digestive Enzyme Activity

The activity of digestive enzymes *viz* protease, lipase, and amylase are shown in Figure 1a–c. A significant increase was reported for intestinal protease activity in fish fed with 5% ($p < 0.001$) and 10% ($p < 0.05$) *Spirulina* powder. Amylase activity increased significantly ($p < 0.001$) in all treatment groups compared to the control. Intestinal lipase activity increased only in fish fed with 1% ($p < 0.05$) and 5% ($p < 0.001$) *Spirulina* powder (Figure 1c).

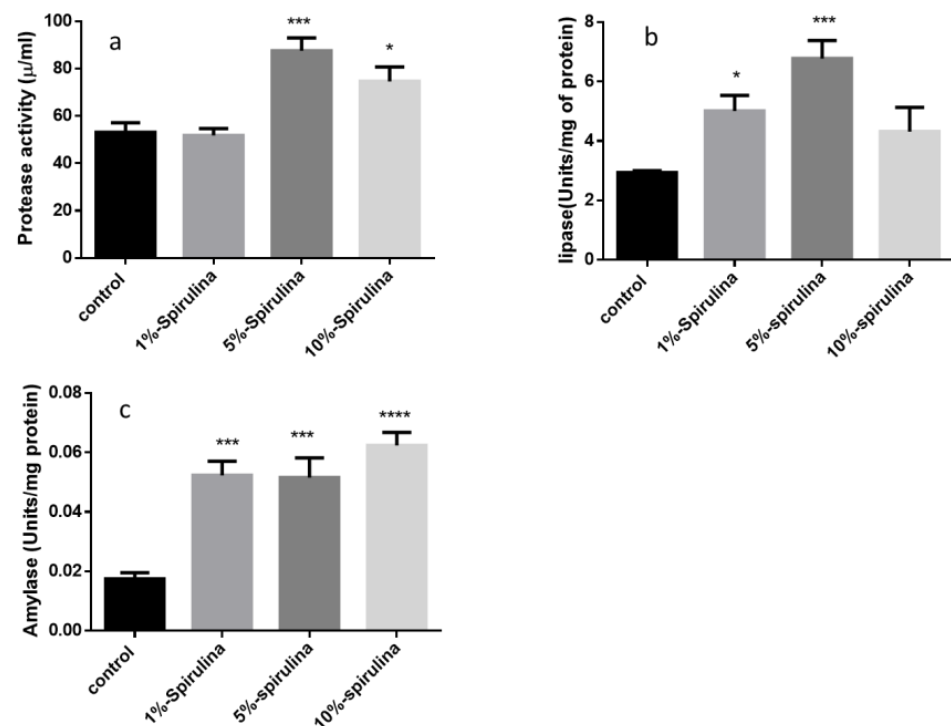


Figure 1. Activity of protease (a), lipase (b) and amylase (c) in the intestine of grass carp fed with various levels of *Spirulina* supplemented diet for 90 days. Statistical difference $p < 0.05$ is represented with a single asterisk (*), while triple (***) asterisks represent statistical difference of $p < 0.001$, (****) represent statistical difference of $p < 0.0001$.

3.4. Hepatic Anti-Oxidants

Hepatic lipid peroxidation decreased significantly in all groups, and a significant decrease was recorded in fish fed with 1 and 5% *Spirulina* powder (Figure 2a). Hepatic superoxide dismutase increased in all groups fed with *Spirulina*, however, a significant increase was only observed in fish fed with 10% *Spirulina* powder (Figure 2b). The activity of reduced glutathione increased significantly in the liver of fish fed with 1 and 10%

Spirulina powder (Figure 2c). A significant increase in glutathione-S-transferase activity was recorded in fish fed with 1 and 5% *Spirulina* powder (Figure 2d). Catalase activity was increased only in groups fed with the lowest level of *Spirulina* (2e) while a non-significant decrease was observed in the liver of fish fed with 5 and 10% *Spirulina* powder for 90 days.

