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Effects of dietary supplementation of bovine lactoferrin on antioxidant status, immune response and disease resistance of yellowfin sea bream (*Acanthopagrus latus*) against *Vibrio harveyi*

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ABSTRACT

This study investigated the effect of the dietary supplementation of bovine lactoferrin (LF) on growth performance, hematological and immunological parameters, antioxidant enzymes activity and disease resistance against *Vibrio harveyi* in yellowfin sea bream (*Acanthopagrus latus*) fingerling. The fish with initial body weight 10 ± 0.3 g were randomly distributed at 10 fish per each 250 L fiberglass tank, and fed with four experimental diets (a control basal diet and three supplemented diets with 400, 800 and 1200 mg LF kg^{-1} diet) for 8 weeks. The obtained results showed that fish fed with LF supplemented diets had significantly higher final body weight as compared to control diet ($P < 0.05$). There were no significant differences between LF-treatments and the control group in white blood cell counts, red blood cell counts, hemoglobin and hematocrit. Total protein and complement activity (ACH50) in the serum of yellowfin sea bream were enhanced with increasing the dietary LF supplementation level ($P < 0.05$). The mucus lysozyme activity in fish fed on 800 and 1200 mg LF kg^{-1} was significantly higher than those fed on 400 mg LF kg^{-1} and control fish ($P < 0.05$). None of the antioxidant enzymes (catalase, glutathione reductase, glutathione S-transferase) was affected by LF supplementation ($P > 0.05$). Fish fed with dietary LF had a significantly higher survival rate than those fed with the control diet after challenge with *Vibrio harveyi* ($P < 0.05$). These results revealed that diet supplementation in *A. latus* especially with 1200 mg LF kg^{-1} improve fish growth performance and immune parameters, as well as survival rate against *Vibrio harveyi*.

1. Introduction

Global production of fish from aquaculture has grown rapidly over the past four decades, contributing significant quantities to the world's supply of fish for human consumption [1]. This increase in production has been achieved through the expanding of intensive fish farming methods. Intensive culture systems are a highly stressful environment for fish, and consequent suppression of the immune response results in an increased susceptibility to diseases [2]. 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 [3].

The use of immunostimulants to enhance activities in the immune system has been given considerable attention in aquaculture in order to increase disease resistance in farmed fish [4,5]. A large number of putative immunostimulants have been investigated in fish, which include killed pathogens and their products, fungal β-glucans, chitin, unidentified animal extracts, synthetic chemicals and plant molecules and nutrients [5–7].

Lactoferrin (LF) is an 80 kDa iron-binding glycoprotein present in secretory fluids of mammals consisting of a single peptide chain with two globular lobes; each containing one iron-binding site [8]. It is thought to play a pivotal role in the activity of the innate immune system [9]. Several biological functions have been attributed to LF, such as regulation of iron absorption, antibacterial activity by depriving the bacteria of iron needed for growth [8], growth promotion of different types of animal cells including lymphocytes [10], enhanced

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production of hydroxyl radical by human neutrophils [11], and increased phagocytosis and killing of an intracellular parasite by murine macrophages and human monocytes [12,13].

Orally administrated LF enhanced the number of granulocytes and lymphocytes in red sea bream (*Pagrus major*) [14], serum lysozyme level in Asian catfish (*Clarias batrachus*) [2], mucus secretion in orange spotted grouper (*Epinephelus coioides*) [15], and bacterial clearance efficiency in giant freshwater prawn (*Macrobrachium rosenbergii*) [16].

Yellowfin sea bream, *Acanthopagrus latus*, is a protandrous hermaphrodite fish distributed widely in the Indian ocean including Persian Gulf [17]. This fish occupies tropical and temperate coastal waters and has high potential for aquaculture in the Indo-Pacific region because of its high market value, easy adaptation to captivity and availability of production technology [18,19]. The yellowfin seabream has a good potential for mariculture in Iran, so many studies have been conducted to evaluate the nutritional requirements and the effect of various dietary supplements in this species. Although several studies have investigated the effects of dietary lactoferrin on some fish species [2,14–16], no data are available on the dietary lactoferrin in this fish. Moreover, other aspects of this immuno supplement such as antioxidant status which mediate host benefits are poorly understood. Therefore, this study was carried out to evaluate the effects of different levels of dietary bovine lactoferrin on growth performance, immune response, antioxidant enzymes activity and disease resistance of yellowfin seabream fingerling.

