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Effects of dietary bovine lactoferrin on growth, haemato-biochemical parameters, immune functions and tolerance to air exposure stress in the African cichlid *Sciaenochromis fryeri*

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Abstract

The effects of dietary bovine lactoferrin (LF) on the growth, haemato-biochemical parameters, non-specific serum immunity and stress responses were investigated in the African cichlid, *Sciaenochromis fryeri*. Four isocaloric diets containing 0 (control), 200, 400 and 800 mg LF kg^{-1} diet were formulated and fed to juvenile fish for 8 weeks. The treatments had no effects on the survival rate, growth performance or haematological parameters. Nor were the serum levels of total protein, albumin and globulin significantly influenced. Serum iron levels and total iron binding capacity (TIBC), however, changed vigorously as the result of dietary LF. Dietary LF led to significant reductions in serum levels of alanine transaminase and alkaline phosphatase. The highest lysozyme and bactericidal activities were observed in the fish fed the diets with 200 and 800 mg/kg of LF, respectively, while no changes were observed in their ACH_{50} levels. Recovery rates were measured after 10 min of air exposure stress. The rate was about twice higher (36% vs 20%) in fish fed on 400 mg LF than in those fed the control diet. Results revealed that adding 400 mg/kg LF could be beneficial as an iron discriminator, for liver function as well as an immune and stress resistance promoter.

KEYWORDS

iron metabolism, lysozyme, recovery rate, serum liver enzymes

1 | INTRODUCTION

Immunostimulants have found wide applications in the aquaculture industry for improving upon the immune system of farmed fish and to enhance their health conditions (Sakai, 1999). A number of such immunostimulants have been administered as food additives both to regulate the non-specific immunity of fish and to assist in their protection against unwanted environmental conditions. These same additives may simultaneously have beneficial impacts on the physiological functions of fish that contribute to more successful aquaculture programmes (Wang, Sun, Liu, & Xue, 2016).

Lactoferrin (LF) comprises a family of iron-binding glycoproteins with a molecular weight of 80 kDa. It is derived from such secretions as mammalian milk and mucus and is categorized as an immunostimulant with numerous biological functions (Lönnerdal & Iyer, 1995). The clear mechanisms about the effects of LF on immunological parameters such as lysozyme activity are not fully known yet. However, some findings show that LF can affect the receptors of immune cells, promote the function of white blood cells and connect to the bacterial cell walls (Actor, Hwang, & Kruzel, 2009; González-Chávez, Arévalo-Gallegos, & Rascón-Cruz, 2009). Iron binding is an important property of LF, which allows LF to bind actively with iron and/or other metals (Welker, Lim, Yildirim-Aksoy, & Klesius, 2007). Among the other properties of LF, one can refer to its bactericidal, antifungal and antiviral activities (González-Chávez et al., 2009). Dietary LF has been reported to improve growth and stress responses in such fish species as goldfish (*Carassius auratus*) and Japanese flounder (*Paralychthys olivaceus*) (Kakuta, 1996; Yokoyama et al., 2005). Recent research has

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indicated the positive effects of dietary LF on certain physiological parameters of Siberian sturgeon, *Acipenser baeri* (Eslamloo, Falahatkar, & Yokoyama, 2012).

Aquarium keeping is among the hobbies most popular with millions of enthusiasts worldwide. A vast majority of ornamental fishes in the aquarium trade are of freshwater origin and farm-raised. The African cichlid (*Sciaenochromis fryeri*) known as the electric blue hap is indigenous to the Malawi Lake, Africa, and belongs to the cichlidae family which has many important representatives in the aquaculture industry not only as an ornamental fish but also as a table fish all around the world. This species has been introduced to many other countries as an ornamental species, such as the USA, Singapore, the Philippines and Iran (Konings, 1993).

Based on the knowledge presently available on the effects of dietary LF on several fish species, it seems that LF impacts could be variable depending on the fish species, fish age/size, LF dose and timing of administration. Hence, recommending LF as a regular feed supplement to the aquaculture industry relies on the prior determination of its biological effects on different aquatic species under specific conditions. This study was carried out to evaluate the effects of dietary LF on the growth performance, haemato-biochemical and immunological parameters, and stress responses of the African cichlid, *S. fryeri*, under laboratory conditions.

