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Hematite-biochemical and immune response of Caspian brown trout (*Salmo troutta caspius*, Kessler, 1877) juveniles fed different levels of spirulina (*Spirulina platensis*)

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Abstract

The effects of dietary *Spirulina platensis* on haemato-biochemical and immunity responses of Caspian brown trout (*Salmo troutta caspius*) juveniles was investigated. For this purpose, diets with five *S. platensis* inclusion levels (control, 2%FMR (13.2 g kg^{-1} spirulina in diet), 4%FMR (26.4 g kg^{-1} spirulina in diet), 6%FMR (39.6 g kg^{-1} spirulina in diet), and 8%FMR (52.8 g kg^{-1} spirulina in diet) were prepared. Six hundred juveniles with an average initial weight of 11±1.0 g were assigned to 15 experimental tanks. The experiment lasted for 10 weeks. At the end of the experiment, growth performance, haemato-biochemical parameters including white and red blood cell counts, neutrophils lymphocytes counts, hematocrit, hemoglobin, glucose, albumin, total protein, aspartate amino transferase (AST), alanine amino transferase (ALT), triglyceride, cholesterol, as well as immunity parameters including lysozyme, C3, C4, Immunoglobulin (IgM), ACH50 and respiratory burst activity were assessed. The results indicated that fish fed diets supplemented with 6%FMR and 8%FMR had a significantly higher weight gain (26.13 g and 25.88 g) and specific growth rate (1.74 %bw day^{-1} and 1.71 %bw day^{-1}) compared with control. Furthermore, 6%FMR and 8%FMR treatments had statistically higher protein efficiency (0.76 and 0.78), lipid efficiency (1.89 and 1.94) and statistically lower feed conversion ratio (2.91 and 2.84) compared to the other treatments respectively (p<0.05). The physiological and immunological factors were improved when fish were fed a high level of *S. platensis* supplement. *S. platensis* inclusion also increased activity of Lysozyme C3, C4, IgM and ACH50 and respiratory burst activity and reduced AST and ALT formation. These results indicate that *S. platensis* supplement is promising for disease prevention in *S. trutta caspius* juveniles, at an optimum dietary level of 6% in diet.

Keywords: Spirulina, *Salmo trutta caspius*, Growth performances, Haemato-biochemial parameters, Immunity.

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Introduction

Growth performance and disease control are the most important priorities in current aquaculture. Nowadays, use of antibiotics and chemotherapeutics has increased significantly, due to intensification of culture practices and followed by high prevalence of infectious diseases (Andrews *et al.*, 2011). Accumulation of antibiotic residues in fish tissues and environment causes human and animal health issues and led to an urgent need for alternative disease preventive substances and use of immune-stimulants in aquaculture (Andrews *et al.*, 2011; Krishnaveni *et al.*, 2013). Several types of stimulants with different mechanisms and functions such as bacterial products, complex carbohydrates, vaccines, immune enhancing drugs, nutritional factors, animal and plant extracts are considered, and their abilities to activate the innate immunity have been studied (Raa, 1996; Sakai, 1999; Shahbazi and Bolhassani, 2016).One supplement source, with ease of production, processing and distribution along with a wide range of macro- and micronutrients of health benefits is spirulina (Ravi *et al.*, 2010). Research has shown that this alga contains a wide variety of compounds such as phycobiliproteins, carotenoids, phycocyanin, polysaccharides, unsaturated fatty acids, superoxide dismutase, different vitamins, and other elements which by bring improvement in body coloration, growth and immunity of fish (Nakagawa *et al.*, 2007). Spirulina (*Spirulina platensis*), a blue-green filamentous, fresh water and multi-cell microalgae is gaining more attention from medical scientists as a nutraceutical and source of potential pharmaceuticals. There are several new peer reviewed scientific studies about spirulina's ability to inhibit viral replication, strengthen and enhancing both the cellular and humoral arms of the immune system (Kozlenko *et al.*, 1998; Hirahashi *et al.*, 2002; Andrews *et al.*, 2011). It has been received the most research and public health attention due to its bioactive compounds including vitamins, essential amino acids, minerals, essential fatty acids (gamma linolenic acid), and antioxidant pigments such as phycocyanine (Belay *et al.*, 1996; Regunathan and Wesley, 2006; Ragap *et al.*, 2012); Ceballos *et al.*, 2006). Additionally, spirulina has high protein content (60–70% by dry weight) and is the richest natural source of vitamin B_{12} (Estrada *et al.*, 2001). This alga contains a whole spectrum of natural mixed carotene and xanthophylls phytopigments (Belay *et al.*, 1996; Estrada *et al.*, 2001; Andrews *et al.*, 2011). It has been confirmed that the addition of small amounts of algae to fish feed can exert pronounced effects on growth, lipid metabolism, body composition and physiological response to stress and disease (Mustafa *et al.*, 1994; Mustafa and Nakagava, 1995; Palmegiano *et al.*, 2008; Abdel-Tawwab and Ahmad, 2009; Teimouri *et al.*, 2013). Spirulina as an immune modulator not only stimulates the immune system, but also strengthens the body's ability to produce new blood cells (Andrews *et al.*, 2011). Important

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parts of the immune system, such as bone marrow stem cells, macrophages cells (number of cells and phagocytosis), T cells (lymphocyte), NK (non-specific cytotoxic), spleen as well as thymus show tangible activities when treated with spirulina extract (Henrikson, 1998; Watanuki *et al.*, 2006; Tongsiri *et al.*, 2010; Yong-Chin *et al.*, 2010; Shahbazi and Bolhassani, 2016). Recently, spirulina has been speculated to be associated with modulation of the host immune system (Hironobu *et al.*, 2006).

