Growth performance, hemato-immunological responses and digestive enzymes activities in silvery-black porgy (*Sparidentex hasta*) fed dietary bovine lactoferrin

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Abstract

An eight-week study was conducted to evaluate three different diets supplemented with bovine lactoferrin (LF) at 0 (control), 800 and 1200 mg LF kg^{-1} diet on somatic growth, hemato-immunological parameters, antioxidant status and digestive enzyme activities in silvery-black porgy (*Sparidentex hasta*) juveniles. Fish fed the 800 mg LF kg^{-1} diet had higher growth performance and feed utilization parameters than the other groups. Hematological and liver antioxidant parameters were not affected by dietary LF supplementation. Fish fed the 800 mg LF kg^{-1} diet had higher plasma lysozyme activity values than the other groups. Total protease activity was higher in fish fed LF supplemented diets than the control group. Results indicated that diet supplemented with 800 mg kg^{-1} for 8 weeks enhanced somatic growth performance, lysozyme activity and proteolytic digestive enzyme activities in *S. hasta*, as well as improving feed efficiency parameters like the protein efficiency and feed conversion ratios.

Keywords

Feed utilization, immunostimulants, non-specific immune response, antioxidant status, Sparidae

Introduction

The use of antibiotics in controlling infection diseases in the aquaculture industry has several undesirable side effects such as threatening public health and environment safety through their bioaccumulation in aquatic animals, as well as inducing acquired drug resistance that may result in the development of antibiotic resistant strains of pathogenic microorganisms [1]. In this regard, the replacement of antibiotics with environmentally friendly and efficient biological agents is a prerequisite for a prosperous and sustainable aquaculture industry. Immunostimulants as harmless, environmentally safe and bio-friendly agents have become one of the most promising applied feed additives in aquafeeds as alternative treatments for antibiotics, since they have been reported to control pathogens by activating the immune system of farmed aquatic animals [2]. Different classes of immunostimulants such as polysaccharides (*i.e.* β-glucans and chitosan), herbal extracts (*i.e.* essential oils), vitamins (*i.e.* vitamin C and E) and carotenoids, probiotics and their secondary metabolites, prebiotics, hormones (thyroxin) and biological factors (*i.e.* lectin, lactoferrin and bactericidal peptides) have been proved that not only can induce potent immune responses and disease resistance, but they can also promote growth performance in the host [1, 3].

Lactoferrin (LF) is an 80 kDa iron-biding glycoprotein, which is a part of the transferrin protein family that is found in high concentrations in mammal's exocrine secretions such as the mucus, saliva and milk, as well as in specific granules of polymorphonuclear immunocytes [4]. Lactoferrin has various biological functions including: antioxidant [5], bactericidal, antifungal, antiviral, antiparasitic, enzymatic, anti-inflammatory and anticarcinogenic activities [4, 6]. Concerning fish species, dietary LF

supplementation has been reported to enhance mucus secretion [7], growth performance [8], non-specific defense systems (*i.e.* lysozyme, haemolytic activity, complement, antiprotease and macrophage respiratory burst activities) [9, 10, 11, 12], reduced stress-induced immune suppression [7, 13] and increased disease resistance [9, 14, 15, 16, 17].

Because of its rapid growth, good adaptation to captivity and high market price, the silvery-black porgy, *Sparidentex hasta* is recognized as one of the most promising candidate for mariculture activities in the Persian Gulf and the Oman Sea regions [18]. In this context, considerable research has been conducted on establishing the nutritional requirements of *S. hasta* and optimizing diet formulation [19]. Regarding dietary immunostimulant supplementation, Morshedi et al. [20, 21] reported that dietary inclusion of 400 and 800 mg LF kg^{-1} did not improve growth performance, feed utilization, digestive enzyme activities (*i.e*. α-amylase, lipase and total protease), hematological and humoral immune responses (*i.e.* lysozyme, complement and bactericidal activities) in *S. hasta* fingerlings (initial body weight = 7.6 ± 0.3 g). Considering these contradictory results in *S. hasta* with regard to the available literature on the use of this immunostimulant in different fish species, authors decided to test again this additive at higher levels. Thus, the aim of the current study was to evaluate the inclusion of LF at 800 and 1200 mg kg^{-1} on growth performance, hemato-immunological and antioxidant responses, as well as digestive enzymes activities in *S. hasta* juveniles.

Materials and Methods

Experimental design

A basal diet containing 487 g crude protein and 187.2 g fat per kg of dry matter (DM) was formulated with fish meal, beef gelatin, wheat gluten, wheat meal, and fish oil in order to meet the nutritional requirements for *S. hasta* juveniles [19]. This basal diet was supplemented with LF (0, 800 and 1200 mg kg^{-1}) at the expense of wheat meal and named as LF_0, LF_{800} and LF_{1200}, respectively. Diets were prepared by mixing all ingredients for 30 min, after which, oil and sufficient distilled water were added to form a soft dough, and then mechanically extruded to obtain pellets of the desired size (3 mm). Pellets were dried in a convection oven at 25 °C and stored in re-sealable plastic bags at −20 °C until use.