2 | MATERIAL AND METHODS

2.1 | Fish and feeding trial

The electric blue hap, *S. fryeri*, was obtained from a local commercial supplier. The fish were first identified according to Konings (1993) and acclimatized. They were then fed the formulated plain diet (the control diet) for 2 weeks prior to the experiment. At the beginning of the experiment, the fish were subjected to a 1% saline bath for 30 min for disinfection (Post, 1983). After acclimation, fish (about 5 g/individual) were pooled and randomly distributed among 12 aquariums (groups of 50 fish) with each dietary treatment allocated to three aquariums (i.e., three replicates for each treatment). A practical isocaloric diet (10.8 KJ/g diet) containing 375 g crude protein, 122 g fat, 63 g fibre and less than 80 g moisture per kilo feed (National Research Council (NRC), 1973) was formulated with fish meal, soybean oil, soybean meal, corn, corn gluten and fish oil supplemented with 0 (control), 200, 400 and 800 mg of LF (Morinaga Milk Industry, Tokyo, Japan, 99% purity) per 1,000 g of the diet. The materials were formed into pellets, allowed to dry at room temperature and stored at 4°C until use; the moisture content was less than 100 g per kilo feed. The fish were hand-fed ad libitum, three times a day at 10:00, 14:00 and 18:00. At each feeding round, all the food was consumed with no leftover. The experiment lasted for 56 days (8 weeks) and about 30% of the water was changed every two to 3 days to maintain the water quality parameters at the desired levels. Temperature (25.5 ± 1°C), pH (7.9 ± 0.5), dissolved oxygen (6.5 ± 0.6 mg/L), EC (464.75 ± 10.22 μS/cm) and total hardness (249.33 ± 3.22 mg/L $CaCO_3$) were monitored at daily or weekly intervals.

2.2 | Growth performance

At the end of the experiment, survival rate (SR), mean final weight (MFW), mean weight gain (MWG), specific growth rate (SGR), feed conversion ratio (FCR), condition factor (CF) and hepatosomatic index (HSI) were determined using the following relations (Bahrami Babaheydari, Paykan Heyrati, Akhlaghi, & Dorafshan, 2014):

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

MWG (g) = (mean final weight − mean initial weight);

SGR (% /day) = 100 × $((\ln W_1 - \ln W_0)/t)$, where W_0 and W_1 are average initial and final body weights, respectively, and t is time (days);

FCR = food consumed (g)/weight gain (g);

CF (g/cm) = weight (g)/(length (cm))^3.

2.3 | Haemato-biochemical analysis

2.3.1 | Haematological analysis

Ten fish from each replicate (30 from each treatment) were starved for 24 hr at the end of the experimental period when they were anaesthetized with MS_{222} (100 ppm). Blood samples were then obtained from the caudal vein and diluted with the Dice solution (Daneshvar, Ardestani, Dorafshan, & Martins, 2012). Red and white blood cell counts (RBC and WBC, respectively) were manually measured using a haemocytometer (Paul Marienfeld Gmbh, Lauda, Koenigshofen, Germany). Haematocrit (Hct: %) was measured after spinning at 3500 × g for 10 min. Haemoglobin concentration (Hb: g/dl) was evaluated using the cyanmethaemoglobin method (Daneshvar et al., 2012).

2.3.2 | Biochemical analysis

At the end of the feeding trial, blood samples from 30 fish in each treatment were collected from the caudal vein after 24 hr of starvation. Serum samples were separated by centrifugation (3000 × g for 10 min) and used for biochemical analysis. Total protein (TP), albumin (Alb), aspartate transaminase (AST), alkaline phosphatase (ALP), alanine transaminase (ALT) and serum iron levels were determined using an automated analyser for serum chemistry (Roche, COBASMIRA Plus CC), in accordance with the manufacturer's manual. Serum globulin (Glb) levels were calculated by subtracting albumin from total protein levels. Serum total iron binding capacity (TIBC) was evaluated based on Tietz & Andresen, (1986) using the magnesium carbonate sediment method (Magnetic TIBC kit, Tehran, Iran).

2.4 | Serum immunological parameters

Lysozyme activity was measured as described in Cha, Lee, Song, Lee, and Jeon (2008) based on the lysis of *Micrococcus lysodeikticus* cells

that are known to be lysozyme-sensitive gram-positive bacteria depending on their lysozyme content (Cha et al., 2008).

Complement activity was assayed via the alternative pathway (ACH_{50}) using rabbit red blood cells (RaRBC) according to the method described in Yano (1992) (Yano, 1992).

Serum bactericidal activity against *Aeromonas hydrophila*, which is responsible for ordinary bacterial infections in fish, was determined using the method described in Rao, Das, Jyotyrmayee, and Chakrabarti (2006). Briefly, a bacterial culture of *A. hydrophila* was centrifuged and resuspended in a phosphate-buffered saline (PBS). After serial dilutions with PBS, the suspensions were incubated at 37°C for 60 min. Finally, the nutrient agar plate was cultured for 24 hr at 37°C and the colonies were counted (Rao et al., 2006).