2. Materials and methods

2.1. Fish and rearing conditions

Yellowfin seabream (*A. latus*) fingerling were provided from the Marine Research Station of the Persian Gulf University, Boushehr, Iran and acclimated for 2 weeks before the onset of the nutritional trial. Fish were hand fed to apparent satiety with commercial dry pellets without bovine lactoferrin prior to the start of the experiment. One hundred and twenty fish (mean initial body weight ± SD: 10 ± 0.3 g) were stocked (10 fish per tank) into each of the 12 tanks (300 L), which were distributed in four treatment groups in triplicate using a complete randomized design. Feeding was performed two times a day until satiation at 08.00 and 16.00 h throughout the 56- days feeding trial and daily feed intake was recorded.

About 25% of the water was changed and uneaten feed and feces were collected daily by siphon from the bottom of each tank. Each tank was provided gentle aeration by an airstone, which was placed on the bottom of the tank. Water temperature and dissolved oxygen were measured daily, while pH, total ammonia–nitrogen and nitrite were measured once a week. During the experiment water temperature was maintained at 28 ± 0.5 °C, salinity between 39 and 41 ppt and pH between 8 and 8.2. Photoperiod was set on a 12 h light: 12 h dark schedule.

2.2. Experimental diets and sampling

Four experimental diets were provided from a commercial pellet diet (crud protein = 51%, lipid = 14%, ash = 15% and fiber = 2%) containing 0 (Control), 400 (LF400), 800 (LF800) and 120 (LF1200) mg bovine lactoferrin (LF) (Morinaga Milk Industry, Tokyo, Japan, 99% purity) per kg diet. Supplemented diets were prepared by spraying the bovine lactoferrin solutions (dissolved in distilled water) uniformly on the commercial feed. Then, gelatin (powder form) was dissolved in warm distilled water and, when its temperature was lower than 30 °C, was coated on pellets. The non-supplemented diet (Control) was prepared by spraying the feed with distilled water alone and then coated by spraying gelatin. The feeds were then dried at 40 °C for 24 h and stored at −20 °C until used.

After termination of the 56-day feeding trial, fish were starved for 24 h, and then all fish were anaesthetized with 0.05 ml l^{-1} 2-phenoxyethanol (Sigma–Aldrich) for weighing and counting. The fish were bulk weighed and the growth performance including weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR) and condition factor (CF) were calculated according to the following formulae:

WG (g $fish^{-1}$) = final body weight (g) - initial body weight (g)

SGR (% day^{-1}) = [Ln (final body weight) – Ln (initial body weight)] × 100/ days of the feeding trial

FCR = feed intake (g)/weight gain (g)

CF = (body weight (g)/total $length^3$ (cm)) × 100

SUR (%) = (total number of fish − number of dead fish)/100

HSI (%) = (liver weight/body weight) × 100

2.3. Collection of blood and skin mucus

At the end of the feeding trial, fish were anaesthetized with 2-phenoxyethanol (0.05 ml L^{-1}) 24 h after the last feeding. Blood samples (3 fish each tank) were obtained from the caudal vein using heparinized disposable-syringes (5000 IU sodium heparinate). Non-heparinized disposable-syringes were also applied to collect blood for serum analysis (2 fish from each tank). Blood was immediately centrifuged at 5000 g for 10 min at 4 °C, and plasma was stored at −80 °C until analyses. Skin mucus from each fish at the same area on the body surface was collected with a small piece of sterilized cotton, and immediately suspended in 1 ml of phosphate buffered saline (PBS, pH = 7.2) and centrifuged (2000 × *g*, 10 min, 4 °C).

2.4. Hematological assays

Hematocrit (Hct; %) was measured in 3 fish from each treatment by microcentrifugation method and determination of the percentage of packed cell volume after centrifuging in standard heparinized micro-hematocrit capillary tubes (3500 × *g* for 10 min) [20]. Hemoglobin concentration (Hb; g dl^{-1}) was spectrophotometrically measured by cyanmethemoglobin method (Drabkin). Number of red (RBC) and white blood cell (WBC) were counted manually by hemocytometer [20], using improved neubauer after diluting blood samples with adding Hayem solution for RBC and Turk solution for WBC. Differential blood counts containing lymphocyte, neutrophil, eosinophil and monocyte was manually carried out and calculated through a microscopic view (Blaxhall 1973). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Ref. [21].

2.5. Blood biochemical analysis

Serum samples were collected by centrifugation (3000 × *g* for 10 min, 4 °C) and used for biochemical analysis. Total protein (TP) and albumin (Alb) were determined using an autoanalyzer (Roche Diagnostics, Basel, Switzerland), in accordance with the manufacturer's manual. Serum total iron binding capacity (TIBC) was measured based on [22] using the magnesium carbonate sediment method (Magnetic TIBC kit, Tehran, Iran). Hemoglobin was determined by a SPOTCHEM EZ SP- 4430 system [23]. Plasma haemolytic activity of alternative complement activity (ACH50) was assayed following the procedure of [24]. The total protein content was assessed according to the method of [25], using bovine serum albumin as a standard. All biochemicals, including substrates were provided by sigma (Darmstadt, Germany) and were of the reagent grade.

