Fish and Shellfish Immunology 94 (2019) 916–923

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Lactobacillus fermentum and/or ferulic acid improved the immune responses, antioxidative defence and resistance against *Aeromonas hydrophila* in common carp (*Cyprinus carpio*) fingerlings

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ARTICLE INFO

Keywords:
Aeromonas hydrophila
Common carp
Ferulic acid
Immune response
Oxidative status
Probiotics

ABSTRACT

This study investigates the possible effects of using *Lactobacillus fermentum* (LF) and/or ferulic acid (FA) in common carp (*Cyprinus carpio*) on some immunological parameters as well as resistance against *Aeromonas hydrophila*. Four diets were prepared including control diet and three diets supplemented with LF (10^8 CFU/g), FA (100 mg kg^{-1}) or LF + FA (10^8 CFU/g + 100 mg kg^{-1}). After 8 weeks, fish fed LF or/and FA had significantly higher final body weight, weight gain, and specific growth rate when compared to control group ($P < 0.05$). The feed conversion ratio of fish fed LF or/and FA were noticeably lower than control ($P < 0.05$). No alterations were observed in case of haematological parameters except red blood cells (RBCs), white blood cells (WBCs), hemoglobin (Hb), and hematocrit (HCT) which were significantly ($P < 0.05$) increased in fish fed FA or those fed both LF and FA. Also, the WBCs of fish treated with LF or/and FA were noticeably higher than control ($P < 0.05$). Feeding on LF and FA notably increased the serum total protein and albumin levels ($P < 0.05$). The serum respiratory burst and lysozyme activity were also enhanced ($P < 0.05$) in fish fed both LF or/and FA. In addition, evaluation of the serum antioxidant enzymes (catalase, glutathione peroxidase (GPX), and superoxide dismutase (SOD)) activity showed significant ($P < 0.05$) increase in fish fed FA or both LF and FA as compared to the control. Fish fed LF and FA supplemented diet had highest survival rate after experimental challenge with pathogenic *A. hydrophila*. The obtained results revealed that LF and/or FA can be used as beneficial feed additive to improve the immune responses and disease resistance in early stages of common carp culture.

1. Introduction

The adverse rearing conditions can negatively affect the fish immunity and consequent infection by naturally occurring microorganisms [1]. The overuse of antibiotics in fish production system aimed at disease treatment causes accumulation in aquatic environment and fish fillet, suppression of immune system as well as antibiotic resistance [2]. The concerns on the detrimental effects of antibiotics to the environment and human health by residual antibiotic related issues resulted in development of safe alternatives such as immunostimulants and probiotics [3]. Probiotics employ fewer complex approaches most likely to prevent early and late onset of disease without risk of drug resistance in animals [4,5]. They have several benefits including growth and immune enhancement to host against pathogens while sustaining health and environmental stability in fish [1,6,7].

In aquaculture, a probiotic is a live microbial adjunct that confers benefits to host by modification of host microbial community [8–10]. The ability of probiotics to promote and/or improve fish health is related to their capacity to stimulate immune response and inhibit growth of pathogenic bacteria [7,11–13]. Probiotics regulate allergic response and modulate host immune system [14]. The benefits of probiotics include immune modulation and production of inhibitory compounds to

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https://doi.org/10.1016/j.fsi.2019.10.019
Received 6 July 2019; Received in revised form 1 October 2019; Accepted 7 October 2019
Available online 09 October 2019
1050-4648/

disrupt quorum sensing mechanisms in pathogen [6,15,16].

It has been reported that *Lactobacillus* sp. have the strong capability of adherence and colonization that make them efficient probiotics to be used in aquaculture [17,18]. *Lactobacillus fermentum* (LF) is a common bacteria strain, which has been used as feed additive [17]. The wide occurrence and high antagonistic effects against pathogens made *L. fermentum* a good potential in testing for their probiotic ability [19]. *L. fermentum* has been reported to improve the growth performance and health status of Snakehead (*Channa striatus*) [18]. However, no information available about the possible effects of *L. fermentum* on fish or shellfish antioxidant defence, immune responses, and disease resistance. This make the strain as novel probiotic to be tested on different aquatic species.