2.5 | Air exposure stress

An air exposure test as one of the harshest stresses was carried out to evaluate fish tolerance of the out-of-water environment; the test period was designated as the "exposure time" (Yokoyama et al., 2006). First of all, in order to determine the appropriate "length of time" for air exposure test, a pretest was carried out. Briefly, some fish that were not included in the main experiment were exposed to the air for different periods of time (5- to 15-min, 5-min intervals). The best length of time was determined as 10 min based on relative survival rates (data are not shown). Then, 15 fish from each treatment were randomly caught. The body surface of each fish was dried using a piece of cotton before they were all exposed to air on a black coloured plastic net for 10 min and finally transferred back into well-aerated water. Dead fish, as judged by the lack of gill movements, were recorded within 60 min after their transfer to water. The test was repeated three times per treatment to include a total of 180 fish. Finally, recovery rate was calculated using the following formula:

Recovery rate (%) = $(N_i - N_d/N_i) \times 100$,

where N_i and N_d are the numbers of fish at the beginning of the test and the number of dead fish, respectively.

2.6 | Statistical analysis

The data thus obtained were reported as means ± standard error of mean (*SEM*) of three replicates (each aquarium was considered as one experimental unit) and subsequently subjected to one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) to find out significant differences among the treatments at $p < .05$. Statistical analysis was performed using SPSS 22.0.

3 | RESULTS

3.1 | Survival rate and growth performance

Survival rates (SR) ranged from 88.5%to 91.5% without any significant differences among the treatments (Table 1). Mean final weight (MFW), MWG, SGR and CF as growth parameters were not affected by the graded levels of dietary LF ($p > .05$; Table 1). Neither was FCR or HSI significantly ($p > .05$) influenced by the dietary supplementation of LF (Table 1).

3.2 | Haemato-biochemical parameters

3.2.1 | Haematological parameters

The effects of different concentrations of orally administered LF on African cichlid observed through haematological assays are summarized in Table 2. Compared to the control, no statistically significant changes occurred in the parameters (RBC, WBC, Hct and Hb) with varying concentrations of LF (200, 400, 800 mg LF kg^{-1} diet) ($p > .05$).

3.2.2 | Serum biochemical parameters

Dietary LF had no effects on serum TP, Alb or Glb levels ($p > .05$; Table 3). Serum ALP and ALT measurements showed significant reductions as a result of LF supplementation, especially at the higher doses of 400 and 800 mg LF kg^{-1} diet, as compared to the control ($p < .05$; Table 4). This is due to the fact that serum levels of AST were not influenced by LF levels ($p > .05$; Table 4). Compared to the control, the lowest serum iron content was measured in the fish on the diet supplemented with 400 mg LF kg^{-1} diet ($p < .05$; Table 4). The results also indicate that TIBC levels ranged over 282.67420.67 μg/dl, with the highest value recorded for the group of fish receiving the 400 mg LF kg^{-1} diet ($p < .05$; Table 4).

TABLE 1 Survival rate and growth performance of the African cichlid, *Sciaenochromis fryeri*, fed different levels of lactoferrin (LF) for 8 weeks

Variable (Unit)	0	200	400	800
SR (%)	88.5 ± 2.1	91.3 ± 1.4	91.0 ± 2.2	91.5 ± 2.5
MFW (g)	8.42 ± 0.29	9.63 ± 0.13	8.76 ± 0.30	8.96 ± 0.29
MWG (g)	3.53 ± 0.29	4.04 ± 0.13	3.64 ± 0.30	3.76 ± 0.29
SGR (%/day)	0.80 ± 0.06	0.91 ± 0.02	0.81 ± 0.06	0.85 ± 0.06
CF (g/cm^3)	1.24 ± 0.03	1.28 ± 0.04	1.49 ± 0.05	1.35 ± 0.04
FCR	1.85 ± 0.17	1.70 ± 0.22	1.93 ± 0.18	1.76 ± 0.19
HSI (%)	2.72 ± 0.33	2.05 ± 0.05	2.38 ± 017	2.17 ± 0.16

No significant changes were observed ($p > .05$).
Data are presented as means ± *SEM*.
SR, survival rate; MFW, mean final weight; MWG, mean weight gain; SGR, specific growth rate; CF, condition factor; FCR, feed conversion rate.