Fish maintenance and feeding

This study was carried out at the Mariculture Research Station of the South Iranian Aquaculture Research Center (SIARC), Sarbandar, Iran. One hundred and thirty five juveniles of *S. hasta* were randomly distributed into 9 cylindrical polyethylene tanks (volume = 250 l), and each tank stocked with 15 fish [initial body weight (BW_i) = 38.0 ± 0.1 g, mean ± standard deviation]. Fish were acclimated for 2 weeks before the onset of the nutritional trial. Tanks were supplied with filtered running seawater (1 l min^{-1}); salinity ranged between 47 and 49 ‰ (48.2 ± 2.0 ‰) and temperature between 21 and 28 °C (25.1 ± 1.6 ºC) during the experimental period. Average values for dissolved oxygen and pH were 6.8 ± 0.4 mg l^{-1} and 7.7 ± 0.1, respectively, and the photoperiod was 16L:8D (light:darkness). Diets were tested by triplicate; fish were fed by hand to visual satiation two times per day (0800h and 1500h) for 56 days. Uneaten food was removed from the

bottom of the tank by siphoning 1 h after feeding, dried in an oven (60 °C for 24 h) and weighed to determine feed intake values.

Fish growth and feed utilization

Fish BW, and the weight of the liver, intraperitoneal fat and viscera were measured to the nearest 0.1g, and standard length (SL) was measured to the nearest 1mm. The following formulae were used to assess growth performance, feed utilization and other parameters: weight gain (WG, %) = $[(BW_f - BW_i) / BW_i] \times 100$; specific growth rate (SGR, % day^{-1}) = $[(\ln BW_f - \ln BW_i) / t] \times 100$, where t is experimental period (56 days); survival (S, %) = (number of fish in each group remaining on day 56 / initial number of fish) × 100; hepatosomatic index (HSI, %) = (liver weight (g) / BW_f) × 100; viscerosomatic index (VSI, %) = (visceral weight (g) / BW_f) × 100; intraperitoneal fat index (IPF, %) = (intraperitoneal fat weight (g) / BW_f) × 100; Fulton's condition factor (K) = $(BW_f / SL^3) \times 100$; feed conversion ratio (FCR) = feed intake (g) / weight gain (g).

Sample collection

At the end of the trial, fish were fasted for 24 hours before being anaesthetized (2-phenoxyethanol at 0.5 ml l^{-1}; Merck, Schuchardt, Germany) and individually weighed (BW_f). Four specimens from each replicate were anaesthetized with 2-phenoxyethanol and then sacrificed with an overdose of this anaesthetic to evaluate their hepatosomatic index (HSI), viscerosomatic index (VSI) and intraperitoneal fat (IPF). For the assessment of digestive and liver enzyme activities, 2 fish per tank (n = 6 fish per dietary treatment, n = 2 fish per replicate) were randomly sampled, euthanized with the anaesthetic, and

immediately eviscerated on an ice surface (0-4 ºC). The alimentary tract and liver were dissected, adherent adipose and connective tissues carefully removed, and placed in individually marked plastic test tubes and stored at -80 °C until their analysis. Blood was collected from the caudal vein (n = 6 fish per diet treatment, n = 2 fish per replicate) with heparinized syringes (1300–1700 µl per specimen) and pooled together (each pool contained the blood from two fish), and it was aliquoted into three parts of 1 ml each. An aliquot of blood was used for hematological factors and the other aliquots centrifuged (4,000 *g*, 10 min at room temperature) and plasma separated and stored at -80 °C until their analysis.

Hemato-immunological and antioxidant status

Hematocrit (Hct; %), hemoglobin concentration (Hb; g dl^{-1}), the number of red blood cells (RBC) and white blood cells (WBC) counts, as well as differential WBC percentage (lymphocyte, monocyte, neutrophil and basophil portions as WBC%) were assessed according to methods described by Blaxhall and Daisley [22]. The hemolytic activity of the plasma was determined using rabbit red blood cells (RaRBC) as the target cells according to the procedure described by Andani et al. [23]. Following washing of the RaRBC three times, the absolute lysis value was prepared by adding 100µl of the RaRBC to 3.4 ml distilled water. The lysate was then exposed to cold centrifugation and the turbidity of the aqueous phase was determined at 414 nm. Subsequently, the plasma specimens were diluted in the buffer and 250 µl of adjusted volume plasma was added to 100µl of RaRBC in test tubes. The prepared solution was kept at room temperature for 90 min with repeated mixing. Then, 3.15 ml of NaCl solution (0.85%) was added to all

samples and the tubes were centrifuged for 10 min and the absorbance of the supernatant was quantified again. The level of cell lysis was determined and hemolysis curve was drawn through plotting the hemolysis degree against the volume of plasma added on a log/log-scaled graph. The volume producing 50% hemolysis was considered for determining the hemolytic activity of the plasma samples and was expressed as mU mL^{-1}. The levels of lysozyme in plasma were determined using a turbidimetric assay according to Ellis [24] by measuring the lytic activity of plasma against lyophilized *Micrococcus lysodeikticus* (Sigma, St Louis, MO, USA). A volume of 135µl of *M. lysodeikticus* at a concentration of 0.2 mg ml^{-1} (w/v) in 0.02 M sodium citrate buffer (SCB), pH 5.8 was added to 15µl of plasma sample. As a negative control, SCB was replaced instead of plasma. Results were expressed in mg of lysozyme mL^{-1} of plasma. Hen egg white lysozyme (Sigma) in phosphate-buffered saline was used as a standard. A unit of lysozyme activity was defined as the amount of plasma causing a reduction of absorbance of 0.001 per min at 450 nm at 22 °C.