Ferulic acid (FA) is presented in different kinds of grains, vegetables and fruits, make it the most abundant hydroxycinnamic acid in the plant kingdom. In addition, previous studies suggested that the FA derivatives are more biologically active than FA [20]. Nomura et al. [21] showed the anticarcinogen effects of a novel FA derivative including ferulic and gallic acids; which was more efficient than the original phytochemicals. Likewise, Kikuzaki et al. [22] noticed that the antioxidant effects of Alkyl esters of FA, such as octyl ferulate, was more than FA. FA and its derivatives have strong free radical-scavenging capability and other physiological activities. The studies on domestic animals confirmed improvement of growth, antioxidant capacities as well as productive traits following dietary administration of FA [23,24]. Likewise, the study on tilapia revealed similar findings as those observed with domestic animals [25].

Previously, different types of feed additives have been tested at different concentrations in common carp to identify their effects mostly on growth, immune parameters, and disease protection [3]. However, to the best of our knowledge, there is no published data regarding the effects of singular or combined administration of FA and LF on carps. Thus, the present study performed to investigate the possible effects of FA or/and LF on the growth performance, antioxidant defence, immune responses, and disease resistance in common carp.

2. Material and methods

2.1. Fish and husbandry condition

Common carp were purchased from a local farm (Sari, Mzandaran, Iran) and transferred to Technical and Vocational Center of Gorgan. All fish were treated with sodium chloride bath (2% for 15 min) upon arrival and kept in four 500 L tanks (for 2 weeks) for acclimation. Fish health status was visually checked such as normal coloration, the absence of cysts, spots or patches over the body and gills and normal behavioral signs (swimming and feeding reflexes). Afterwards, fish (3.90 ± 0.2 g) were stocked in twelve 100-L tanks at density of 16 fish per tank (triplicates). To maintain water quality, the tanks water was continuously aerated and daily water exchange (50%) was considered. The water quality parameters were measured regularly and maintained at following range: temperature, 24.20 ± 2.13 °C; dissolved oxygen, 6.6 ± 0.5 mg L^{-1} and pH, 7.3 ± 0.54, respectively.

2.2. Experimental diets and feeding protocol

L. fermentum PTCC 1638 were purchased from the culture collection at Iran Institute of Industrial and Scientific Research. The strains were reactivated in the MRS broth (Oxoid, UK) at 37 °C for 48 h. The mold cultures were cultivated on yeast extract dextrose chloramphenicol agar (Lab M, UK) slants for 9 days at 25 °C. After reviewing the dosages used in previous reports, we performed dosage tests for LF (1×10^8 CFU g^{-1}) and FA (100 mg kg^{-1}) as the optimal dosages [25,26]. The basal diet was supplemented with ferulic acid (FA, Sigma-Aldrich®, Chemie, Steinheim, Germany) and *L. fermentum* (PTCC 1638) [26]. Four diets were prepared by supplementing the basal diet with LF, FA and combination of them (LF + FA).

Table 1
Ingredients and chemical composition of the basal diet.

Ingredients	%	Composition	%
Fish meal	40	Dry matter	89.50
Wheat flour	21	Crude protein	38.22
Soybean meal	13.5	Crude lipid	10.24
Wheat gluten	5.5	Ash	3.45
Soybean oil	6	Fiber	11.20
Fish oil	6		
Mineral permix[a]	3		
Vitamin permix[a]	2		
Binder[b]	2		
Anti fungi[c]	0.5		
Antioxidant[d]	0.5		

[a] Permix detailed by Hoseinifar et al. [31].
[b] Amet binder™, Mehr Taban-e-Yazd, Iran.
[c] ToxiBan antifungal (Vet-A-Mix, Shenan-doah, IA).
[d] Butylated hydroxytoluene (BHT) (Merck, Germany).