TABLE 2 Haematological assays of the African cichlid, *Sciaenochromis fryeri*, fed graded levels of lactoferrin (LF) for 8 weeks

Variable (Unit)	0	200	400	800
RBC ($\times 10^3$ /mm^3)	1400.00 ± 360.5	1533.33 ± 338.3	1440.00 ± 230.0	1413.33 ± 59.2
WBC ($\times 10^3$ /mm^3)	10.83 ± 2.20	11.50 ± 1.08	10.57 ± 3.32	11.20 ± 1.13
Hct (%)	27.33 ± 2.73	25.00 ± 2.31	26.67 ± 4.18	25.33 ± 0.67
Hb (g/dl)	8.67 ± 0.34	8.30 ± 0.31	8.60 ± 1.20	8.17 ± 0.34

No significant changes were observed ($p > .05$).
Data are presented as means ± *SEM*.
RBC, red blood cells; WBC, white blood cells; Hct, haematocrit; Hb, haemoglobin.

TABLE 3 Serum protein, albumin and globulin levels in the African cichlid, *Sciaenochromis fryeri*, fed graded levels of lactoferrin (LF) for 8 weeks

Variable (g/dl)	0	200	400	800
Total protein	4.83 ± 0.15	5.30 ± 0.35	5.17 ± 0.23	4.90 ± 0.12
Albumin	1.43 ± 0.27	1.33 ± 0.22	1.30 ± 0.26	1.33 ± 0.23
Globulin	3.40 ± 0.26	3.97 ± 0.33	3.87 ± 0.32	3.57 ± 0.12

No significant changes were observed ($p > .05$).
Data are presented as means ± *SEM*.

TABLE 4 Serum levels of hepatic enzymes and Iron-dependent indices of the African cichlid, Sciaenochromis fryeri, fed graded levels of lactoferrin (LF) for 8 weeks

Variable (unit)	0	200	400	800
ALP (IU/L)	69.00 ± 8.08^a	50.00 ± 2.08^b	46.00 ± 4.04^b	49.67 ± 2.73^b
ALT (IU/L)	45.67 ± 1.76^a	35.50 ± 5.50^{ab}	22.67 ± 1.20^b	27.33 ± 5.93^b
AST (IU/L)	228.00 ± 26.00^a	262.33 ± 43.88^a	234.00 ± 32.58^a	157.00 ± 43.82^a
Iron (μg/dl)	150.00 ± 10.26^a	120.00 ± 15.72^{ab}	86.00 ± 14.00^b	104.67 ± 8.21^{ab}
TIBC (μg/dl)	282.67 ± 21.40^b	295.00 ± 52.20^b	420.67 ± 59.40^a	345.00 ± 26.46^{ab}

ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; TIBC, total iron binding capacity.
In each row, groups with different superscript are statistically different ($p < .05$).
Data are presented as means ± *SEM*.

3.3 | Serum immunological parameters

Serum lysozyme activity was observed to improve as a result of LF supplementation in the diet, with its highest (677.10 ± 11.10 unit min./ml) observed in the fish fed the diet containing 200 mg LF kg^{-1} diet followed by those receiving the diet containing 400 mg LF kg^{-1} diet ($p < .05$; Figure 1a).

ACH_{50} levels ranged over 333–392 unit/ml without any significant variation across the treatments ($p > .05$; Figure 1b).

Improvements were observed in the bactericidal activity of only those fish that received the highest level of LF supplementation ($p < .05$; Figure 3c). Lower levels of LF, however, did not lead to improved serum bactericidal activity compared to that observed in the control treatment ($p > .05$; Figure 1c).

3.4 | Air exposure stress

Results of the air exposure stress test revealed that recovery rates were in the range of 20.00%–36.6% (Figure 2). The fish on the treatment with 400 mg/kg of LF showed the highest recovery rates of about two times those of the fish on the control diet ($p < .05$).

4 | DISCUSSION

The results of the present study revealed that dietary LF had no effects on the survival and growth performance of the African cichlid under the culture conditions specified. These results are in agreement with those of previous studies that reported dietary administration of LF had neither positive nor negative effects on the survival rates or growth performance of such varied fish species as common carp, *Cyprinus carpio* (Kakuta, 1998); Atlantic salmon, *Salmo salar* (Lygren, Sveier, Hjeltnes, & Waagbø, 1999); Japanese flounder (Yokoyama et al., 2005); Nile tilapia, *Oreochromis niloticus* (Welker et al., 2007); and Siberian sturgeon (Eslamloo et al., 2012). In contrast to our results, Kakuta (1996) reported that dietary LF of up to 1000 mg/kg diet in commercial feeds improved growth performance in goldfish. It may be hypothesized that the interaction between LF and some other unknown food ingredients such as iron might have affected LF absorption to improve its functions (Yokoyama et al., 2005). It has also been suggested that culture conditions and species-specific capability to exploit dietary LF might have contributions to improving growth performance (Eslamloo et al., 2012). Finally, LF dose, feeding duration and feed processing conditions are among the other important factors