Caspian brown trout (*Salmo trutta caspius*, Kessler 1877), is one of the world's nine subspecies of brown trout (Quillet *et al.*, 1992; Habibi *et al.*, 2013) and attains the greatest size and growth rate of all brown trout (Sedgwick, 1995; Rajabi *et al.*, 2016). This species is a critically endangered anadromous species distributed in southern region of the Caspian Sea. In 1999, this species was declared at risk according to IUCN conditions (Kiabi *et al.*, 1999; Coad, 2000; Kalbassi *et al.*, 2006). Artificial propagation and releasing fingerlings to the natural waters is an approach to prevent brown trout's extinction (Rajabi *et al.*, 2016). In addition, in the recent years, this species has attracted interest for aquaculture in cage and raceways in intensive culture systems (Kalbassi *et al.*, 2006). *S. trutta caspius*, like many other species is sensitive to stressors and pathogenic agents. Thus, strong defense mechanisms or immune system are needed against pathogens to improve the health of the fish. Despite the importance of Caspian salmon as an endangered species, however, little is known about their nutritional requirements especially in the field of increasing immunity of species and types of immune-stimulants. Therefore, the main objective of this study was to evaluate the effects of S. *platensis* meal as feed additive on immune system, biochemistry and haematology in *S. trutta caspius* as a valuable species.

Materials and methods

Experimental diets

Five artificial diets were formulated using five levels of microalgae *S. platensis* (Sina microalgae Co., Qeshm, Iran) and in three replicates were examined in a randomized design. The experimental diets were formulated by partially replacing fishmeal in the basal diet (65.89% in control) with spirulina powder at inclusion levels of 0, 2%, 4%, 6% and 8%, respectively. Diet formulations were performed using Lindo software (Lindo copyright, release, 6.11998), and fishmeal and *S. platensis* powder were used as a protein source. Test diets provided, which were iso-nitrogenous (45-46% crude protein) and iso-caloric (20 MJ kg^{-1}) were analyzed in this regard, only varied in terms of fish meal and *S. platensis* content. The proximate composition of experimental diets was measured according to the standard methods of Association of Official Analytical Chemists (AOAC, 1995) for moisture, protein, fat, and ash determination. Briefly, Moisture content was estimated by drying the samples to constant weight at 95 $^{\circ}$C in a drying oven (GCA, model 18EM, Precision Scientific

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Group, Chicago, IL, USA). N content was measured using the Labconco Micro Kjeldahl Apparatus (Labconco Corporation, Kansas, MO, USA) and crude protein was estimated by multiplying N content by 6.25.

Lipid content was determined by acetone extraction using the extraction Soxhlet apparatus (Lab-Line Instruments, Inc., Melrose Park, IL, USA) for 16 hours, and ash was determined by combusting dry samples in a muffle furnace (Thermolyne Corporation, Dubuque, IA, USA) at 550 °C for 6 hours. Carbohydrate (i.e., nitrogen-free extract plus fiber) was calculated by the difference: (100-(protein+fat+ash+moisture)) (Aksnes and Opstvedt, 1998).

Gross energy was calculated using the coefficient of 23.6, 39.5, and 17.2 (kJ g^{-1}) for protein, fat, and carbohydrates, respectively (NRC, 1993). The experimental diet formulation and proximate composition are showed in Table1, respectively. Dietary feed ingredients were ground using a laboratory grinder (Philips HR7628, Finland) and then blended into a homogenous doughy matter by adding water and transferred to a meat grinder with a 2 mm-mesh (CGT Company, 2mec, Rome, Italy). After drying, diets were broken by hand to fit fish mouths and placed in a grinding machine for breakdown into small pieces. The resulting diets were then dried in food dryer and stored in plastic bags in a refrigerator at -2 °C until further use.

Table 1: Dietary formulation and proximate composition of experimental diets.

Ingredient (g kg^{-1} dry weight)	Experimental Diets				
	0%	**2%**	**4%**	**6%**	8%
Fish meal [1]	658.9	645.7	632.4	619.4	606.2
Spirulina[2]	0	13.2	26.5	39.5	52.7
Wheat flour	115.9	117.8	119.4	120.9	122.4
Fish oil[3]	45	45	45	45	45
Soybean oil[4]	50.3	51.3	52.3	53.3	54.5
Lethitin[5]	5	5	5	5	5
Filler(sand)	60	57.1	54.6	52.1	49
Mineral mixture[6]	25	25	25	25	25
Vitamin mixture[7]	15	15	15	15	15
Anti-oxidant[8]	0.2	0.2	0.2	0.2	0.2
Choline Chloride[9]	1	1	1	1	1
Mono calcium phosphate[10]	5	5	5	5	5
Anti fungal[11]	2.5	2.5	2.5	2.5	2.5
Lysine[12]	5	5	5	5	5
Methionine[13]	5	5	5	5	5
Vitamin C[14]	1	1	1	1	1
Chemical analysis (%)					
protein	45.1	45.2	44.89	45.07	45.12
lipid	17.96	17.75	18.1	18.2	18.1
moisture	7.9	8.02	8.00	7.9	8.1
ash	8.9	8.8	8.7	8.9	9.1
carbohydrate	12.14	11.93	12.21	11.73	11.58
Gross Energy(MJ kg^{-1})	19.83	19.73	19.84	19.84	19.8