Ingredients (i.e. fishmeal, soybean meal, wheat gluten, mineral and vitamin premixes, antioxidant, anti-fungi, and binder) used for the basal diet preparation are shown in Table 1. The diet formulation (two times) and storage condition were taken into account by following Hoseinifar et al. [27]. Briefly, desired amounts of ingredients were weighed and then 300 mL kg^{-1} distilled water and fish oil and soybean oil were added and mixed thoroughly. LF and FA were added to 1000 g of the dry ingredients mixture to achieve the desired concentration levels at 0, 10^8 CFU g^{-1} LF, 100 mg kg^{-1} FA and combination of them (10^8 CFU g^{-1} + 100 mg kg^{-1}) (Control, LF, FA and LF + FA). Finally, a 2-mm diameter, fixed die pelleting machine was used to make pellets containing 10% moisture at room temperature. Formulated diets were then air dried and stored at −02 °C. The feeding rate was 3% of body weight and fish were visually fed three times per day (09:00; 12:00; 15:00), during feeding trial which lasted for 8-weeks. Fish were weighed every 2 weeks to the feeding rate was adjusted.

2.3. Blood sample collection

To study the hematological parameters, six 24-h starved fish were randomly selected from each experimental tank (6 fish per tank). After anaesthetization, blood samples were obtained from the caudal vein and transferred to non-heparinized sterile tubes (1–1.5 mL^{-1}/tube). At the same time of blood sampling, smears were prepared for Giemsa staining and 3 drops of each blood sample were collected in heparinized Eppendorf tubes for respiratory burst activity assay and hematological parameters. The blood samples were left at room temperature for 1 h, centrifuged at 6000 × *g* for 10 min and the sera were separated and frozen at − 20 °C until used for further analysis.

2.4. Blood haematology and biochemistry

The cells haemocytometer was used to count the red (RBC) and white blood cells (WBC). The cyanmethemoglobin technique was followed to measure the hemoglobin (Hb) level by using spectrophotometer and reading absorbance at 540 nm. Also, the hematocrit (HCT) level was calculated based on microcentrifuge technique, using standards heparinized microhematocrit capillary tubes (75 mm at 7000 g for 10 min) [28]. To calculate the differential WBC, blood smears were prepared and stained with Wrighte-Giemsa [29]. The obtained smears were first air dried, fixed in 96% ethanol for 30 min, stained by Giemsa staining for 30 min and were examined for leucocyte differential count under compound microscope. In addition, the hematological parameters including, mean cell hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular volume (MCV) were calculated using the total RBC count, Hb

concentration and HCT [30].

The levels of serum albumin, glucose, triglyceride, cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were determined using auto analyzer (Eurolyser, Belgium) [31].

2.5. Serum immune parameters

Serum total immunoglobulin (IgM) levels were determined by following Siwicki [32]. Briefly, serum total protein was analyzed using microprotein method (C-690; Sigma), then the immunoglobulin molecules precipitated down by 12% solution of polyethylene glycol (Sigma) and the protein level were re-measured.

The lysozyme activity was determined as described by Ellis [33]. The results were expressed in units of lysozyme ml^{-1}. Briefly, test serum (0.1 ml) was added to 1.9 ml of a suspension of *Micrococcus luteus* (Sigma, USA) in a 0.05 M sodium phosphate buffer (pH 6.2). The reaction was carried out and absorbance was measured at 530 nm after 0.5 and 4.5 min in a spectrophotometer at room temperature. One unit is defined as the amount of sample causing a decrease in absorbance of 0.001 min^{-1} at 530 nm compared to the control.

Respiratory burst activity was measured using the chemiluminescence assay (CL), with adaptations and PMA as stimulus [34]. The CL response was measured on an automated luminometer (LUMI skan Ascent T392, Finland). Substrate used to assess the CL response was luminol. Luminol was stored at 0.01 M in DMSO and the working solution was obtained by diluting the stock solution at 1:1000 in HBSS. Into polystyrene tubes, 10 mL luminol solution 10^{-5} M, 280 Ml leukocytes suspension (6×10^5 cells) and 10 mL PMA 10^{-5} M were mixed. The volume was adjusted to 1 mL with HBSS/gelatin 0.1% and PMA was the last component added. For each sample, there was a negative control without PMA addition. Subsequently, tubes were put into the luminometer at 22 °C. The course time of CL response was followed by counting the rate of photons emitted at constant time interval (10 min) and registered in millivolts. The results of light emission are expressed in the form of relative light units per second (RLU s^{-1}).

2.6. Antioxidant enzymes activity

Glutathione peroxidase activity (GPX) was analyzed by following the rate of NADPH oxidation by the coupled reaction with glutathione reductase at 340 nm using an extinction coefficient of 6.22 mM^{-1} cm^{-1} [35].