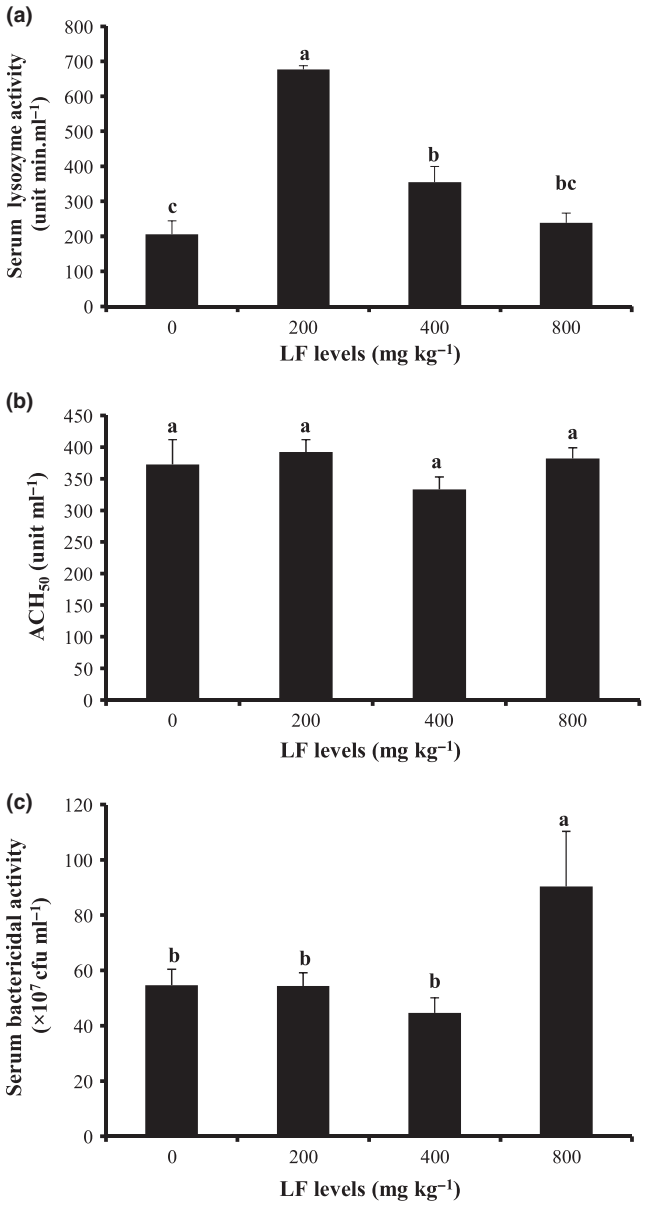

FIGURE 1 Serum non-specific immune parameters of the African cichlid, *Sciaenochromis fryeri*, fed graded levels of lactoferrin (LF) for 8 weeks: (a) lysozyme activity, (b) ACH_{50} and (c) bactericidal activity. Bars with different superscripts are significantly different ($p < .05$). All the data are reported as means ± *SEM*

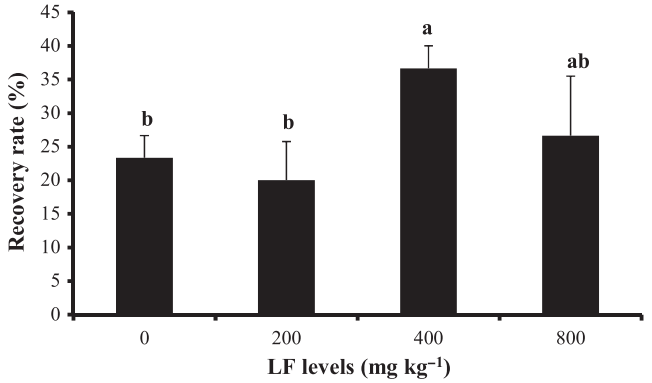

FIGURE 2 Recovery rate (%) of the African cichlid, *Sciaenochromis fryeri*, fed graded levels of lactoferrin (LF) for 8 weeks after exposure to air for 10 min. Bars with different superscripts are significantly different ($p < .05$). All the data are reported as means ± *SEM*

likely to be involved in the growth performance of fish supplemented with dietary LF.