2.6. Serum and mucus lysozyme activity

After 8 weeks of LF feeding, blood was gathered with a syringe from the caudal vein from the fishes, kept at 4 °C for 3 h after clotting and centrifuged at 4500 g for 5 min at 4 °C. Sera obtained were stored at −80 °C until further analysis. Lysozyme activity of serum and mucus was measured with turbidimetric assays [26]. For this, a turbidometric method was used based on lysis of *Micrococcus lysodeikticus* (Sigma) cells as lysozyme-sensitive gram-positive bacteria by lysozyme present in serum or mucus. *M. lysodeikticus* was suspended in phosphate buffer saline (PBS, pH = 7.2) at a concentration of 0.2 mg ml^{-1}. Twenty five microlitre of serum, diluted with an equal volume of PBS, were added to 125 ml of the substrate solution at room temperature and the optical density at 450 nm was measured after 1 and 5 min at room temperature. One unit of lysozyme activity was determined as a reduction in absorbance of 0.01 min^{-1}.

2.7. Determination of skin mucus protein

Skin mucus protein content was determined using a dye binding kit (BioRad, Hercules, California).

2.8. Antioxidant enzyme assay

For the determinations of antioxidant enzyme activities in liver, the frozen pooled samples were first defrosted and homogenised (10 strokes) in 9 vol of 0.05 M phosphate buffer pH = 6.6 containing 1% Triton X-100, using a homogenizer (Potter-Elvehjem, France). The homogenates were centrifuged (10 min, 10,000 × *g*, 4 °C) and the supernatants were used for enzymatic determinations.

Catalase (CAT) activity was measured in quartz cuvettes by the decrease of H_2O_2 absorbance at 240 nm [27]. Final concentrations in the cuvette were 50 mM KH_2PO_4/K_2HPO_4 pH = 6.5 and 50 mM H_2O_2. Glutathione reductase activity was measured spectrophotometrically at 25 °C by the modified method of [28]. The assay system contained 50 mM Tris–HCl buffer pH = 8.0, containing 1 mM EDTA, 1 mM oxidized form glutathione disulfid (GSSG) and 0.1 mM NADPH. One enzyme unit was defined as the amount that oxidizes 1 μmol NADPH per min under the assay conditions. The activity of glutathione S-transferase (GST: EC 2.5.1.18) was measured according to Ref. [29] using 1-chloro-2,4 dinitrobenzene (CDNB) as substrate. The rate of oxidation of NADPH by GSSG at 30" was used as a standard measure of enzymatic activity [30]. The reaction system of 1 ml contained: 1.0 mM GSSG, 0.1 mM NADPH, 0.5 mM EDTA, 0.10 M sodium phosphate buffer (pH = 7.6), and a suitable amount of the glutathione reductase sample to give a change in absorbance of 0.05 to O·SO/min. The oxidation of 1 pmol of NADPH/min under these conditions is used as a unit of glutathione reductase activity. Total protein of the homogenate was determined by the Coomassie brilliant blue dye-binding technique [25], with bovine serum albumin as standard.

2.9. Natural haemolytic complement activity

The activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets [31]. Aliquots (100 μl) of test serum as complement source, serially diluted in HBSS with Mg^{2+} and EGTA (ethylene glycol-bis β-aminoethyl ether), were added to 100 μl of SRBC (final serum concentrations from 10 to 0.078%). After incubation for 1 h at 22 °C, the samples were centrifuged (800 g, 5 min, 4 °C) to remove non-lysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 540 nm in a chemiluminometer. The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 100 μl of distilled water or HBSS to 100 μl samples of SRBC, respectively. The degree of haemolysis (Y) was estimated and the lysis curve for each specimen was obtained by plotting Y/(1-Y) against the volume of serum added (ml) on a log-log scaled graph. The volume of serum producing 50% haemolysis (ACH_{50}) was determined and the number of ACH50 units ml^{-1} was obtained for each experimental group.