[1] Herring meal, produced by Mirood, Mazandaran province, Iran.
[2] Produced in Sina micro algae, Qeshm. Hormozgan province, Iran.
[3] Produced by Mirood, Mazandaran province, Iran.
[4, 5] Prepared of Mazandaran aquatic food factory, Sari, Mazandaran province, Iran.
[6] Mineral premix consisted of (mg kg^{-1} premix): Man=2600mg, Cu=600mg, Fe=6000mg, Zn=4600mg, Se=100 mg, I=100 mg, Co=50 mg and carrier up to 1 kg.
[7] Vitamin premix consisted of (mg kg^{-1} premix) : A=1200000 IU, D3=400000 IU, E= 3000IU, K3=1200mg, C= 5400mg, B1=200mg, H2=200 mg, B2=3360mg, B12=4mg, B9=600mg, B5=9000mg, B7=7200mg
[8] Preparation of the Mazandaran aquatic food factory and its combination including BHT,Etoxyquin ,Synergist Propylgallate.
[9] Choline Chloride, 60%, Prepared of livestock and poultry pharmaceutical factory Aras Bazar.
[10, 11, 12, 13] Prepared of pharmaceutical factory Aras Taban, Amol, Mazandaran province, Iran.
[14] Prepared of pharmaceutical factory Aras Bazar, Amol, Mazandaran province, Iran.
[15] Aqutic vitamin C, production of pharmaceutical factory Aras Bazar, Amol, Mazandaran province, Iran.

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Fish rearing

Fish juveniles (n= 600; mean individual initial weight 11±1.0 g) were obtained from Cold Water Breeding and Restocking Hatchery, Shahid Bahonar Center in Kelardasht, Mazandaran, Iran, and then were transferred to NIAC multi-purpose co-operative farm fish in Baghbankola village, Amol, Mazandaran, Iran. Fish were allowed to acclimatize for two weeks in indoor fiberglass tanks prior to the experiment and during this period were fed a commercial diet twice daily. After the acclimation period, fish were randomly divided into five equal groups, each comprising three replicates (40 fish per replicate) in 15 separate 400 L fiberglass tanks. The fish were hand-fed three times daily (8:00, 12:00 and 16:00 h) to apparent satiation by visual observation for ten weeks (Sotoudeh *et al.,* 2015) Water temperature, dissolved oxygen and pH were monitored daily (during the experiment, temperature, dissolved oxygen and pH were 15±2 °C, 8.3±1 mg L^{-1} and 7.6±0.3 respectively). Uneaten feed and fecal matter were siphoned off every day. Due to the sensitivity of these juveniles, and to prevent mortality, biometry was performed once at the beginning and once at the end of this period.

Growth parameters

Growth performance was determined and feed utilization was calculated as following (Goytortua-Bores *et al.,* 2006):

Weight gain (WG %)=100×(final body weight–initial body weight)/initial body weight.

Specific growth rate (SGR (%))=100 (ln W2–ln W1)/T; where W1 and W2 are the initial and final weight, respectively, and T is the number of days in the feeding period;

Feed conversion ratio (FCR) = feed consumed (g dry weight)/weight gain (g);

Condition Factor (CF) = Weight/Total length ^{3}×100;

Survival rate (%) = (Number of fish at the beginning/Number of fish at the end) ×100.

Sampling

At the end of our experiments, to evaluate haemotological parameters, fish were fasted for 24 hours immediately prior to blood sampling and then six fish from each tank were sampled randomly. To prevent sampling stress, the fish were anesthetized with a stock solution (50 ppm) of clove oil and blood samples were collected quickly (approximately one min/fish) by puncturing the veins in the caudal peduncle with a sterile 5 ML syringe (Esmaeili *et al.,* 2017). The collected blood sample was divided into two portions. One portion was transferred into Eppendorf tubes containing heparin anti-coagulant (500 U L^{-1}) for haematological tests and the second portion of blood sample was also transferred into Eppendorf tubes, left to clot at 4 °C and centrifuged at 5000 rpm for 5 min at room temperature for biochemical and Immunological tests.

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Haemotology tests

Red blood cells (RBC) and white blood cell (WBC) counts were determined using Neubaeur haemocytometer following the methods of Blaxhall and Daisley (1973). Blood was diluted to 1:200 with Race solution. Five center cells of middle square in Neubauer chamber were used for counting RBC and multiplied by 10,000 (Esmaeili *et al.*, 2017). For counting WBC, four marginal squares in Neubauer chamber were used after blood dilution (1:50) with Race solution and the results were multiplied by 50. Then, blood samples were fixed in methanol and stained using Wright–Giemsa stain for determination of the differential WBC (lymphocyte and neutrophil portions of total WBC) count (Houston, 1990). At least 200 WBCs were counted for differential WBC determinations. Thin slices were used for counting WBC counting was done according to their shape and reported as percentages. To measure the Hemoglobin (Hb) levels were obtained by the Cyanmethemoglibin method (Houston, 1990). In this method, the RBC are haemolized and hemoglobin is converted to Cyanmethemoglobin. Twenty microliters of blood samples with 50-µl Drabkin's reagent were mixed and placed in dark place for10 min to form Cyanemethemoglobin. Then, absorbance was read at 540 nm in a UV with spectrophotometer (Jenway 6800, UK) and finally, hemoglobin value was calculated. Haemotocrit (Ht) was measured using the standard micro-haemotocrit method (Subhadra *et al.*, 2006) and reported as percentages. Microhematocrit capillary tubes were centrifuged at 2500 rpm for 5 min, and then hematocrit value was calculated using graded plate. To calculate others haematological indices, MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), the relevant formula (Bain *et al.*, 2011) was used as follows:

MCV (fl)=hematocrit/RBC(million per mm^3)×10

MCH (pg)=hemoglobin/RBC(million per mm^3) × 10

MCHC=hematocrit/hemoglobin×100

Blood biochemical parameters

Total protein (modi-biuret method, Tietz, 1986) and Albumin (romocresol green binding method, Doumas and Peters, 1997) assays were performed with Zist Chemistry kits (Zist chemistry Company, Tehran, Iran).Total protein content (at wavelength of 560-520 nm) and Albumin (at wavelength of 630 nm) were determined using an auto analyzer system (Thecnicon, RA 1000, NewYork, NY, USA). Globulin levels of the samples were calculated from the difference in albumin from total protein (Kumar *et al.*, 2005). Total cholesterol, triglyceride and glucose measurements were performed using Pars Azmoon commercial kits (Pars Azmoon Company, Tehran, Iran) according to the company's protocol and using an auto-analyzer (Thecnicon, RA 1000, New York, NY, USA). Briefly, in this method, 10 µl of plasma sample had mixed with 1000 µl of reagent and incubated for 20 min in room temperature. Then, the absorbance of

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the sample against the blank (10 µl of distilled water mixed with 1000 µl of reagents) had measured at a wavelength of 546 nm. Similar approach had done for other parameters, but with appropriate sample amount and wavelength according to each parameter's protocol (Esmaeili *et al.*, 2017). The Kinetic method was used to measure liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), using Pars Azmoon commercial clinical investigation kits (Parsazmon Company, Tehran, Iran) and were analyzed with an auto analyzer (Technicon RA-1000; Technicon Instruments, New York, NY, USA).

Immunological parameters

The lysozyme activity in serum was determined turbidometrically using the method described by Ellis (1999). In this method, *Micrococcus luteus* bacteria was used (Sigma, M0508) with phosphate buffer (30 ml, 0.1 M, pH=7) as substrate. For this purpose, activated bacteria were used and cultivated at a concentration in 0.6–0.8 nm adjusted with spectrophotometer (Jenway 6800, UK). Then, 1990 µl of substrate and 10 µl of the sample were added to cuvette. The decrease of absorption at 450 nm each min for a period of 6 min had measured through spectrophotometer (Tukmechi *et al.*, 2011) with chicken egg white (Sigma, A5503) standard. Turbidometric method and Pars Azmoon commercial clinical investigation kits (Pars Azmoon Company, Tehran, Iran) were used to measure C3 and C4 serum complement system (Tang *et al.*, 2008). The serum C3 and C4 reacted with the antibodies contained in the kit, and then OD had measured at a wavelength of 340 nm using an auto-analyzer (Thecnicon, RA 1000, New York, NY, USA), compared to the standard in kits and finally, the C3 and C4 amount was calculated based on mg L^{-1}. ACH50 (the activity of the complement system) was determined according to the indirect ELISA method using a commercial kit (Wielsa, comple 300 total complement functional screen kit, Sweden). The volume of the serum complement producing 50% hemolysis (ACH50) was determined, and the number of ACH50 units in ml was calculated for each experimental fish.

Total immunoglobulin levels were determined according to the method described by Siwicki and Anderson (1993). In this method, analysis of total Ig level in plasma is based on the biuret colorimetric method. First the immunoglobulins were separated from plasma by precipitation with polyethylenic glycol 10,000 (Sigma) and remaining supernatant was read. That number was subtracted from the total protein to give total immunoglobulin. Protein readings from supernatant gave the amount of protein taken out by absorption to polyethylene glycol. To calculate total immunoglobulin, subtracted these readings from total protein on individual samples and compared with standards for calculation of protein.

The production of oxygen radicals by leukocytes was determined by the reduction of Nitro Blue Tetrazolium

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(NBT, Sigma-Aldrich Chemical, St. Louis, MO, USA) according to Rook *et al.* (1985). Absorbance was converted to NBT units based on a standard curve of NBT diformazan per milliliter of blood.

Statistical analysis

All the data have been analyzed using the SPSS (version 17, SPSS, Richmond, VA, USA) statistical package as described by Dytham (1999). Mean values of all the parameters were subjected to one-way analysis of variance (ANOVA) to study the treatment effect and comparison of any mean values had done by Duncan's Multiple Range Test (DMRT). Comparisons were made at the five percent probability level (p<0.05).

Result

The growth performance of Caspian brown trout juvenile fed diets containing different levels of spirulina are presented in Table 2. Overall, the optimum growth performance has been obtained at 6%FMR and 8%FMR treatments, whereas the fish fed control diet had the lowest growth performances. According to the results, fish fed diet supplemented with 6%FMR and 8%FMR had a significantly higher weight gain (26.13 g and 25.88 g) and specific growth rate (1.74 %bw day^{-1} and 1.71 %bw day^{-1}) compared with those fed the control diet (18.18 g and 1.37% bw day^{-1}). Furthermore, 6 %FMR and 8 %FMR treatments had statistically higher protein efficiency (0.76 and 0.78), lipid efficiency (1.89 and 1.94) and statistically lower feed conversion ratio (2.91 and 2.84) compared with other treatments (p<0.05). Moreover, there was no significant difference in the condition factor among experimental treatments. In addition, fish fed control, 2 %FMR and 4 %FMR diets exhibited equivalent FCR, protein efficiency and lipid efficiency. Survival rate in all treatments was 100%.