Superoxide dismutase (SOD) was measured by the percentage reaction inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using a SOD Assay Kit (Sigma-Aldrich, 19160) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min reaction at 37 °C. The inhibition percentage was normalized by mg protein and presented as SOD activity units [36].

Catalase (CAT) assay was performed using spectrophotometric determination of hydrogen peroxide (H_2O_2) which forms a stable complex with ammonium molybdate that absorbs at 405 nm [37]. 50 µl serum was added to 1 ml substrate (65 µmol ml^{-1} H_2O_2 in 60 mmol l^{-1} phosphate buffer, pH 7.4) and incubated for 60 s at 37 °C. The enzymatic reaction was stopped with 1 ml of 32.4 mmol l^{-1} ammonium molybdate ((NH_4) $6Mo_7O_{24}$ 0.4 H_2O) and the yellow complex of molybdate and hydrogen peroxide measured at 405 nm against a reagent blank. One-unit of CAT decomposes 1 µmole of hydrogen peroxide l^{-1} minute under assay conditions. CAT activities are expressed as kilo unit per liter (U l^{-1}).

2.7. Challenge test

A. hydrophila (ATCC 7966) was obtained from Iranian Biological Resource Center And prepared as described elsewhere [38]. After 8 weeks of feeding with the experimental diets, 10 fish were randomly sampled from each experimental unit and exposed to *A. hydrophila* (10^8) via intraperitoneal injection as described by Hoseinifar et al. [27]. The dead fish from each tank was counted and removed daily. The mortality (%) of fish in each treatment was calculated after 15 days post-challenge. Moreover, the cumulative mortality and relative percent survival (RPS) values in each group were calculated over 15 days as follows: RPS = 100- [(treatment mortality/control mortality) × 100].

2.8. Growth parameters and statistical analysis

At the end of the feeding trial all fish in each tank were weighed to calculate the growth parameters of common carp fed the experimental diets based on the following formula:

Weight gain (g) = W2 (g) – W1 (g); Specific growth rate (SGR) = 100 (ln W2 – ln W1)/T; Feed conversion ratio (FCR) = feed intake (g) / weight gain (g)

Where W1 is the initial weight, W2 is the final weight, T is the duration of the feeding trial.

The statistical significance difference between treatments was assessed by the statistical package for social sciences (SPSS) software Ver. 19.0 and one-way analysis of variance (ANOVA). The values were given as mean ± standard error (SEM). To compare the means, the output data were subjected to LSD analysis with the least square difference and at $P < 0.05$ the differences were assumed significant.

3. Results

3.1. Growth performance and feed utilization

Final body weight and specific growth rate significantly increased ($P < 0.05$) in fish fed both LF or/and FA compared to the control, while the feed conversion ratio was significantly decreased ($P < 0.05$) in fish fed on LF or/and FA diets (Table 2). The weight gain was significantly higher in LF and LF + FA groups when compared with the control group ($P > 0.05$). However, no significant difference was noticed between LF and LF + FA groups and FA group (Table 2). The highest final body weight, weight gain, and specific growth rate as well as the lowest feed conversion ratio were observed in fish fed both LF

Table 2
Growth performance and feed utilization of fish fed the test diets for 8 weeks.

	Control	LF	FA	LF + FA
Initial weight	3.91 ± 0.20	3.88 ± 0.10	3.95 ± 0.13	3.93 ± 0.17
Final weight (g)	9.09 ± 0.26^{a}	10.15 ± 0.42^{b}	9.98 ± 0.37^{b}	10.95 ± 0.34^{c}
Weight gain (g)	5.42 ± 0.32^{a}	6.57 ± 0.34^{b}	6.05 ± 0.50^{ab}	6.53 ± 0.43^{b}
Specific growth rate	1.51 ± 0.05^{a}	1.72 ± 0.04^{b}	1.66 ± 0.01^{b}	1.83 ± 0.03^{c}
Feed conversion ratio	2.22 ± 0.14^{c}	1.84 ± 0.10^{b}	1.91 ± 0.16^{ab}	1.64 ± 0.10^{a}
Survival	100	100	100	100

*Values expressed as means ± SE. Different superscript letters indicate significant differences for each pairwise comparison between treatments.