Plasma total immunoglobulin (Ig) was measured using the method described by Siwicki et al. [25]. Primary separation of immunoglobulins from the plasma was achieved by precipitation with polyethylene glycol (PEG) and the resulting supernatant analyzed. To perform the assay, 100µl of plasma were combined with 100µl 12% PEG and incubated at room temperature for 2 h in continuous agitation. Following the incubation time, the mixture was centrifuged (400 g, 10 min at room temperature) and total protein concentration in the supernatant determined by the biuret method. The total Ig levels were calculated considering total protein values less the quantity of protein in the supernatant.

Superoxide dismutase (SOD) activity in liver samples was assayed according to Kono [26]. The homogenate (2 ml, 10% w/v) was prepared in sodium carbonate buffer (pH 10.0) and centrifuged at 10,000 rpm (20 min at 4 °C). The 2.5 ml of assay mixture consisted of 1.3 ml of carbonate buffer, 0.5ml of NBT, 0.1 ml of TritonX-100, 0.1 ml of hydroxylamine hydrochloride and 0.5ml of enzyme extract. The increase in absorbance was recorded at 540 nm. The activity of catalase (CAT) was determined using the method described by Koroluk et al. [27]. Briefly, 5 µl of tissue supernatant or plasma were incubated for 10 min in 150µl of reaction mixture containing 10 mM hydrogen peroxide in 0.05 mM Tris–HCl buffer (pH 7.8, 25 °C). The enzymatic reaction was stopped with 50 µl of ammonium molybdate 4% and the concentration of the yellow complex of molybdate and hydrogen peroxide was measured spectrophotometrically (λ= 410 nm). Results were expressed in U mg protein^{-1}. Total antioxidant capacity (TAC) was assayed in liver samples according to the method of the ferric reducing ability of plasma [28]. Briefly, the FRAP reagent contained 5 ml of a 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl plus 5 ml of $FeCl_3$ (20 mM) and 50 ml of acetate buffer (0.3 M, pH 3.6). Aliquots of 100 µl supernatant or plasma were mixed with 3 ml of the FRAP reagent. The conversion rate of the ferric tripyridyl-striazine (Fe^{3+}-TPTZ) complex to ferrous tripyridyl-s-triazine (Fe^{2+}-TPTZ) at pH 3.6 and 25 °C is directly proportional to the concentration of total antioxidants in the sample. Fe^{2+}-TPTZ has an intensive blue color that can be monitored for up to 5 min at 593 nm. Calculations were performed using a calibration curve of $FeSO_4 \cdot 7H_2O$ (100 to 1000 µml^{-1}). Results were expressed in mmol mg protein^{-1}.

Digestive enzymes

Samples were processed and handled following the indications of Solovyev and Gisbert [29]. The digestive tracts pooled, homogenized (1–2 min at 0–4 °C; 3 volumes v/w of 50 mM 2 mM Tris–HCl buffer, pH 7.0) [30]. Samples were centrifuged (10,000g, 20 min at 4 °C) and the supernatant was collected, aliquoted and frozen at −80 °C for the determination of selected pancreatic enzymes. Total alkaline proteases were assayed by the azo-casein method described by Garcia-Carreño et al. [31] using 50 mM Tris-HCl buffer (pH 9.0). Total alkaline protease activity (U) was defined as the mg of substrate hydrolyzed during 10 min ml^{-1} of the extract at 25 °C at 440 nm. Bile salt-activated lipase (E.C. 3.1.1) activity was assayed for 30 min at 30 °C using p-nitrophenyl myristate as substrate dissolved in 0.25 mM Tris-HCl (pH 9.0), 0.25 mM 2-methoxyethanol and 5 mM sodium cholate buffer. The reaction was stopped with a mixture of acetone:n-heptane (5:2), the extract centrifuged (6,080g, 2 min at 4°C) and the absorbance of the supernatant read at 405 nm. Lipase activity (U) was defined as the 1 μmol of p-nitrophenyl myristate hydrolyzed min^{-1} ml^{-1} of extract [32].

The soluble protein of crude enzyme extracts was quantified by means of the Bradford's method [33] using bovine serum albumin as standard. All the assays were made in triplicate (methodological replicates). All oxidative stress condition parameters and digestive enzymes activities were measured by a microplate scanning spectrophotometer (PowerWave HT, BioTek®, USA).

Statistical analyses

Data were analyzed using SPSS ver.19.0 (Chicago, Illinois, USA). All data are presented as mean ± standard error of the mean calculated from three biological replicates. Arcsine

transformations were conducted on data expressed as percentage. One way ANOVA was performed at a significance level of 0.05 following confirmation of normality and homogeneity of the variance. Duncan's procedure was used for multiple comparisons when statistical differences were found among groups by the one-way ANOVA.

Results

Survival and growth performance

No mortality occurred throughout the experiment (Table 2). Weight gain in fish fed the LF_{800} diet was 29.2 and 19.1% higher than fish fed the control and LF_{1200} diets ($P < 0.05$). Somatic indices including VSI, HSI and PFI were not affected by the dietary inclusion of LF ($P > 0.05$). Fish fed LF supplemented diet had higher FI than the LF_0 group ($P < 0.05$). Regarding feed efficiency parameters, fish fed the LF_{800} diet had the lowest FCR (1.3 ± 0.0) and the highest PER (1.6 ± 0.1), respectively.