Table 2: Mean growth performance of *Salmo trutta caspius* fed different levels of *Spirulina platensis.*

Growth performance	Experimental Diets				
	0% control	2%	4%	6%	8%
W_1 (g)	11.18±0.23	11.91±1.23	11.16±0.56	10.91±1.61	11.19±0.33
WG (%)	62.73±12.3^{a*}	93.93±11.31^{ab}	101.41±14.29^{ab}	163.35±67.22^{c}	131.96±35.27^{bc}
SGR (% / day)	0.81±0.13^{a}	1.1±0.1^{ab}	1.16±0.12^{ab}	1.58±0.16^{c}	1.39±0.26bc
CF	1.33±0.05^{a}	1.28±0.03^{a}	1.38±0.06^{a}	1.27±0.04^{a}	1.31±0.14^{a}
FCR	3.93±0.74^{b}	3.95±0.49^{b}	2.73±0.06^{a}	2.11±0.49^{a}	2.04±0.56^{a}
Survival rate (100%)	100	100	100	100	100

W1: Primary weight
WG%= Weight gain
FCR: Feed conservation ratio
SGR: Specific growth ratio
Conditional Factor(CF)
*Values are the least square means±standard errors of the means of triplicate samples. *P* values determined with one-way ANOVA are also provided for the main factors and their interactions. Means marked with the same letter are not significantly different (p>0.05).Letters a, b, c, d, and e indicate significant differences in treatments, according to Duncan's test.

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The mean haemotology parameters of *S. trutta caspius* juveniles fed with diets containing different levels of *S. platensis* are shown in Table 3. Based on obtained results, spirulina had a significant impact on haemotological parameters (RBC, WBC, Hct, Hb, lymphocytes, neutrophils) in *S. trutta caspius* juveniles. RBC increased with increasing levels of dietary *S. platensis* to 6%, but in 8%, the rate decreased (p<0.05). WBC, Hct, Hb, differential white blood cell percent, lymphocyte and neutrophil increased with increasing levels of dietary *S. platensis*. So, the lowest and the highest levels for these parameters (with exception of neutrophils) were observed in the control and 8% treatments, respectively. Other haematological indices such as average volume of red blood cells (MCV), mean hemoglobin in red blood cells (MCH), average concentration of hemoglobin in a red blood cells (MCHC) also have increased with increasing levels of dietary *S. platensis* (p<0.05).

Table 3: Haemotological parameters of *Salmo trutta caspius* fed with different levels of *Spirulina platensis*.

Haemotological parameters	Experimental Diets				
	0% control	2%	4%	6%	8%
RBC ($\times 10^6$ n mm^{-3})	0.77±0.17 [a*]	1.22±0.05 [c]	1.25±0.16 [cd]	1.27±0.09 [d]	1.12±0.15 [b]
WBC (cell ml^{-1})	9316±3521.6 [a]	10100±1649.24 [b]	11800±2415.7 [c]	13500±2485.4 [d]	15400±2256.5 [e]
Hct (%)	24.66±1.98 [a]	35.83±1.94 [b]	37.17±1.83 [c]	37.66±0.81 [c]	37.83±1.26 [c]
Hb (g dl^{-1})	4.32±0.61 [a]	7.95±0.36 [bc]	7.59±0.75 [b]	8.16±0.31 [c]	8.9±0.51 [d]
LYM (% of WBC)	85.5±3.39 [a]	89.5±1.76 [b]	92.66±7.28 [c]	98.16±1.16 [d]	98.66±0.81 [d]
Neut (% of WBC)	1.16±0.75 [a]	1.16±0.75 [a]	1.33±0.81 [a]	1.67±0.81 [c]	1.5±0.83[c]
MCV(fl)	280.81±6.96 [a]	298.76±25.03 [ab]	300.98±26.45 [b]	332.15±17.8 [c]	337.21±12.2 [c]
MCH(pg)	52.37±1.3 [a]	56.1±1.72 [a]	61.23±4.17 [a]	64.57±2.3 [ab]	80.13±6.7 [b]
MCHC (%)	17.63±1.77 [a]	19.98±0.71 [b]	20.4±1.18 [b]	21.68±1.04 [b]	23.78±2.13 [c]

*Values are least square means±standard errors of the means of triplicate samples. p values determined with two-way ANOVA tests are also provided for the main factors and their interactions. Means marked with the same letter are not significantly different (p>0.05). Letters a, b, c, d, and e indicate significant differences in treatments, according to Duncan's test.

The mean blood biochemical parameters of *S. trutta caspius* juveniles fed with diets containing different levels of *S. platensis* are summarized in Table 4&5. Based on obtained results, with increasing levels of dietary *S. platensis* up to 6%, ALT and AST enzymes decreased (p<0.05). Total protein and globulin decreased and then increased in 8% treatment (p<0.05). Other blood biochemical parameters such as glucose, cholesterol and triglycerides have significantly decreased with increasing levels of dietary *S. platensis* (p<0.05).

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Table 4: Liver enzymes of *Salmo trutta caspius* fed different levels of *Spirulina platensis.*

Liver enzymes	Experimental Diets				
	0% control	2%	4%	6%	8%
ALT(U L^{-1})	323±45.2^{d*}	305.4±27.1^{bc}	290±20.1^{b}	214.2±28.7^{a}	212.3±16.2^{a}
AST(U L^{-1})	25.4±4.5^{d}	23.7±3.8^{cd}	22.9±6.6^{bc}	21.3±4.3^{b}	12.2±2.5^{a}

*Values are least square means ± standard errors of the means of triplicate samples. *p* values determined with two-way ANOVA tests are also provided for the main factors and their interactions. Means marked with the same letter are not significantly different (p>0.05). Letters a, b, c, d, and e indicate significant differences in treatments, according to Duncan's test.