2.10. Bacterial challenge test

At the end of the feeding experiment, five fish from each tank were randomly selected and moved to the challenge facilities. Fish from each replicate were randomly collected and injected intraperitoneally with 0.1 ml (100 μl of suspension Vibrio harveyi CFU mL^{-1}) virulent strain of Vibrio harveyi. The challenge trial lasted for 15 days during which the mortality rate was recorded. Dead fish were removed once a day. Vibrio harveyi was grown in 10 ml of tryptic soy broth (TSB) supplemented with 2% NaCl (w/v) in a shaking incubator for 24 h at 30 °C. After incubation, the bacteria were harvested by centrifugation (2000 g), then it was washed and re-suspended in filter sterilized (0.22 μm pore size) sea water (FSSW) and adjusted to the final concentration of 2×10^8 CFU mL^{-1}. The relative percent of survival (RPS) was calculated using the following formula:

RPS = (1-(% experimental mortality/ % control mortality)) × 100

2.11. Statistical analysis

All statistical analyses were performed using the SPSS 16.0 for Windows software package (SPSS Inc., Chicago, IL, USA). The normality was assessed using the Kolmogorov–Smirnov test. Data were tested for homogeneity of variances using Levene's test. Then, data were subjected to one-way ANOVA to evaluate the effect of lactoferin supplementation. Differences between means were tested using Turkey's multiple range test as a post hoc test. All Data are presented as mean ± standard error of mean. Differences were reported as significant if $P < 0.05$.

3. Results

3.1. Growth performance

Effects of different dietary Bovine lactoferrin levels on the growth performance and feed utilization are shown in Table 1. Compared to the control treatment, fish fed on LF supplemented diets displayed higher

Table 1
Growth performance and feed utilization of *A. latus* fingerling fed experimental diets containing different levels of LF (mean ± SE, n = 3).

Parameters[2]	Control	LF400	LF800	LF1200
Initial body weight (g)	12.03 ± 0.32	11.76 ± 0.75	11.56 ± 0.66	11.86 ± 0.75
Final body weight (g)	21.29 ± 0.45[b]	23.22 ± 0.88[a]	23.02 ± 0.22[a]	22.49 ± 0.9[a]
WG (%)	92.04 ± 11.01	98.19 ± 20.29[a]	89.99 ± 19.43	1 ± 15.76
CF	1.80 ± 0.26[ab]	1.54 ± 0.13[b]	2.09 ± 0.20[a]	2.01 ± 0.27[a]
SGR (% day^{-1})	1.01 ± 0.11	1.21 ± 0.18	1.22 ± 0.16	1.17 ± 0.11
FCR	1.59 ± 0.06	1.71 ± 0.09	1.66 ± 0.06	1.44 ± 0.08
PER	1.18 ± 0.12	1.10 ± 0.09	1.13 ± 0.18	1.25 ± 0.22
HSI (%)	0.62 ± 0.05	0.60 ± 0.1	0.67 ± 0.33	0.60 ± 0.17
Survival (%)	100	100	100	100

A different superscript in the same row denotes statistically significant differences ($P < 0.05$).
Abbreviations: WG, weight gain; CF, Condition factor; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio; HSI, hepatosomatic index.

Table 2
Hematological parameters of *A. latus* fingerling fed experimental diets containing different levels of LF (mean ± SE, n = 3).

Parameters	Control	LF400	LF800	LF1200
WBC ($\times 10^3$ µl^{-1})	5.96 ± 1.62	6.63 ± 1.05	7 ± 2.25	5.9 ± 8.5
RBC ($\times 10^6$ µl^{-1})	2.54 ± 6.13	2.85 ± 8.12	2.85 ± 4.83	2.84 ± 4.28
Hct (%)	17.33 ± 1.52	17 ± 4.35	24.33 ± 5.13	20.33 ± 1.52
Hb (g dl^{-1})	7.53 ± 1.02	8.10 ± 0.1	8.26 ± 0.37	8.06 ± 0.23
Lymphocyte (%)	71.5 ± 0.5	73 ± 0.57	73 ± 0.57	71.33 ± 1.2
Eosinophil (%)	2 ± 0.10	1.33 ± 0.33	1.66 ± 0.66	2 ± 0.57
Neutrophil (%)	21.66 ± 0.88	20.66 ± 1.20	20.66 ± 0.88	21.33 ± 0.33
Basophil (%)	3.5 ± 0.50	2.33 ± 0.33	2.5 ± 0.50	4 ± 1.00

A different superscript in the same row denotes statistically significant differences ($P < 0.05$).
Abbreviations: WBC, white blood cell counts; RBC, red blood cell counts; Hct, hematocrit; Hb, hemoglobin.

final body weight ($P < 0.05$). Weight gain (WG), feed conversion ratio (FCR), specific growth rate (SGR), protein efficiency ratio (PER), hepatosomatic index (HSI) were not affected by the dietary inclusion of LF ($P > 0.05$). Condition factor (CF) was significantly increased at dose of 800 and 1200 mg in compared to control group. CF in fish fed the LF800 and LF1200 diets was 2.09 and 2.01 higher than fish fed the control and LF400 diets, respectively ($P < 0.05$). There was no mortality or abnormally behaved fish in each dietary group during a feeding trial.