Hemato-immunological and antioxidant parameters

Hematological parameters including RBC, Hb, Hct, WBC counts and differential WBC percentage, as well as hematological indices (MCV, MCH and MCHC) were not affected by the inclusion of LF in the basal diet (Table 3). In contrast, fish fed the LF_{800} diet had the highest plasma lysozyme activity (Fig. 1a; $P < 0.05$); however, plasma total Ig content (Fig. 1b) and hemolytic activity (Fig. 1c) were not affected among different experimental groups ($P > 0.05$). In addition, there were not any significant differences in the measured liver antioxidant parameters, including SOD (Fig. 2a), CAT (Fig. 2b) and TAC (Fig. 2c) among experimental groups ($P > 0.05$).

Digestive enzyme activity

In the present study, fish fed both LF supplemented diets had the higher total protease activity than the control group (Fig. 3a; $P < 0.05$); however, bile salt-activated lipase activity was not affected by dietary LF supplementation (Fig. 3b; $P > 0.05$).

Discussion

In the current study, fish fed the diet containing 800 mg LF kg^{-1} (LF_{800} diet) showed higher somatic growth rate and feed efficiency values than the other experimental groups. A similar result has been also reported in goldfish (*Carassius auratus*) fed diets supplemented with LF up to 1000 mg kg^{-1} compared with the LF-free group [8]. Taking into account the dose-response results in terms of growth performance from the current study with those previously published by Morshedi et al. [20], it seems that the somatic growth response to dietary inclusion of LF may be quadratic rather than linear. Multiple factors such as the synergistic effects between LF and some other unknown feed ingredients [7], feed processing [36], species-specific ability to dietary LF absorption [37], and/or experimental design (*i.e.* dietary LF dose, feeding duration and culture conditions) [12] might be differentially involved in promoting growth performance in fish and be accountable for such differences between both trials run in *S. hasta*. However, in most of the consulted studies, LF administration did not have positive effects neither on growth performance nor feed utilization in other fish species such as orange spotted grouper (*Epinephelus coioides*) [7], Japanese flounder (*Paralichthys olivaceus*) [13], Atlantic salmon (*Salmo salar*) [16], Nile tilapia (*Oreochromis niloticus*) [17], gilthead seabream (*Sparus aurata*) [34] and Siberian sturgeon (*Acipenser baerii*) [35].

The results from the current study showed that the dietary LF did not significantly affect hematological parameters in *S. hasta* juveniles as the same also reported in rainbow trout (*Oncorhynchus mykiss*) [11], African electric blue cichlid (*Sciaenochromis fryeri*) [12], Nile tilapia [17] and Siberian sturgeon [37]. In contrast, Welker et al. [38] reported an increase in blood Hct of channel catfish, *Ictalurus punctatus* fed diets supplemented with 800 and 1600 mg kg^{-1} LF. Moreover, using LF and iron-supplemented diets improved the hematological parameters of iron-deficient anemic rats [39], whereas dietary LF supplementation in piglets has been reported to be associated with a reduction in neutrophils and an increase in lymphocytes [40]. These differences may be attributed to the fact that animal health condition and iron concentration in the diet may responsible for ability of LF to improve hematological parameters [39].

The immunostimulatory effect of LF in fish is mediated by activating the non-specific immune response, which provides protection against a wide range of pathogens [9]. Regarding humoral immune responses, fish fed the LF_{800} diet showed the highest plasma lysozyme activity in comparison to the other groups. Similarly, LF supplemented diets increased serum lysozyme activity in Asian catfish (*Clarias batrachus*, effective dietary LF dose = 50–100 mg kg^{-1}, feeding duration = 1 week) [9], Japanese eel, (*Anguilla japonica*, effective dietary LF dose = 500 mg kg^{-1}, feeding duration = 3 weeks, [41], rainbow trout (effective dietary LF dose = 100–400 mg kg^{-1}, feeding duration = 8 weeks) [11] and African electric blue cichlid (effective dietary LF dose = 200 mg kg^{-1}, feeding duration = 8 weeks) [12]. In contrast, Morshedi et al. [20] reported that dietary LF administration (dietary LF tested = 400 and 800 mg kg^{-1}, feeding duration = 6 weeks) did not affect plasma lysozyme activity in *S. hasta* fingerlings. The contradictory result

between our study and that of Morshedi et al. [20] may result of difference in LF feeding period (8 *vs* 6 weeks) and/or fish growth stage (juvenile *vs* fingerling stage). In addition, the decrease in plasma lysozyme activity in fish fed the 1200 mg LF kg^{-1} diet might be related to a negative feedback system to excessive and/or long term administration of LF [42], which would exhaust immune responses, which may also correlated to the quadratic growth response observed [20] as it has been also described in other fish species [9, 12, 42, 43]. In this context, it has been documented that LF and its bioactive peptide domain, called lactoferricin (LFcin, originated by the pepsin hydrolysis of LF during the gastric transit), can block the classical complement pathway [44], which would support the absence of statistical differences in hemolytic activity between fish fed the LF_0 and LF-supplemented diets. The hemolytic activity in plasma in our study did not significantly change among experimental groups, which was in line with results observed in different fish species [9, 12, 16, 17, 37]. On the contrary, Rahimnejad et al. [11] reported higher serum hemolytic complement activity in rainbow trout fed 100 and 400 mg LF kg^{-1} diet than fish fed a LF-free diet for 6 weeks. These discrepancies in the literature may related to fish species-specific intracellular proteases and gastric pepsin activities, which affected their capacity to digest dietary LF into LFcin, and consequently, reduced the bilological availability of LFcin and by extension, influenced the non-specific humoral response of the organism [45, 46].