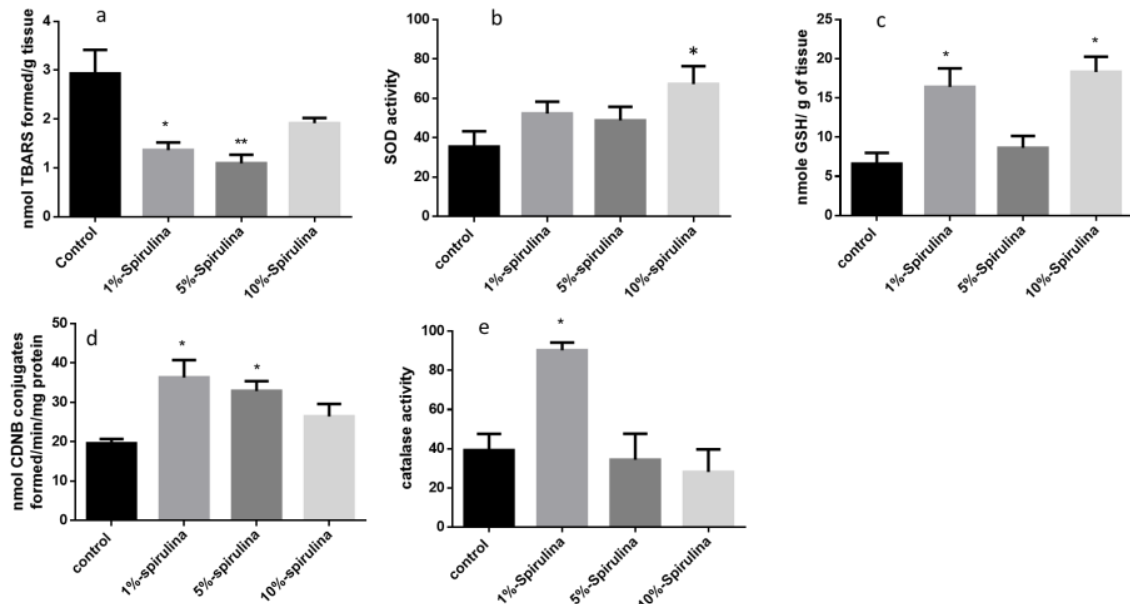


Figure 2. Hepatic antioxidant activity of grass carp fed with 0,1,5 and 10% *Spirulina* for 90 days. Data expressed as mean $\pm$ S.E.M *—represent significant difference $p < 0.05$. **—represent statistical differences of $p < 0.01$. (a) Lipid peroxidation is expressed as nmol of thiobarbituric acid reactive substance (TBARS) formed. (b) Superoxide dismutase activity. (c) Reduced glutathione level. (d) Glutathione-S-transferase activity is expressed as nmol of 1-chloro-2,4-dinitrobenzene (CDNB) conjugates formed. (e) Catalase activity.

3.5. Gene Expression

Expression of immune-related genes was recorded in kidney tissue of grass carp after 90 days of feeding with different levels of *Spirulina*. The mRNA expression of *tumor necrosis factor- α* increased significantly in the group fed with 1 and 10% *Spirulina* powder (Figure 3a). A decrease in the mRNA level of *interleukin-8* was recorded in the head kidney of fish fed with 1% *Spirulina*, while no significant difference was observed in groups fed with 5% *Spirulina* powder, however, the inclusion of 10% *Spirulina* resulted in a significant increase in mRNA expression of *il-8* (Figure 3b). Expression of *interferon- γ* increased significantly in the head kidney of fish fed with 1 and 5% *Spirulina* powder (Figure 3c).

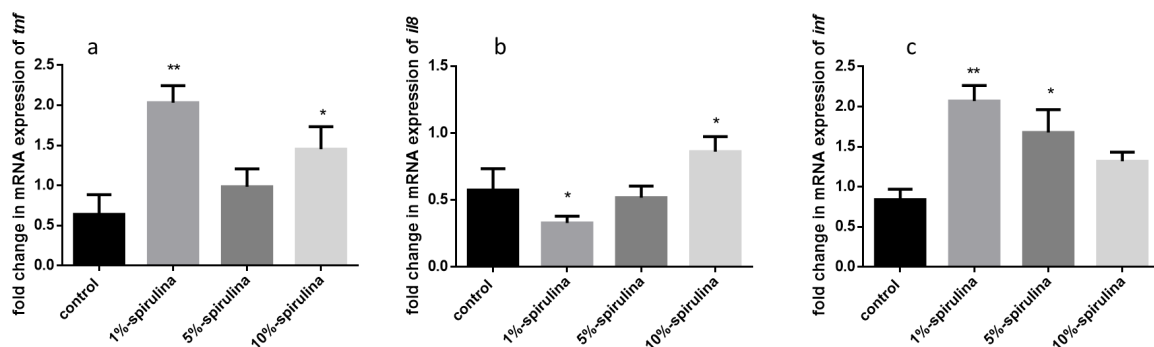


Figure 3. Relative mRNA expression of *tnfr- α* (a), *il-8* (b) and *ifn- γ* (c) in head kidney of grass carp fed with 0, 1, 5 and 10% *Spirulina* for 90 days. Values are presented as mean $\pm$ S.E.M. * = $p < 0.05$; ** = $p < 0.01$.

4. Discussion

Many algal species, plants, and plant extracts (aqueous, methanolic, ethanolic) are now being successfully used in the aquaculture industry and had a positive effect on fish growth, antioxidant status, immunity, and resistance against water-borne pathogens [26,27,57,63–68].