Table 3
Blood hematological parameters of fish fed the test diets for 8 weeks.

	Control	LF	FA	LF + FA
HCT (%)	22.80 ± 2.28^{a}	24.40 ± 1.95^{ab}	26.40 ± 2.97^{bc}	28.60 ± 2.07^{c}
Hb (g/100 ml)	4.18 ± 0.34^{a}	4.32 ± 0.29^{a}	4.43 ± 0.29^{ab}	4.89 ± 0.52^{b}
RBC (× 10^{6}/μl)	1.10 ± 0.24	1.23 ± 0.21^{ab}	1.16 ± 0.30^{a}	1.34 ± 0.26^{b}
WBC (× 10^{3}/μl)	5.74.00 ± 0.28^{a}	6.38.00 ± 0.22^{b}	6.52.00 ± 0.44^{b}	7.12.00 ± 0.43^{c}
Lymphocytes (%)	95.80 ± 0.84	96.80 ± 0.84	97.60 ± 0.55	98.60 ± 0.89
Neutrophil (%)	3.00 ± 0.71	2.40 ± 0.89	1.80 ± 0.84	1.00 ± 1.00
Monocytes (%)	1.20 ± 0.84	0.80 ± 0.84	0.60 ± 0.89	0.40 ± 0.55
MCV (fL)	215.00 ± 54.50	203.63 ± 40.04	234.81 ± 37.69	221.45 ± 49.34
MCH (pg)	39.16 ± 8.13	35.70 ± 4.73	39.81 ± 7.68	37.16 ± 4.34
MCHC (g/dl)	1.86 ± 0.32	1.77 ± 0.15	1.70 ± 0.24	1.72 ± 0.27

*Values expressed as means ± SE (n = 3). Different superscript letters indicate significant differences for each pairwise comparison between treatments.

and FA. The survival rate was recorded 100% in all groups.

3.2. Blood haematological and biochemical parameters

No alterations were observed for the measured blood indices except for the RBCs, WBCs, Hb, and HCT (Table 3). HCT showed significantly ($P < 0.05$) increased values in fish fed FA or those fed both LF and FA. WBCs and Hb values showed significantly increased values in fish fed both LF and FA ($P < 0.05$). WBCs increased significantly ($P < 0.05$) in fish fed both LF or/and FA over the control, while the highest WBCs was observed in case of fish fed both LF and FA.

Similarly, no abnormal signs were observed in the measured blood biochemical parameters (Table 4).

3.3. Serum immune responses

The serum total protein level was significantly increased ($P < 0.05$) in fish fed both LF and FA compared other treatments. There were no significant difference between other treatments ($P > 0.05$) (Fig. 1A). Albumin levels showed the same trend with significant ($P < 0.05$) values in fish fed LF or both LF and FA as compared to the control (Fig. 1B). The serum total IgM level significantly increased ($P < 0.05$) in fish fed both LF or/and FA over the control (Fig. 1C). The respiratory burst activity was significantly ($P < 0.05$) higher in FA and LF + FA groups than the control group (Fig. 1D). Lysozyme activity was significantly increased ($P < 0.05$) in fish fed both LF or/and FA over the control, while the highest value was observed in case of fish fed both LF and FA (Fig. 1E).

3.4. Antioxidant enzymes activity

CAT levels showed significantly improved values ($P < 0.05$) in fish fed FA or both LF and FA as compared to the control (Fig. 2A). The serum GPX activity of fish fed LF or/and FA was significantly higher ($P < 0.05$) than control (Fig. 2B). Similarly, the SOD activity was significantly ($P < 0.05$) increased in fish treated LF and FA compared with those in fish fed control diet (Fig. 2C).

3.5. Survival rate of common carp after A. hydrophila challenge

After 4 days of infection with *A. hydrophila,* fish showed almost similar survival rates without significant differences among the groups as shown in Fig. 3. The survival rate started to decrease after 8 days from infection and fish fed on LF or/and FA exhibited higher survival rates than the control. After 15 days post challenge, fish fed LF and FA (LF + FA) showed highest survival rate followed by those fed LF or FA, while the lowest survival rate was observed in fish fed the control diet.