Table 5: Blood biochemical parameters of *Salmo trutta caspius* fed different levels of *Spirulina platensis.*

Biochemical factors	Experimental Diets				
	0% control	2%	4%	6%	8%
Total protein (g dl^{-1})	2.6±0.4^{a*}	2.6±0.4^{a}	2.8±0.7^{b}	3.1±0.3^{c}	4.3±0.5^{d}
Albumin (g dl^{-1})	1.8±0.3^{a}	1.8±0.3^{a}	1.7±0.1^{a}	2±0.3^{a}	2±0.3^{a}
Globulin (g dl^{-1})	0.8±0.1^{a}	0.8±0.1^{a}	1.1±0.2^{b}	1.1±0.3^{b}	2.3±0.3^{c}
Glucose (mg dl^{-1})	172.5±16.3^{e}	176±7.8^{d}	121.5±12.3^{b}	133.1±15.9^{c}	94.5±8.2^{a}
Cholesterol (mg dl^{-1})	438.6±45.1^{d}	412.5±47.3^{c}	401.4±26.2^{b}	346.8±42.2^{a}	394.8±21.6^{b}
Triglyceride (mg dl^{-1})	369.9±5.3^{e}	356.4±40.5^{d}	326.9±45.8^{c}	322.7±58.9^{b}	253.8±61.4^{a}

*Values are least square means ± standard errors of the means of triplicate samples. *p*values determined with two-way ANOVA tests are also provided for the main factors and their interactions. Means marked with the same letter are not significantly different (p>0.05). Letters a, b, c, d, and e indicate significant differences in treatments, according to Duncan's test.

The mean immunity parameters of *S. trutta caspius* juveniles fed with diets containing different levels of *S. platensis* are shown in Table 6 and 7. Based on obtained results, lysozyme enzyme activity increased with increasing dietary *S. platensis* levels up to 6% and then decreased in 8% treatment. Complement C3, C4, ACH50, free radical oxygen and immunoglobulin were significantly affected by dietary *S. platensis* levels. The lowest and the highest values for these parameters have been observed in the control and 8% treatments.

Table 6: Lysozyme activity of *Salmo trutta caspius* fed different levels of *Spirulina platensis*. .

Lysozyme activity	Experimental Diets				
	0% control	2%	4%	6%	8%
After 15 sec (µg ml^{-1})	1.44±0.82^{a*}	4.3±0.75^{b}	4.95±0.32^{c}	7.33±0.22^{d}	5.87±0.93^{e}
After 180 sec (µg ml^{-1})	6.22±0.35^{a}	6.32±0.38^{a}	6.71±0.28^{b}	8.59±1.06^{c}	7.95±0.44^{d}

*Values are least square means ± standard errors of the means of triplicate samples. *p* values determined with two-way ANOVA tests are also provided for the main factors and their interactions. Means marked with the same letter are not significantly different (p>0.05).Letters a, b, c, d, and e indicate significant differences in treatments, according to Duncan's test.

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Table 7: Immunological parameters of *Salmo trutta caspius* fed different levels of *Spirulina platensis.*

Immunological factor	Experimental Diets				
	0% control	2%	4%	6%	8%
C_3 (mg ml^{-1})	21.1±3.3 ^{a*}	21.1±3.3 ^{ab}	23.4±2.5 ^{b}	25.7±3.2^{c}	35.7±6.9^{d}
C_4 (mg ml^{-1})	13.9±3.6 ^{a}	14.7±2.7^{a}	15±2.4^{a}	19.2±1.8^{b}	20.8±1.5^{b}
ACH50(unit ml^{-1})	220.1±3.87 ^{a}	225.16±4.9^{b}	228.23±7.1^{c}	234.33±8.73^{d}	247±4.77^{e}
Igm(mg dl^{-1})	183.4±25.4^{a}	183.7±21.7^{a}	194.6±19.2^{b}	321.5±13.6^{c}	334.5±25.8^{d}
O^{-}(RLU s^{-1})	384.96±21.05^{a}	401.5±19.38^{b}	415.51±29.73^{c}	429.28±23.31^{d}	471.03±23.77^{e}

*Values are least square means±standard errors of the means of triplicate samples. *p* values determined with two-way ANOVA tests are also provided for the main factors and their interactions. Means marked with the same letter are not significantly different (p>0.05).Letters a, b, c, d, and e indicate significant differences in treatments, according to Duncan's test.