Evaluation of growth performance is one of the important parameters to assess the efficacy of feed additives [36]. In the present work, weight gain, %WG, SGR, and TCR was highest in fish fed with a 1 and 5% *S. platensis* supplement diet for 90 days. A similar growth-promoting effect was observed in other fish species [49,53,62] fed with *Spirulina*-supplemented diets (2.5–10%). Food components are also assessed by their ability to be digested and absorbed in the gut, thus promoting growth [69]. The degradation of food in the digestive tract is performed with the assistance of enzymes. Assessment of intestinal enzymes (proteases, lipases, amylases, trypsin, and chymotrypsins) provides information about the physiological status of the gut [70]. The intestinal enzyme activity of grass carp was enhanced after the inclusion of *Spirulina* in the diet. Previous studies also reported that the inclusion of *Spirulina* in the diet of aquatic organisms significantly enhanced the digestion and absorption process [47]. An increase in digestive enzymes leads to better nutrient absorption that can explain the growth-promoting effect of *Spirulina* [47,71] as observed in the present study.

Unfavorable water quality parameters, high stocking density, and other environmental factors can induce oxidative stress in fish. Several antioxidant compounds (glutathione) and enzymes (catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, etc.) make up the body's anti-oxidant defense system that detoxifies reactive oxygen and nitrogen species through a series of reaction cascades. Recently, a great deal of research focused on supplementing the fish diet with additives that can enhance the natural anti-oxidant level and alleviate oxidative stress. In the present study, fish were not challenged with any stress but it is generally accepted that an enhanced and better anti-oxidant system will provide better resistance against oxidative stress. In the present study, the level of lipid peroxidation decreased in the fish fed with 1 and 5% *Spirulina* diets. The activity of catalase, glutathione-S-transferase, and glutathione levels increased significantly ($p < 0.05$) in fish livers fed with 1% *Spirulina*-supplemented diets. *Spirulina* is a rich source of bioactive compounds such as catechins, phycobiliproteins, allophycocyanin, and phycocyanins [72]. Catechins have the ability to chelate metal ions, scavenge reactive oxygen species, and produce antioxidant enzymes [73]. Similarly, phycocyanin and allophycocyanin have the properties of antioxidants [74]. The presence of these bioactive components in *Spirulina* may be responsible for the improved antioxidant status of grass carp.

The innate immune response provides protection against pathogens. Fish skin mucus is the first line of defense against pathogens. Feeding fish with a diet containing various additives that can enhance the immunity of a mucosal surface [1,5,24,27,33,67,71] has become an active area of research in the last decade. Innate immune enzymes and molecules present in fish skin mucus are lysozymes, esterases, proteases, antiproteases, anti-microbial peptides, and complement proteins [75]. These mucosal enzymes and molecules have strong anti-microbial activity against both Gram-positive and -negative bacteria, thus, improved status of these molecules in mucus may protect the fish against water-borne pathogens.

The grass carp fingerlings fed with 5% *Spirulina* diets had significantly increased mucosal lysozyme, protease, antiprotease, and peroxidase activities. A similar increase in skin mucosal innate immune biomarkers was reported in other fish species when fed with diets containing *Spirulina* and our results are consistent with previous studies on other fish species [47,52,76,77]. Many plants and algae are rich in antimicrobial peptides, essential oils, polysaccharides, saponins, and phenolic compounds that are effective against infections. These secondary metabolites modulate the active sites of enzymes and also modulate the receptor sites hence, enhancing immunity [78].

Our gene expression analysis revealed that fish fed with a diet containing 1 and 5% *Spirulina* powder had higher expression of *tnf- α* and *inf- γ* . Tumor necrosis factor- α is a pro-inflammatory cytokine and is used as a biomarker of innate immune status in fish.

Tnf- α actively recruits lymphocytes to fight infection and stimulate the cellular and humoral immune response [79]. Similar up-regulation of *tnf- α* was reported in Nile tilapia, common carp, and rainbow trout when fed with diets supplemented with *Spirulina* [5,47,51,79,80]. Mast cells are considered an important source for the synthesis and release of cytokines, such as *tnf- α* [81]. Active compounds such as C-phycocyanin present in *Spirulina* [82] can influence mast cells which may be responsible for increased expression of *tnf- α* as observed in the present study. Tumor necrosis factor- α along with interferon- γ and interleukin-8 activate natural killer cells, macrophages, and cytotoxic-T cells and also augment phagocytosis that leads to the inactivation of viruses and eradication of pathogens [23]. An in vitro study revealed that *Spirulina* induced the secretion of interferon-gamma in the peripheral blood mononuclear cells [83]. Polysaccharides found in many microalgae can increase the expression of pro-inflammatory cytokines, thus acting as immunostimulating agents in aquaculture [84].

In conclusion, the results of the study displayed that the inclusion of *Spirulina* (up to 5%) is effective for improvement in growth performance, antioxidant, digestive enzymes, and innate immune biomarkers in grass carp.

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