Haematological analysis is typically carried out to detect fish health conditions. The results of this study did not indicate any effects of dietary LF on the main haematological parameters of African cichlid (*S. fryeri*). Nor had those of rainbow trout, *Oncorhynchus mykiss* (Rahimnejad, Agh, Kalbassi, & Khosravi, 2012; Sakai, Kobayashi, & Yoshida, 1995), Nile tilapia (Welker et al., 2007) or Japanese eel, *Anguilla japonica* (Ren et al., 2007), been reportedly affected by dietary LF. In contrast, LF supplementary diet when combined with iron was found capable of improving the haematological parameters of anaemic rats (Kawakami, Hiratsuka, & Dosako, 1988) or those of humans stricken with colorectal cancer (Moastafa, El-Sissy, El-Saeed, & Koura, 2014), suggesting that the physiological conditions of the animal as well as the availability of iron are responsible for the LF potency to boost haematological indices. Hence, it is essential to investigate the capacity of dietary LF to improve blood parameters in such unhealthy fish as anaemic ones.

Total protein and its major components, albumin and globulin, play a key role in the activity of the immune system in different species including fish. Our results indicated no significant effects of dietary LF on elevating the levels of total blood protein and its major components in the African cichlid although slight increments were observed in these parameters in the fish fed the LF-supplemented diet rather than the LF-free one. This finding is in agreement with those reported by Ren et al. (2007) studying the Japanese eel and Eslamloo et al. (2012) experimenting with the Siberian sturgeon. Both studies found that oral administration of LF did not lead to any improvements in these serum biochemical parameters. Nevertheless, a positive relation may be assumed to exist between serum total protein and dietary LF levels because of the LF structure as a glycoprotein and its resistance to proteolysis. Hence, future research may be directed towards using different levels of dietary LF supplementation to verify such a relationship.

Another finding of the present study indicated that dietary LF supplementation generally led to reduced serum levels of liver enzymes including ALT and ALP. Some previous finding indicates that unusual increase in serum liver enzymes can be considered as symptoms of liver dysfunction (Shi, Li, Zhuang, Nie, & Long, 2006). Proper functioning of the liver is representative of vital functions of the body. The decline observed in ALT and ALP levels may reflect the potential positive effects of dietary LF on the liver. Generally speaking, our present knowledge of the effects of dietary LF on serum liver enzymes in fish is scant. Japanese flounder fed diets supplemented with LF rather than LF-free ones reportedly showed significantly higher levels of heat shock proteins (HSP70s), which is regarded as a general primary cellular event in the liver tissue in response to stress (Iwama, Vijayan, Forsyth, & Ackerman, 1999; Yokoyama et al., 2005). HSP acts as a regulator of liver functioning under stressful conditions (Lee et al., 2004). Consequently, it is expected that higher levels of synthesized

HSP contribute to the normal functioning of the liver, lowering serum liver enzymes and improving whole-body vital functions. Studies of other mammals such as pigs and rats have indicated the positive role of orally administrated LF on liver functions (Burrin, Wang, Heath, & Dudley, 1996; Tsubota et al., 2008). Recently, dietary LF has been shown to improve liver and kidney functions in humans (Moastafa et al., 2014). This is because dietary LF may protect liver against damages by reactive oxygen species (ROS) as LF can serve as an antioxidant (Actor et al., 2009; Legrand, Elass, Carpentier, & Mazurier, 2005). From a different perspective, iron is essential as a catalyst for the production of ROS. Therefore, the ability of LF to withhold iron diminishes the harmful effects of ROS in the liver and, thereby, lowers serum liver enzymes (Adlerova, Bartoskova, & Faldyna, 2008). The LF anti-inflammatory effect, due to its antimicrobial activity, prevents liver inflammation. Hence, liver damage in the African cichlid could be considered as another possible explanation for the declining serum liver enzyme. However, further study is required to determine the exact effects of LF on liver functions in fish.

Serum iron and TIBC levels are considered as the two major biochemical parameters in fish receiving dietary LF. It was observed that, compared to the control, the fish receiving 400 mg/kg of LF exhibited a decrease in their serum iron levels but elevated levels of TIBC. These changes are related to the iron-withholding ability of lactoferrin. The role of LF as a transferrin has been clearly demonstrated. Thus, it could be assumed that dietary LF may cause a significant rise in serum TIBC coinciding with decreased blood iron level. Studies of different fish species have, however, yielded no consistent results about the dietary LF effects on iron metabolism. For example, while serum iron levels have been reported to decline and TIBC to rise in Nile tilapia (Welker et al., 2007) and Siberian sturgeon (Eslamloo et al., 2012) as a result of LF supplementation, these same parameters were not significantly differed in rainbow trout (Rahimnejad et al., 2012). These differences could be explained by the LF dose duration and/or the ability of the fish to utilize LF. Serum iron deficiency is considered as a common response of some animals to bacterial infection, as a presumably defensive reaction against microbial invasion (Brown & Holden, 2002). Serum iron deficiency due to the iron-withholding capacity of LF can therefore be regarded as a means of preventing certain diseases in the African cichlid.