3.2. Hematological factors

The results of the hematological factors are shown in Table 2. Hematological factors (white and red blood cell counts and hemoglobin) were not affected by dietary LF levels, except hematocrit that was significantly higher in fish fed the 800 and 1200 mg added LF diets compared with the groups fed the control or 400 mg added LF diets ($P < 0.05$). The differential blood count (including lymphocytes, eosinophils, neutrophils and basophils) also did not vary ($P > 0.05$) in fish fed on different levels of LF.

3.3. Blood biochemical parameters

Blood biochemical analyses of *A. latus* fingerling fed on different levels of dietary LF are shown in Table 3. Total protein in the serum of the fingerling were increased as the dietary LF supplementation level was increased up to 1200 mg LF kg diet^{-1} ($P < 0.05$). Total iron-binding capacity (TIBC) and concentrations of serum iron were significantly affected by supplementation of dietary LF ($P < 0.05$). Serum TIBC of fingerling fed 400 mg LF kg^{-1} was significantly higher than that of the control. Iron levels in serum were significantly higher in fish fed the control diet and declined significantly in group fed 400 mg LF kg^{-1} ($P < 0.05$). Serum albumin was significantly ($P < 0.05$) affected by dietary LF and the highest concentration of albumin was 1.64 (g/dl) in 1200 mg LF kg^{-1}. Skin mucus total protein levels was not significantly influenced by supplementation of lactoferin ($P > 0.05$).

3.4. Serum and mucus lysozyme activity

The serum and mucus lysozyme activity of fish fed dietary LF are shown in Fig. 1. The serum and mucus lysozyme activity were influenced by dietary LF ($P < 0.05$). Serum lysozyme activity was increased with increasing dietary LF supplementation. Fish fed the diets with 1200 mg LF kg^{-1} dietary LF (42 ± 2.64 unit ml/min) had significantly higher lysozyme activity in serum than those fed the diets containing 400 and or control ($P < 0.05$) (Fig. 1). Mucus lysozyme activity of fish fed diets supplemented with 800 or 1200 mg LF was significantly higher than that of fish fed the control or LF400 diets ($P < 0.05$).

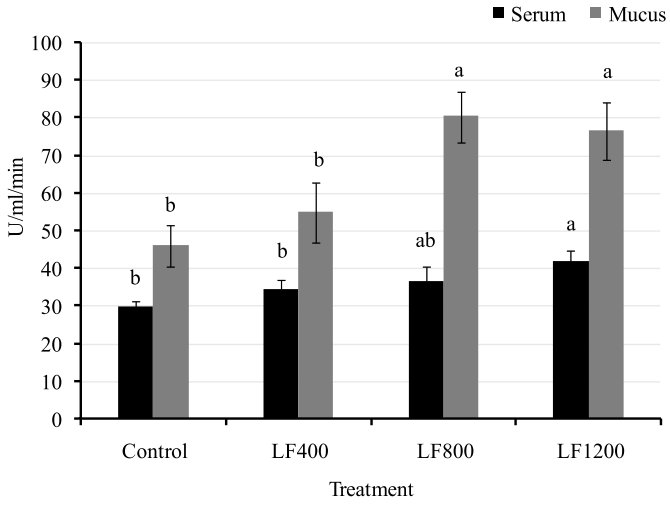

Fig. 1. Serum and mucus lysozyme activity of *A. latus* fingerling fed different LF-supplemented diets for 8 weeks. Data are expressed as mean ± SE. Bars different letters indicate significant differences ($P < 0.05$).

3.5. Alternative complement pathway activity

Significantly higher levels of ACH50 were recorded in fish receiving LF1200 mg LF kg^{-1} diet after 8 weeks in comparison to the control diet (Fig. 2); nonetheless, significant difference was not observed between the LF groups.

Table 3
Serum (total protein, albumin, TIBC: total iron binding capacity) and mucus parameter (total protein) of *A. latus* fingerling fed the experimental diets for 8 weeks (mean ± SE, n = 3).

Parameters	Control	LF400	LF800	LF1200
Serum total protein (g dl^{-1})	4.16 ± 0.21^{b}	4.35 ± 0.16^{b}	4.39 ± 0.20^{b}	5.19 ± 0.27^{a}
Serum albumin (g dl^{-1})	1.29 ± 0.07^{b}	1.37 ± 0.05^{b}	1.31 ± 0.03^{b}	1.64 ± 0.07^{a}
Serum TIBC (µg dl^{-1})	354 ± 8.5^{b}	378 ± 14.1^{a}	371 ± 5.1^{ab}	368 ± 6.4^{ab}
Serum iron (µg dl^{-1})	239 ± 2.5^{a}	227 ± 3.6^{b}	236 ± 4.1^{ab}	233 ± 4.2^{ab}
Mucus total protein (mg ml^{-1})	0.78 ± 0.12	0.86 ± 0.05	0.81 ± 0.07	0.81 ± 0.14

A different superscript in the same row denotes statistically significant differences ($P < 0.05$).