Discussion

The present results indicated that spirulina significantly improved the growth performance of juvenile Caspian brown trout and the fish meal could be replaced with this microalga until 8% without impairments. During the experimental period, no mortality and disease in fish fed spirulina were observed compared to control. In agreement with our research, many studies have demonstrated a positive impact of spirulina on growth performance (Mustafa *et al.*, 1994; James *et al.*, 2006: Palmegiano *et al.*, 2008; Ramakrishnan *et al.*, 2008; Tongsiri *et al.*, 2010; Ungsethaphand *et al.*, 2010; Promya and Chitmanat, 2011; Teimouri *et al.*, 2013; Khanzadeh *et al.*, 2016; Cao *et al.*, 2018; Gogoi *et al.*, 2018). However, contradictory results have been reported with no beneficial effects of dietary spirulina on growth of common carp *Cyprinus carpio* (Nandeesha *et al.*, 1998), Catla *Catla catla* (Nandeesha *et al.*, 2001) and Nile tilapia *Oreochromis niloticus* (Lu and Takeuchi, 2002). It is likely that for carnivorous fish, this microalga has had better growth performance and better fish muscle quality and our study is in agreement with this hypothesis. Growth improvement to dietary spirulina may be due to the improved feed intake and nutrient digestibility. Some researchers have been reported that spirulina improved intestinal microbial balance, leading to better growth by improving food absorption, digestive enzymes activities and fats transport system (James *et al.*, 2006; Teimouri *et al.*, 2013). In addition, the bio-compounds in spirulina, delayed absorption of dietary nutrients and improved carbohydrate and protein utilization in fish. Moreover, there are several nutrients especially vitamins, minerals, essential amino acids, fatty acids in spirulina, that may be beneficial to fish growth promotion and might activate metabolism and act as growth stimulants (Mustafa *et al.*, 1994). Generally, in the current study growth performance in the 6%FMR and 8%FMR treatments were higher than the other dietary groups. Differently, similar spirulina content to the 8%FMR (52.8 g kg^{-1}) has been reported to decrease growth in rainbow trout (*Oncorhynchus mykiss*) (Teimouri *et al.*, 2013) and Nile tilapia (Olvera-Novoa *et al.*, 1998). There are no reports about anti-nutritional factors in spirulina (Vonshak *et al.*, 2014).

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However, the slightly lower growth in 8%FMR compared to 6%FMR is due to the lower mineral content such as phosphorous in spirulina, compared to fish meal (Olvera-Novoa *et al.*, 1998). Perhaps the fish meal content in 6%FMR (620 g kg^{-1} in diet) is optimum for Caspian brown trout growth and higher or lower contents is not enough to reach the highest growth.

There was a significant difference in haemotology parameters among all tested treatments. Our results revealed that *S. platensis* has positive impact on the health status of *S. trutta caspius*. Previous studies such as those by Terry *et al.* (2000) on tilapia, Abdel Tawwab and Ahmad (2009) on tilapia, Yong-Chin *et al.* (2010) on vannamei shrimp (*Litopenaeus vannamei*), Andrews *et al.* (2011) on Nile tilapia, Promya and Chitmanat (2011) on African catfish, Ibrahem *et al.* (2012) on Nile tilapia, Ragap *et al.* (2012) on Nile tilapia, Krishnaveni *et al.* (2013) on catla, Zamini and Azimi (2015) on Carp koi; Salehi Farsani *et al.* (2014) on stellate sturgeon confirmed that *S. platensis* has positive effects on haemotologic parameters. In addition, increasing haemotology indices (e.g., RBC, Hb and Hct) of *S. trutta caspius* with increasing levels of *S. platensis* can result from a variety of reasons including iron compounds, vitamins (B12, A and E), and also the considerable antioxidant capacity contained in *S. platensis*. This high antioxidant capacity is due to the considerable amount of pigments, particularly Phycocyanobilin (e.g., Phycocyanin, Allophycocyanin and Phycoerythrin). In fact, these pigments are responsible for the removal of peroxide radicals in the body and reduce the rate of haemolysis of RBC by oxidants as well as the ability to significantly increase iron absorption (Kop and Durmaz, 2008; Abdel Tawwab and Ahmad, 2009; Andrews *et al.*, 2011).

Reactions caused by WBC are mechanisms of cellular nonspecific or early defense in fish that occur in response to various conditions such as bacterial, viral, fungal, protozoan and parasitic infections, and it is the first indicator to determine the health of every living being (Andrews *et al.*, 2011). Increasing WBC and their differentiate percent with increasing levels of dietary *S. platensis* is due to stimulating the immune system via bioactive substances contained in the microalgae (Tort, 2003). Furthermore, phytocyanins contained in *S. platensis* activate types of WBC (e.g., macrophages and granulocytes) and are responsible for repairing damaged tissues by pathogens (Selmi *et al*., 2011). This bioactive substance with induction of cytokine secretion (molecules involved in the immune system by activating macrophages and lymphocytes.) stimulates the production of new WBC marker molecules (Raa, 1996).

Based on these results, levels of liver enzymes (AST and ALT) decreased with increasing levels of *S. platensis*. Based on accepted hypothesis, the increase of these enzymes in animals (including fish), indicate damage to the liver cells (Jeney and Anderson, 1993).

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Numerous studies have confirmed the effectiveness of *S. platensis* to reduce cholesterol and liver protection (Khan *et al.*, 2005; Pieretti and Meineri, 2011; Kim, 2013; Zeinab *et al.*, 2015). One of the most important approaches of antioxidants is liver cell protection. The antioxidant properties of *S. platensis* have attracted the attention of many researchers. One of the latest investigations Manoj *et al*. (1992) reported that alcoholic extract of *S. platensis* accelerates oxidation of fat significantly (65%) in comparison to the antioxidant chemicals such as alpha-tocopherol (35%), BHA or Butylated Hydroxy Anisole (45%), and beta-carotene (48%).