At least two of the experimental groups of fish receiving dietary LF showed significant improvements in their serum lysozyme levels relative to the control. Those receiving 400 and 800 mg of dietary LF, however, showed significant reductions in their lysozyme levels compared to those on the diet supplemented with 200 mg of LF. Lysozyme is an important component of the immune system in fish with an antibacterial activity, especially against gram-positive bacteria (Chipman & Sharon, 1969). The mechanism underlying the LF effects on lysozyme activity is not yet completely understood. However, LF receptors have been discovered on a wide variety of immune cells in different animals so that LF could be expected to modulate and affect the responses of both innate and adaptive immune systems (Actor et al., 2009). Generally, serum lysozyme activities are linked to phagocytic cells, granulocytes and monocytes. Serum lysozyme is also secreted by tissue macrophages (Alhazmi, Stevenson, Amartey, & Qin, 2014). LF is known to induce changes in the leucocytes of the innate immune system through increasing natural killer cell activity, promoting the function of neutrophils by enhancing phagocytic activity and activating macrophages through increasing cytokine but limiting intracellular pathogen proliferation (Moastafa et al., 2014). Based on the results of this study and the findings of previous studies indicating the positive role of dietary LF on non-specific immune status in different aquatic species such as the Asian catfish (Kumari, Swain, & Sahoo, 2003), rainbow trout (Rahimnejad et al., 2012; Sakai et al., 1995) and Siberian sturgeon (Eslamloo et al., 2012), it may be claimed that fish immune cells are probably activated by dietary LF and/or its bioactive fragments. It is very hard to provide a decisive explanation for decrease in lysozyme activity in fish receiving diets supplemented with 400 and 800 mg of LF relative to those receiving the one supplemented with only 200 mg of LF. Several factors such as nutritional status, seasonal variation, sex and sexual maturation, salinity, pH, water temperature, stresses and infections could be considered as possible parameters involved in the variations in lysozyme activity in vertebrates (Alhazmi et al., 2014; Saurabh & Sahoo, 2008). Indeed, excessive and/or long-term administration of some immunostimulants may lead to the declining response of the defence system or reversion to a previous state by negative feedback systems in fish (Sakai, 1999). Such negative effects have been previously reported not only for LF in several fish species including the Asian catfish (Kumari et al., 2003) and rainbow trout (Zhang, Swain, Bøgwald, Dalmo, & Kumari, 2009) but also for some other active compounds such as medicinal plant extracts in common carp (Bahrami Babaheydari, Dorafshan, Paykan Heyrati, Mahboobi Soofiani, & Vahabi 2014; Bahrami Babaheydari, Paykan Heyrati et al., 2014). These parameters can vindicate the decrease in lysozyme activity in fish fed the diets supplemented with 400 or 800 mg of LF when compared to those receiving 200 mg of LF in their diet.

Based on the results obtained, it was also found that graded dietary levels of LF were not able to promote any changes in serum ACH_{50} in the fish. A similar finding has been reported for the Atlantic salmon (Lygren et al., 1999), Asian catfish (Kumari et al., 2003), Nile tilapia (Welker et al., 2007) and Siberian sturgeon (Eslamloo et al., 2012). But in contrast to our observation, Rahimnejad et al. (2012) observed significantly higher levels of serum ACH_{50} in rainbow trout receiving 100 and 400 mg LF kg^{-1} diet after 6 weeks relative to those recorded by the groups receiving either an LF-free diet or one with 200 mg LF kg^{-1} diet (Rahimnejad et al., 2012). This difference could be ignored at 2, 4 or 8 weeks of feeding trial, possibly indicating the time-dependent effect of dietary LF on ACH_{50}. Evidence has shown the strongly negative effect on the ACH_{50} pathway of lactoferricin (LFcin), a bioactive fragment that is insulated from LF via the proteolytic attack during the gastric transit in some animals (Flanagan & Willcox, 2009). It has also been documented that dietary LF is actively affected by proteolytic cleavage through gastric transit, changing the majority of the dietary LF to LFcin in the rainbow trout (Cecchini & Caputo, 2009). Given that no adverse effects of dietary LF were observed on ACH_{50} in the African cichlid, it is likely that the African cichlid has the ability to absorb LF in its intact form. These differences among the different fish species can be explained by the mutability of enzyme (protease and pepsin) activities.