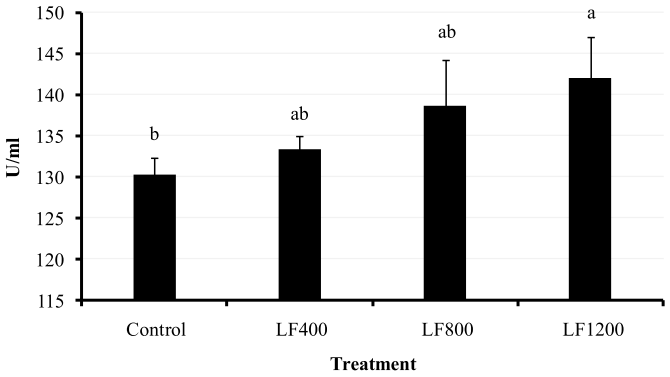

Fig. 2. ACH50 of *A. latus* fingerling fed different LF-supplemented diets for 8 weeks. Data are expressed as mean ± SE. Different letters indicate significant differences ($P < 0.05$).

3.6. Antioxidant enzyme assay

The effects of the different levels of dietary lactoferin on the antioxidant enzyme activity (catalase, glutathione reductase, glutathione S-transferase) of *A. latus* fingerling are shown in Fig. 3. Dietary supplementation with lactoferin did not have a significant ($P > 0.05$) impact on the activity of the antioxidant enzymes.

3.7. Bacterial challenge test

The percent survival of *A. latus* fingerling after challenge with *Vibrio harveyi* at 2×10^8 CFU mL^{-1} are presented in Fig. 4. Fish fed with diets supplemented with different levels of LF (LF400, LF800 and LF1200) showed significantly higher survival rate compared to control group ($P < 0.05$).

4. Discussion

In our study, fish fed on lactoferin (LF) supplemented diets showed higher final body weight than the control group. This is in agreement with Kakuta [32] who reported that dietary lactoferin supplementation of up to 1000 mg kg^{-1} improved growth performance in goldfish (*Carassius auratus*) after 4 weeks. Furthermore, increased growth in fish fed diets supplemented with LF has been observed in Nile tilapia (*Oreochromis niloticus*) [33]. In spite, in most of studies, LF administration did not have positive effects neither on growth performance nor on feed utilization in other fish species such as orange-spotted grouper (*Epinephelus coioides*) [34], Japanese flounder (*Paralichthys olivaceus*) [35], Atlantic salmon (*Salmo salar*) [36], Nile tilapia [37], gilthead seabream (*Sparus aurata*) [38] and Siberian sturgeon (*Acipenser baerii*) [39]. Multiple factors such as the synergistic effects between LF and some other unknown feed ingredients [34], feed processing [40], species-specific ability to dietary LF absorption [41], and/or experimental design (i.e., dietary LF dose, feeding duration, and culture conditions) [42] might be included in the obtained contradictory results in studies on LF supplementation.

Haematological analysis is typically carried out to detect fish health conditions. The results from the current study showed that the dietary LF did not significantly alter hematological parameters in yellowfin seabream fingerling. Some similar results were obtained in African electric blue cichlid (*Sciaenochromis fryeri*) [42], Nile tilapia [37], Siberian sturgeon (*Acipenser baeri*) [41] rainbow trout, (*Oncorhynchus mykiss*) [5,43], or Japanese eel (*Anguilla japonica*) [44]. In contrast, LF supplementary diet when combined with iron was observed capable of enhancing the haematological parameters of anaemic rats [45] or those of humans stricken with colorectal cancer [46], suggesting that the physiological conditions of the animal, as well as the availability of iron, are responsible for the LF potency to improve hematological