The plasma total Ig level of *S. hasta* in our study did not significantly vary among treatments, which in line with results reported in Siberian sturgeon fed a LF- supplemented diet [37]. In addition, it has been reported that LF feeding did not influence serum antibody titre and protection in vaccinated Asian catfish against *A. hydrophila* challenge [9]. In fact,

immunostimulants cannot efficiently trigger specific immune system and antibody production due to the lack of immunologic memory in fish; thus, this type of immune response lasts only for a short duration [47].

The generation of reactive oxygen radicals is induced by metals specially, Fe^{+2}, which are abundant in hemin and non-hemin iron compounds (*i.e.* hemoglobin, ferritin and hemosiderin). It is well documented that LF is an efficient iron chelator and potent antiradical agent, which scavengers' free radicals and decreases biomembrane lipid peroxidation [5]. The results of the current study showed that liver antioxidant status in *S. hasta* was not affected by dietary LF supplementation, which indicated an optimal balance between pro- and antioxidants in experimental diets. In accordance with our results, Lygren et al. [16] reported that liver catalase activity did not change in Atlantic salmon fed the 140 mg LF kg^{-1} diet in comparison with the LF-free group.

To our knowledge, the effect of LF on the activity of digestive enzymes in fish has never been studied before. In our study, total alkaline protease activity was significantly increased in fish fed the LF-supplemented diet, which might be attributed to better growth performance especially in fish fed the LF_{800} diet. It has been reported that dietary LF increased intestinal epithelial cell proliferation and protected intestinal crypt and villous structure in neonatal piglets [48, 49]. These authors proposed that dietary LF promoted the integrity of intestinal mucosa integrity help to intestinal brush boarder stabilization and preventing loss of digestive enzymes to the gut lumen by interacting with LF receptors on intestinal brush border membranes. On the other hand, the higher activity of proteases in fish fed LF-supplemented diets may be due to higher levels of LF in diets, since LF is a glycoprotein that may affect the production of proteases.

In summary, according to the results obtained in the present study it may be concluded that feeding of *S. hasta* juveniles with a diet containing 800 mg LF kg^{-1} additive can promote growth performance, improve feed efficiency, enhance the activity of pancreatic proteases and lysozyme activity, which may increase the immune competence and disease resistance in this species.

Compliance with ethical standards

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Conflict of interest: There is no conflict of interest between authors in the publication of this paper.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Table 1

Ingredient list and proximate composition of the basal experimental

Ingredients (g kg^{-1})	Basal diet
Fish meal[a]	560
Beef gelatin[b]	51
Wheat Gluten meal	120
Wheat meal[c]	99.8–101
Fish oil[a]	105
Soybean lecithin[d]	30
Vitamin premix[e]	15
Mineral premix[f]	15
Antioxidant[g]	3
Bovine lactoferrin[h]	0–1.2
Proximate composition **(g kg^{-1})**	
Dry matter	915.4
Crude protein	487.0
Crude lipid	187.2
Ash	80
NFE[i]	245.8

[a] Fish meal (*Clupeonella* sp.); Parskilka Mazandaran, Iran (635 g kg^{-1} crude protein, 177 g kg^{-1} crude lipid).

[b] Gelatine; Beyza feed mill, Shiraz, Iran. (850 g kg^{-1}crude protein, 42 g kg^{-1}crude lipid).

[c] Wheat meal; Beyza feed mill, Shiraz, Iran. (120 g kg^{-1} crude protein, 30 g kg^{-1} crude lipid).

[d] Behpak Industrial Company, Behshahr, Mazandaran, Iran.

[e] Vitamin premix (mg kg^{-1}) of premix: vitamin A, 5,000,000 IU; vitamin D3, 500,000 IU; vitamin E, 3,000 mg; vitamin K3, 1,500; vitamin B1, 6,000; vitamin B2, 24,000; vitamin B5, 52,000; vitamin B6, 18,000; vitamin B12, 60,000; folic acid, 3,000; nicotinamide 180,000; antioxidant, 500, Damloran pharmaceutical company, Borujerd, Iran.

[f] Mineral premix (mg kg^{-1}) of premix: copper, 3,000; zinc, 15,000; manganese, 20,000; Iron, 10,000; potassium iodate, 300. Microvit®, Razak laboratories, Tehran, Iran.

[g] Butylated hydroxyl toluene, GarmabShimi, Iran.

[h] Biopole SA/NV, Belgium.