4. Discussion

The environmental stressors and the infectious diseases are among the major obstacles for the expansion of the aquaculture industry [39]. Over the past decades, the aquaculture industry was heavily dependent on antibiotics and chemotherapeutics to control the infectious diseases [40]. To date, the effects of dietary immunostimulants and probiotics have been extensively investigated on aquaculture species and their positive effects were acknowledged [41–44].

Supplementation of carp diet with LF and/or FA resulted in improved final body weight, SGR, and feed conversion ratio in common carp. Probiotics can inhabit in fish intestine to improve the digestion and absorption process by secreting the digestive enzymes [3]. The present study is in line with previous studies confirmed that fish fed probiotic showed improved growth rates and feed efficiency [43,44]. In the present study, the feed conversion ratio was significantly decreased in all groups fed with LF and FA. These results were in accordance with previous studies that indicated probiotics and feed additives could be fed to fish and animals without any adverse effect on feed intake. The addition of FA in the feed significantly improved WG and significantly decreased FCR of common carp in the current study. The obtained data seems to be similar to the earlier report that dietary supplementation of FA to Nile tilapia increases growth [25]. The growth-promoting mechanisms of FA are not clear and there are limited studies on application of FA as feed supplement in fish feeding. However, Talbott [45] reported that the growth-promoting effect of FA might be due to the increase in growth hormone and testosterone levels in blood. The excellent lipophilicity of FA derivative increases its ability to cross cell membranes and might cause promotion of growth in fish [25]. Also,

Table 4
Blood biochemical parameters of fish fed test diets for 8 weeks.

	Control	LF	FA	LF + FA
AST (U/l)	173.41 ± 16.28	189.33 ± 12.6	205.46 ± 30.75	180.28 ± 21.93
ALT (U/l)	9.46 ± 1.12	9.50 ± 1.59	9.32 ± 1.57	11.06 ± 2.41
ALP (U/l)	195.56 ± 19.41	218.24 ± 31.11	192.14 ± 29.67	214.04 ± 25.19
Glucose (mg/dl)	138.02 ± 17.22	140.80 ± 13.58	131.54 ± 8.02	139.22 ± 21.51
Cholesterol (mg/dl)	121.14 ± 11.71	123.84 ± 10.07	116.36 ± 14.31	120.56 ± 15.53
Triglyceride (mg/dl)	116.36 ± 9.43	103.72 ± 7.25	111.54 ± 11.62	102.72 ± 41.33

*Values expressed as means ± SE (n = 3). Different superscript letters indicate significant differences for each pairwise comparison between treatments.