In this study, serum total protein, albumin and globulin increased with increasing levels of *S. platensis*. Several studies have been demonstrated the effects of *S. platensis* on blood biochemical parameters (Schaperclaus *et al.*, 1992; Dunkan and Klesius, 1996; Abdel-Tawwab and Ahmad, 2009; Andrews *et al.*, 2011; Hernandez, 2005; Zeinab *et al.*, 2015). Blood serum protein show changes in health conditions affected by internal and external factors. Albumin is a transporter protein or public transporter of many organic and inorganic ligands, such as thyroxine, bilirubin, penicillin, cortisol, estrogen, free fatty acids, calcium, and magnesium during illness, malnutrition and stress, blood albumin is reduced (Misra *et al.*, 2006; Alexander *et al.*, 2011). Glucose, cholesterol and triglyceride levels fluctuate during protein catabolism and glycogenesis. Serum glucose levels are often referred to as a non-specific marker of stress (Sheikhzadeh *et al.*, 2012). In this study, blood glucose, triglycerides and cholesterol decreased with increasing levels of *S. platensis*. Our results are in agreement with data reported by other researchers (Abdel-Tawwab and Ahmad, 2009; Andrews *et al.*, 2011; Hernandez, 2005; Salehi Farsani *et al.*, 2014).

Long-chain omega-3 fatty acids in the liver by reducing the secretion of lipoproteins (VLDL) can be reduced triacylglycerol levels (Harris *et al.*, 1984). *S. platensis* is low fat (5% fat) so, 10 gr. of *S. platensis* has only 36 gr. calorie and no cholesterol. *S. platensis* particularly in terms of alpha-linolenic acid (36% of the total PUFA) is very rich. The most essential fatty acids in *S. platensis* that in other food sources are scarce, is gamma linolenic acid. It has been grown under special lighting conditions in the cells, are also able to enrich and enhance it (Khan *et al.*, 2005). Gamma-linolenic acid is a precursor of prostaglandins (PGE) and has special effect on the control of blood cholesterol levels that leads to rapid cell growth. The role of PGE is blood pressure regulation, cholesterol synthesis control and cell survival (Harris *et al.*, 2002).

In this study, we evaluated the immunity status of *S. trutta caspius* fed with different levels of *S. platensis*, using lysozyme, complement C3 and C4, immunoglobulins, oxygen free radical and haemolytic activity of the complement factor. Based on our results, the activity of lysozyme after 15 and 180 seconds with increasing levels

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of *S. platensis* significantly increased (up to 8%). Effect of *S. platensis* on lysozyme by various researchers have confirmed (Watanuki *et al.,* 2006; Abdel Tawwab and Ahmad, 2009; Andrews *et al.,* 2011; Promya and Chitmanat, 2011; Ragap *et al.,* 2012; Ibrahem *et al.,* 2012; Krishnaveni *et al.,* 2013; Kim , 2013; Shimaa, 2016; Salehi Farsani *et al.,* 2014). Lysozyme by WBCs (monocytes, macrophages, neutrophils) and using circulating the blood in different tissues of the body released and leads to non-specific immune organisms (Saurabh and Sahoo, 2008).

The complement system is a part of the immune system that enhances the ability of antibodies and phagocytic cells to clear microbes and damaged cells from an organism, promotes inflammation, and attacks the pathogen's plasma membrane (Claire *et al*., 2002). Several factors have an impact on immunity, including divalent ions (especially magnesium and calcium), polysaccharides immunogenic, season, sexual maturity, prostaglandins, thromboxane and immunoglobulins (Tort *et al*., 2003). The activity of the complement system and immunoglobulins is increased using microalgae (Ragap *et al.,* 2012; Krishnaveni *et al.,* 2013; Shimaa, 2016). C3 and C4 high levels are related to the health of the fish. High serum complement activity in fish fed *S. platensis* could be due to the presence of pigment carotenoids especially beta-carotene. In addition, lysozyme, antibodies and complement factors due to adhesion and colonization with pathogenic microorganisms, prevent infection and pathogenesis. Free radicals of oxygen (O_2, H_2O and O^{2-}) together with nitric oxides, lysozyme, cytokines and other messenger molecules, (prostaglandins, leukotrienes and thromboxanes) increased phagocytic and Pynocytic capacity of macrophage cells (Taoka *et al.,* 2006). Measuring any of these factors can be effective in determining the activation of macrophages. Several studies have demonstrated a positive impact of immune-stimulants such as probiotics and micro-algae on amount of oxygen free radicals and phagocytic activity (Dunkan and Klesius, 1996; Rengpipat *et al.,* 2000; Li and Gatlin, 2004; Panigrahi *et al.,* 2005; Taoka *et al.,* 2006; Watanuki *et al.,* 2006; Abdel-Tawwab and Ahmad, 2009; Andrews *et al.,* 2011; Ragap *et al.,* 2012; Kim, 2013; Ibrahem *et al.,* 2012). Oxidative stress is the accumulation of ROS (Reactive Oxygen Species, ROS). It may be an important factor in the process of developing a variety of diseases. The immune system is vulnerable to oxidative damage. *S. platensis* also contains other pigments Phycocyanobilin including Phycocyanin, Allophycocyanin and Phycoerythrin that are mainly responsible for antioxidant activity and the removal of peroxide radicals in the body (Ragap *et al.,* 2012)

The results of different impacts levels of *S.platensis* on immune and haemotology parameters in *S.trutta caspius* show that *S.platensis* may be beneficial in improve growth, immunity levels in Caspian Sea salmon and create

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resistance to pathogens. These results indicate that *S. platensis* supplementation is promising for disease prevention in *S. trutta caspius* juveniles and can substitute for up to 8% of the fish meal in the diet, although the optimum performance is obtained with 6% substitution

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