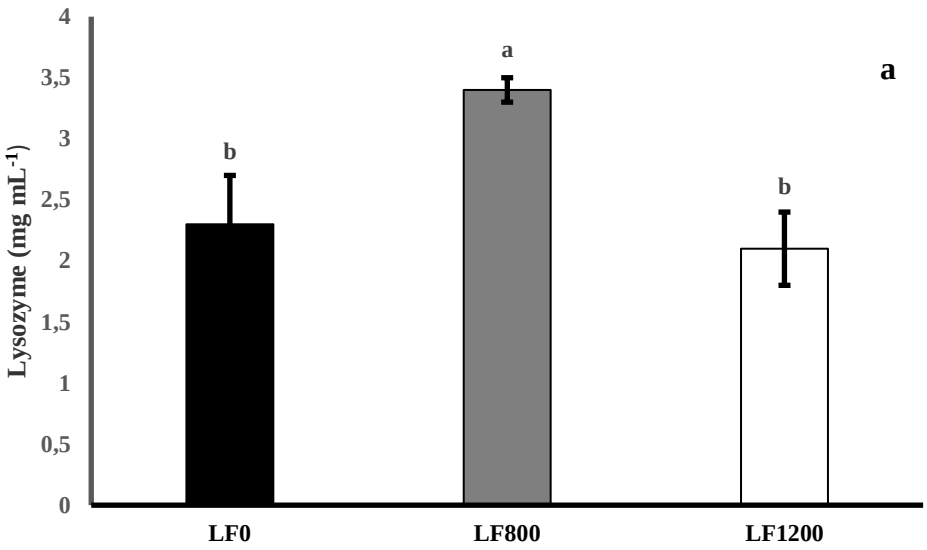
[i] Nitrogen-free extract = 100−(protein+lipid+ash+moisture).

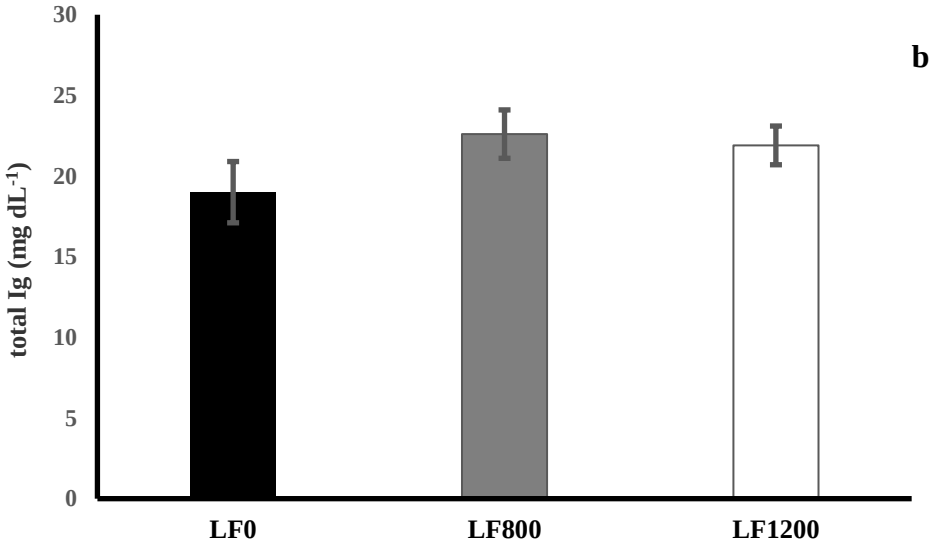
Table 2
Growth in body weight (g), specific growth rate (SGR) and somatic indexes including hepatosomatic (HSI), viscerosomatic (VSI) and intraperitoneal fat (PFI) indexes and feed utilization of *S. hasta* juvenile fed experimental diets containing different LF levels (mean ± SEM, n = 3). A different superscript in the same row denotes statistically significant differences (P <0.05).

	Diets		
Parameter	LF_0	LF_{800}	LF_{1200}
BW_i (g)	38.1 ± 0.1	38.0 ± 0.0	37.9 ± 0.2
BW_f (g)	77.2 ± 2.7^b	88.1 ± 1.4^a	80.6 ± 1.8^b
SGR (%)	1.3 ± 0.1^b	1.5 ± 0.0^a	1.4 ± 0.0^b
SUR (%)	100 ± 0.0	100 ± 0.0	100 ± 0.0
K (%)	3.0 ± 0.1	2.9 ± 0.0	3.1 ± 0.1
VSI (%)	7.8 ± 0.7	8.7 ± 0.4	7.8 ± 0.5
HSI (%)	1.4 ± 0.1	1.7 ± 0.1	1.4 ± 0.1
PFI (%)	3.7 ± 0.6	4.0 ± 0.3	3.3 ± 0.3
FI (g $fish^{-1}$)	60.4 ± 0.8^b	62.9 ± 0.4^a	62.7 ± 0.4^a
FCR	1.6 ± 0.1^b	1.3 ± 0.0^a	1.5 ± 0.1^b
PER	1.3 ± 0.1^b	1.6 ± 0.1^a	1.4 ± 0.1^b

Table 3
Complete blood count including red blood cell counts (RBC), hemoglobin (Hb), hematocrit (%) and hematological indexes (MCV, MCH and MCHC) and differential WBC percentage (lymphocyte, monocyte and neutrophil as WBC%) of *S. hasta* juveniles fed experimental diets containing different LF levels (mean ± SEM, n = 3). A different superscript in the same row denotes statistically significant differences ($P < 0.05$).