The results obtained in the present study concerning the effects of dietary LF on resistance to specific bacterial infections (bactericidal activity) were inconsistent with those reported in other species. In this experiment, the fish receiving maximum LF (800 mg/kg) in their diets exhibited significantly (by up to 150%) improved serum bactericidal activity against *A. hydrophila* as compared to the other treatments including the control. Similarly, the Siberian sturgeon receiving graded levels of dietary LF showed improved serum bactericidal activity when fed diets with the highest level of LF (1600 mg/kg diet) (Eslamloo et al., 2012). Moreover, the positive effects of dietary LF on bacterial resistance have been shown in rainbow trout (Sakai, Otubo, Atsuta, & Kobayashi, 1993) and red sea bream (Kakuta & Kurokura, 1995). Nevertheless, the Atlantic salmon did not show any positive changes in its disease resistance after 19 days of receiving an LF-supplemented diet (Lygren et al., 1999). Indeed, Welker et al. (2007) found no significant changes in the serum bactericidal activity of the Nile tilapia against *Streptococcus iniae* as a result of dietary LF (Welker et al., 2007). The external membrane of *A. hydrophila* can be affected by LF connection to the lipopolysaccharide in the membrane, which could lead to significant pathogen mortality (González-Chávez et al., 2009). It could be demonstrated that LF imbibes into the serum or the combined effects of LF action on the immune factors are responsible for the significant rise in the serum bactericidal activity only in those fish receiving the highest LF dose (i.e., 800 mg LF kg^{-1}). Nevertheless, further study is required to understand the detailed effects of LF on the bactericidal activity of different fish species including the African cichlid.

The air exposure test is a routine stress test in the aquaculture industry. In this experiment, the fish receiving the diet supplemented with 400 mg LF exhibited a significant improvement in their recovery rates when compared to those receiving 0 or 200 mg of LF, suggesting an enhanced tolerance of stress with increasing LF doses. Most published works on different fish species such as the rainbow trout (Kakuta, 1997), the Japanese flounder (Gallardo-Cigarroa et al., 2000) and the orange-spotted grouper (Yokoyama et al., 2006) have revealed the positive effects of dietary LF on recovery rates after exposure to air, high temperatures or high-density stress. Inconsistent with our findings, Kakuta (1998) found that their common carp fish receiving dietary LF showed lower stress responses than their control (Kakuta, 1998). Moreover, dietary LF did not reportedly affect the stress indicators (serum glucose and cortisol levels) in the Siberian sturgeon during the rest period (Eslamloo et al., 2012). The mechanism governing the LF impact of responses to stress is not yet fully understood. Yokoyama et al. (2006) suggested that enhanced tolerance to air exposure and low salinity was due to the stimulation of the non-specific defence mechanism such as HSP and mucus secretion as a result of LF administration (Yokoyama et al., 2006). It may be claimed that exposure to a stressor affects the body surface, that is, skin. Therefore, the high tolerance of the skin to stressors is vital for the resistance of the whole body. It has been reported that oral administration of LF increases mucus secretion in the Japanese flounder (Gallardo-Cigarroa et al., 2000) and Siberian sturgeon (Eslamloo et al., 2012). On the other hand, the serum level of TIBC in this experiment was significantly higher, as was the recovery rate also, in the group receiving the diet containing 400 mg of LF than it was in the control group. Thus, it is likely that the enhanced recovery rate observed in the group receiving 400 mg of supplementary LF was due to the establishment of homeostasis with the iron-withholding ability of lactoferrin.

The most beneficial effects of dietary LF mainly based on serum biochemical and air exposure stress tolerance were found in the group of fish receiving 400 mg LF kg^{-1} diet. However, the highest levels of serum lysozyme and bactericidal activities as main immunological parameters were observed in fish fed on the diet containing 200 and 800 mg LF kg^{-1}, respectively. Certain other parameters such as growth and haematological indices did not, however, exhibit significant changes as a result of LF supplementation. It may therefore be concluded that the levels of dietary LF can differ according to the aim of use. However, further studies are recommended to investigate the iron binding property of LF, other challenge tests such as handling and bacterial infection. Evaluation of different modes of LF application such as short-/long-term baths, especially for ornamental fishes, is highly recommended.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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