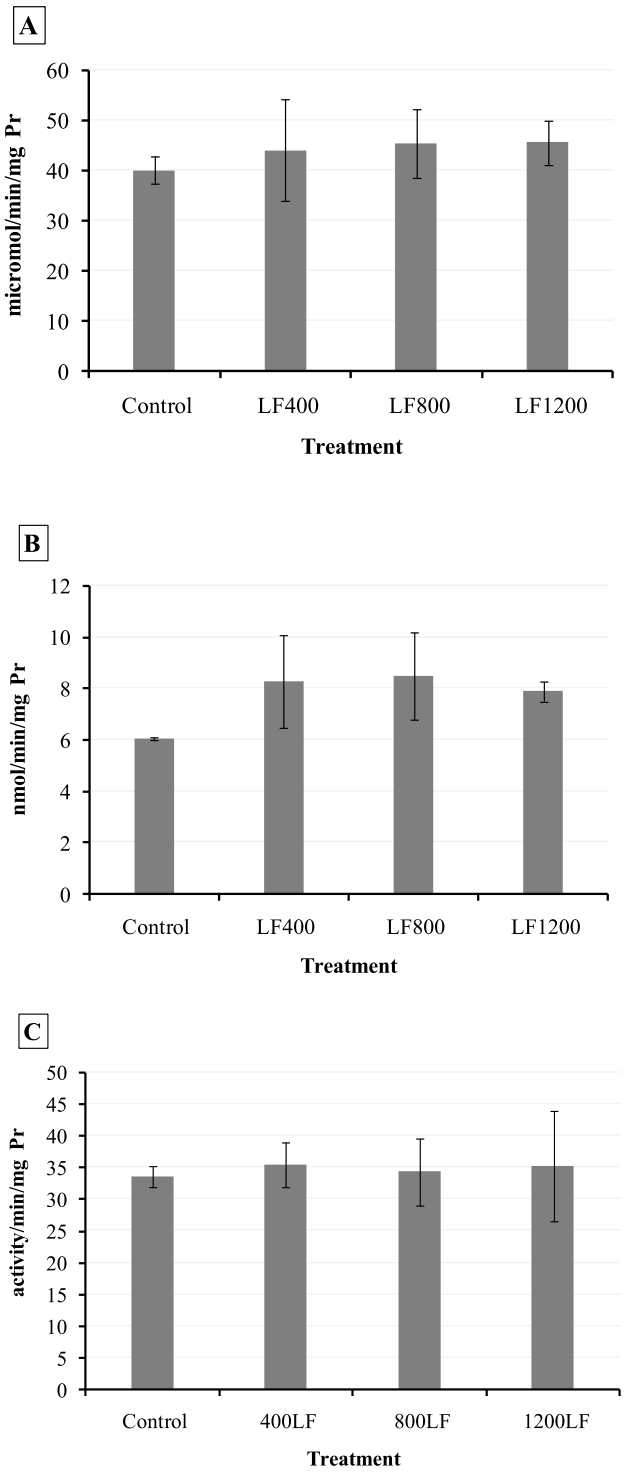

Fig. 3. Specific activities of Catalase **(A)**, glutathione reductase **(B)**, glutathione S-transferase **(C)** in *A. latus* fingerlings fed with different LF-supplemented diets for 8 weeks. Data are expressed as mean ± SE. Different letters indicate significant differences ($P < 0.05$).

indices.

Total protein and its major components, albumin perform a key role in the activity of the immune system in various species including fish [47]. In our study, plasma total protein increased with increasing dietary LF. Similar observations were noted by Chand et al. [16] in giant freshwater prawn fed a diet containing supplemented 100 mg kg^{-1} LF. Plasma iron and total iron binding capacity (TIBC) levels are regarded as the two major biochemical parameters in fish

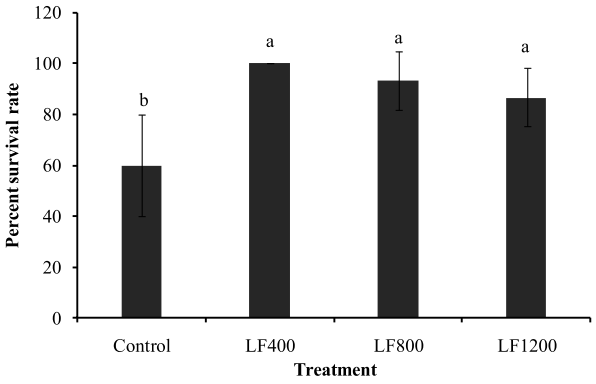

Fig. 4. Percent survival of *A. latus* fingerling upon challenge with *Vibrio harveyi*. Different letters indicate significant differences ($P < 0.05$).