	Diets		
Parameter	LF_0	LF_{800}	LF_{1200}
RBC ($\times 10^6$ μL^{-1})	2.3 ± 0.1	2.2 ± 0.1	2.2 ± 0.0
Hb (g dL^{-1})	7.8 ± 0.4	7.4 ± 0.2	7.5 ± 0.1
Hct (%)	54.3 ± 2.5	51.3 ± 1.2	52.0 ± 0.5
HCV (FL)	231.5 ± 1.8	229.8 ± 1.8	231.7 ± 1.4
MCH (pg)	33.2 ± 0.4	32.8 ± 0.2	33.2 ± 0.4
MCHC (g dL^{-1})	14.0 ± 0.0	14.2 ± 0.2	14.0 ± 0.0
WBC ($\times 10^3$ μL^{-1})	4.9 ± 0.0	5.0 ± 1.0	5.2 ± 0.3
Neutrophilis (%)	24.2 ± 1.2	22.8 ± 1.6	25.3 ± 2.1
Lymphoctes (%)	71.8 ± 1.5	73.8 ± 1.9	71.5 ± 2.4
Monocytes (%)	4.0 ± 0.3	3.3 ± 0.6	3.2 ± 0.3


a
Lysozyme (mg mL-1)
4
3,5
3
2,5
2
1,5
1
0,5
0
b
a
b
LF0
LF800
LF1200


b
total Ig (mg dL-1)
30
25
20
15
10
5
0
LF0
LF800
LF1200

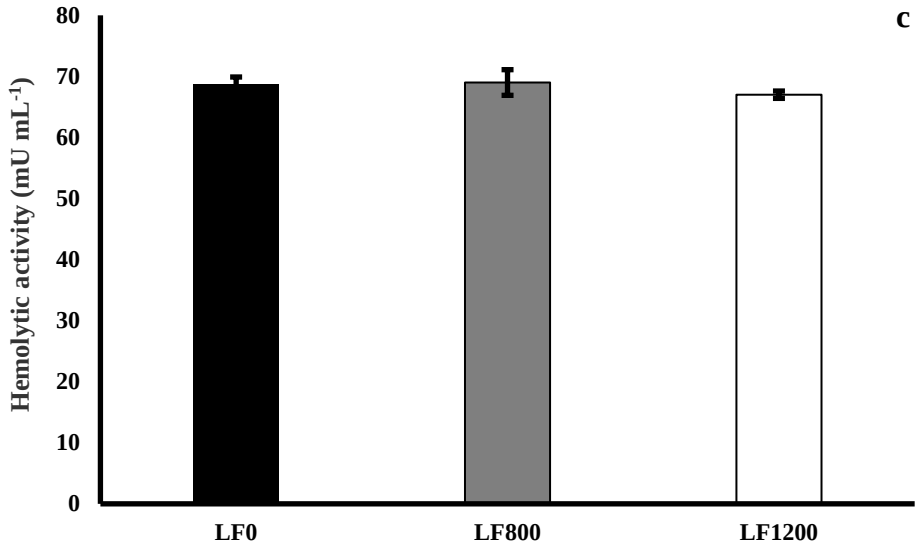
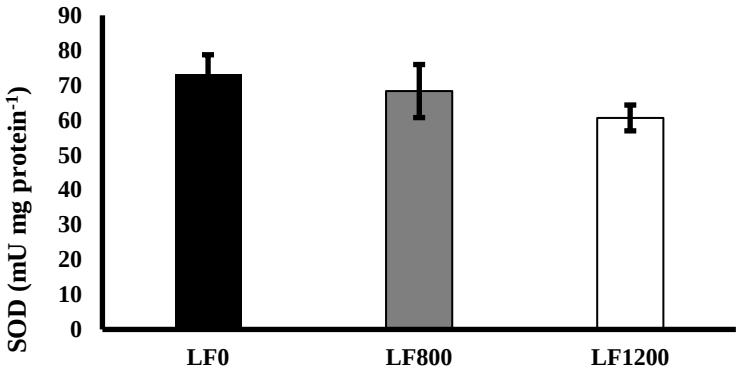
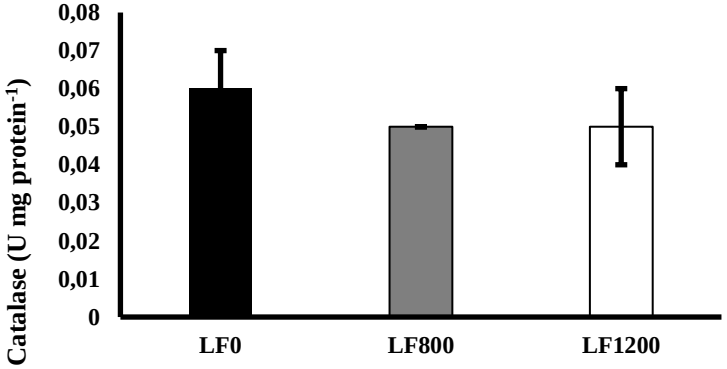


Figure 1. Plasma humoral immune parameters including lysozyme level (mg mL^{-1}) (a) total Ig (mg dL^{-1}) (b), and hemolytic activity (mU mL^{-1}) (c) in *S. hasta* fed experimental diets containing different LF levels (mean ± SEM, n = 3). Different superscripts on the bars denotes statistically significant differences ($P < 0.05$).

a





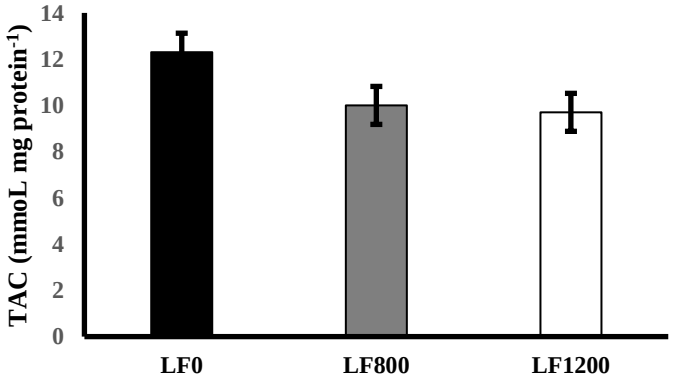


Figure 2. Liver superoxide dismutase (SOD) (mU mg protein^{-1}) (a) and catalase (U mg protein^{-1}) (b) activities and total antioxidant capacity (mmol mg protein^{-1}) (c) in *S. hasta* fed experimental diets containing different LF levels (mean ± SEM, n = 3). Different superscripts on the bars denotes statistically significant differences ($P < 0.05$).

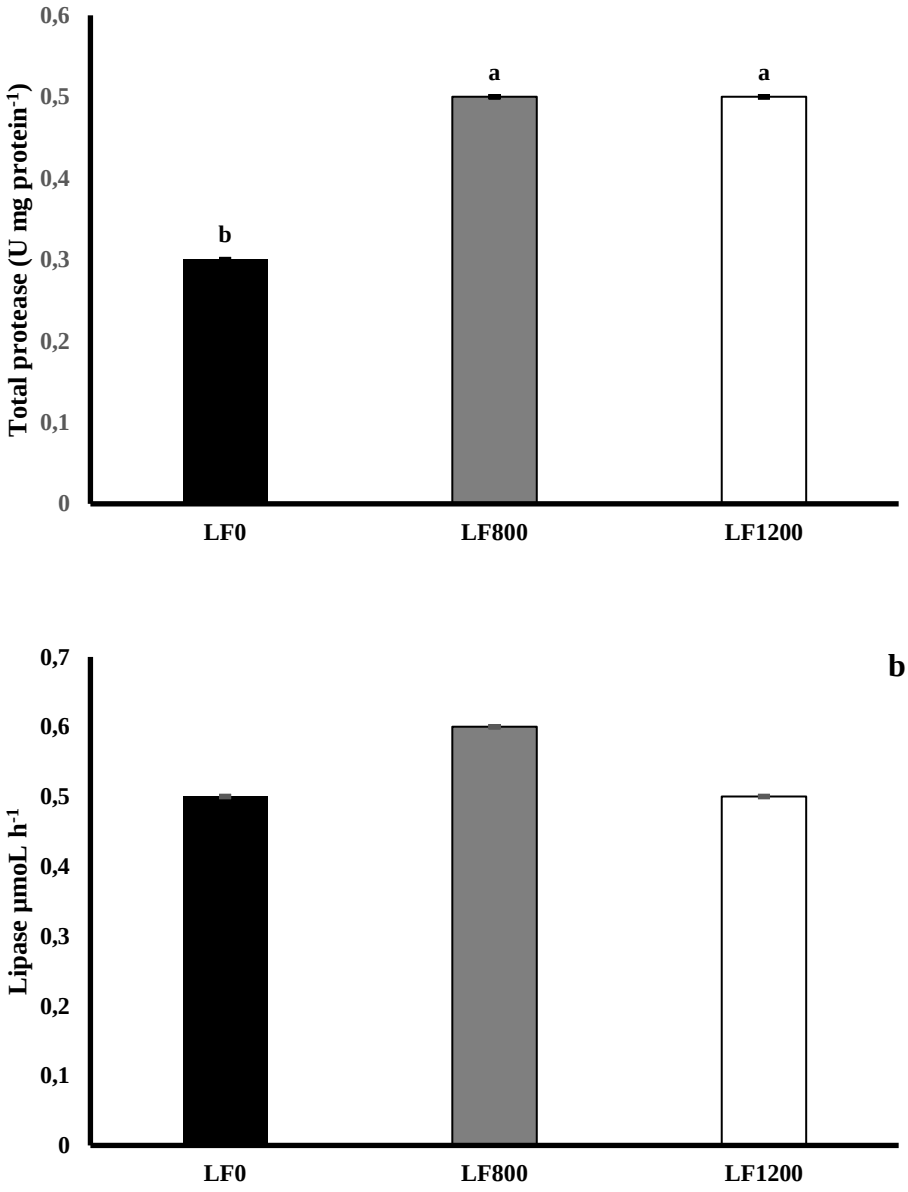


Figure 3. Digestive enzyme activities including total protease (U mg protein^{-1}) (a) and lipase (µmoL h^{-1}) (b) in *S. hasta* fed experimental diets containing different LF levels (mean ± SEM, n = 3). Different superscripts on the bars denotes statistically significant differences ($P < 0.05$).