receiving dietary LF [42]. We observed a decrease in plasma iron concentration and an increase in TIBC with increasing level of LF in diets which recorded the highest survival rate to *V. harveyi* infection. The ability of LF to separate iron, an essential nutrient needed for the growth of bacteria, is considered as one of its key antibacterial properties [48]. McNulty et al. [49] reported a reduction in Hb and RBC concentration in Nile tilapia infected with *S. iniae*. These authors hypothesized that the breakdown of Hb increases circulating iron levels and probably overloads the "iron withholding system" in *S. iniae*-infected tilapia. These findings provide further support that the iron sequestration could aid in the resistance of LF-fed yellowfin seabream to *V. harveyi*. We observed an increase in iron concentration and a decrease in TIBC in plasma with increasing level of LF in diet. According to our results, the influence of dietary LF on plasma iron of *A. latus* increased to a certain extent by increasing the dosage of LF more than 800 mg kg^{-1} these changes may be related to the iron-withholding ability of lactoferrin. Plasma iron deficiency is considered as a common response of some animals to bacterial infection [50]. Findings on the effect of dietary LF on iron metabolism in fish appeared to be inconsistent and sometimes contradictory. For example, serum iron levels have been reported to decline and TIBC to rise in Nile tilapia [37] and Siberian sturgeon [41] by dietary LF. However, different levels of lactoferrin did not significantly affect these parameters in rainbow trout [43]. These differences could be explained by the LF dose, the ability of the fish to utilize LF and different duration of Lf administration.

Lysozyme is an important part of the immune system in fish with an antibacterial activity, particularly against gram-positive bacteria [51]. The mechanism underlying the LF effects on lysozyme activity is not yet thoroughly understood. However, It be expected that LF could modulate and affect the responses of both innate and adaptive immune systems [52]. Humoral immunity such as lysozyme activity functions as an initial defense agent in the preference to cellular defense mechanism when attacked by an invader. In this study, serum and mucus lysozyme activity enhanced with increasing dietary LF. The results of this study and the previous studies indicated that dietary LF have a positive role on non-specific immune status in different aquatic species such as the Asian catfish [2], rainbow trout [5,43] and Siberian sturgeon [41], it may be claimed that fish immune cells are probably activated by dietary LF and/or its bioactive fragments. Conversely Welker et al. [37] showed no effect of LF administration on serum lysozyme activities in *O. niloticus*. The different result obtained in this study may be species specific and/or due to the differences in the LF feeding period.

The generation of reactive oxygen radicals are induced by metals particularly 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 a potent antioxidant factor [53]. The results of this experiment showed that the dietary supplementation of LF had no significant effect on antioxidant enzyme activity (catalase, glutathione reductase, glutathione S-transferase), indicating that there is an acceptable balance between dietary LF- and antioxidants in the experimental groups. In agreement with the present study, Lygren et al. [13] 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.

In the current study, haemolytic complement activity was higher in fish fed LF diet compared to the control group. Complement activity plays a major role in the innate immune response such as to destroy the cell surface membranes of pathogens. It is carried out by producing pores and opsonising pathogens for destruction by enhanced uptake of phagocytes and mediating through ligand-receptor interactions between the surfaces of the two cells [54]. Rahimnejad et al. [43] also reported significantly higher haemolytic complement activity in rainbow trout juveniles fed with 100 and 400 mg LF kg^{-1} diet for 6 weeks than that of control, but no effect of LF at 50 and 200 mg kg^{-1} doses after the same time of treatment. On the contrary, other studies reported no significant effects of dietary LF on haemolytic complement activity of Atlantic salmon *Salmo salar* [36], Siberian sturgeon [41], Asian catfish [2] and Nile tilapia [37]. Therefore, it appears that the response of ACH50 to LF may be species specific, dose and/or time-dependent.

In the present study, mortality rates among the challenged *A. latus* with Vibrio harveyi were significantly lower in the groups fed LF diet compared to the control group. Several studies have similarly observed enhanced disease resistance in fish fed diets supplemented with bovine LF. Kumari et al. [2] observed the increase of resistance of Asian catfish (*C. batrachus*) to *A. hydrophila* when fish were fed 100 mg kg^{-1} dietary LF for 1 week. Also, Welker et al. [37] reported the enhanced survival rate in *O. niloticus* challenged with *S. iniae* when fish were fed 800 mg kg^{-1} dietary LF for 8 weeks. In addition, the positive effects of dietary LF on bacterial resistance have been shown in rainbow trout [55] and red sea bream [14]. Previous studies showed that the increased bacterial resistance after LF treatment is due to the enhanced functions of non-specific immune system as well as the capacity LF to take up the Fe^{3+} ion, limiting of this nutrient by bacteria at the infection site and inhibiting the growth of these microorganisms as well as the expression of their virulence factors [56].

It has been shown that biochemical compounds of carcasses are affected by several factors, which can be related to morphological factors, physiological factors and environmental factors [57].

In conclusion, results of the present investigation indicate that dietary LF supplementation could be enhances the non-specific immunity and diseases resistance in *Acanthopagrus latus* fry. High level of LF (1200 mg kg^{-1} diet) improved growth, ACH50, Serum and mucus lysozyme activity, albumin and total protein parameters. It is therefore concluded that supplementation of LF in the diet up to 400 mg kg^{-1} could enhance the immune ability of *A. latus* and increase its resistance to *Vibrio harveyi*.

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