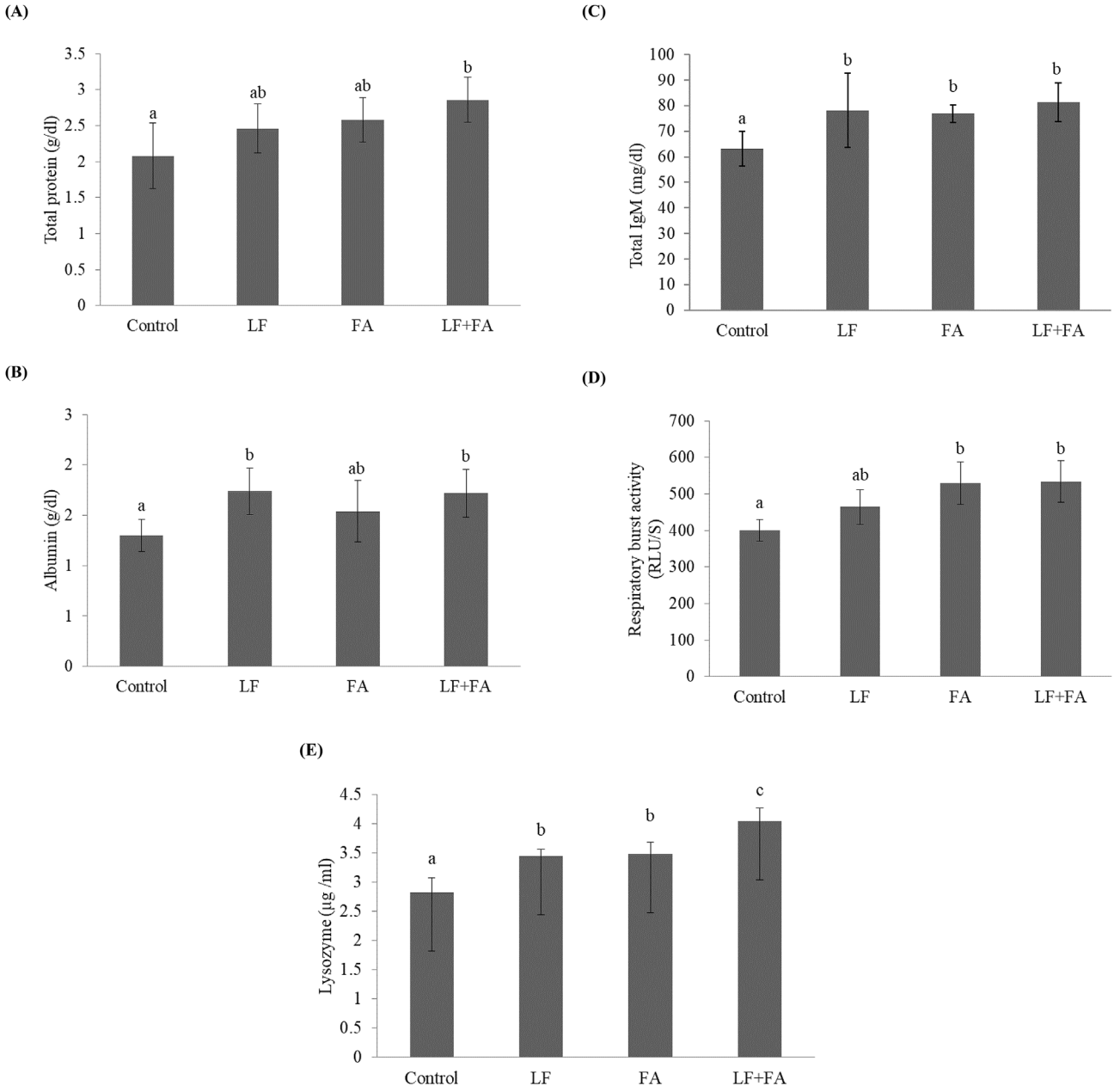

Fig. 1. Blood immune responses of fish fed test diets for 8 weeks. Values are expressed as mean ± SE from triplicate groups. Bars with an asterisk are significantly different from those of control group ($P < 0.05$).

this improvement may be related to the effect of LF and FA in improving the enzymatic digestion which in turn can increase the feed utilization. Thus, the growth performance can be linked to improved digestibility of nutrients and feed efficiency by LF and FA feeding.

The hematological and biochemical parameters are normally used to identify the possible changes in the general health condition in fish following feeding on functional feed additives [46–48]. In this study, obtained results revealed increase in HCT, RBCs, WBCs, Hb, total protein, and albumin in fish fed on LF and/or FA without negative effects on the other measured blood parameters. It has been assumed that dietary probiotics or FA can positively alters most of the blood indices in fish [25,46]. The presence of digested products in LF and FA helped to improve the immune function in fish blood [25,49]. The observed changes in haematological parameters might be because of relatively long-term influence of the probiotic and FA supplementation. Similar results were observed in Oscar (*Astronotus ocellatus*) fingerlings fed diets supplemented with probiotics [50]. The increased total number of RBC in the fingerlings fed LF and FA compared to the control apparently caused by a high, probiotic-driven metabolism with consequent increase in oxygen requirements [50]. The increased RBC, therefore, intensifies the concentration of Hb and eventually leads to a high oxygen-carrying capacity in fish. Such fishes, hence, may be more capable of supplying oxygen to tissues in situations where oxygen is highly required. Irianto and Austin [51] presented evidence that use of probiotic could result in raised RBCs and WBCs levels in rainbow trout. Similarly, the present study revealed significant rise of average total number of WBCs. Elevated number of WBCs is a reinforcement of non-specific immune system resulting from LF and FA consumption [52]. As lymphocytes were the most numerous in WBCs, and because interactions of lymphocytes B and T as well as macrophages is necessary for an immune response to occur [53], it can, accordingly, be concluded that fish immune system can be substantially stimulated by elevation of lymphocytes following an increase in WBCs. In other words, an appropriate dose of LF and FA additives entering the digestive tract of fish is

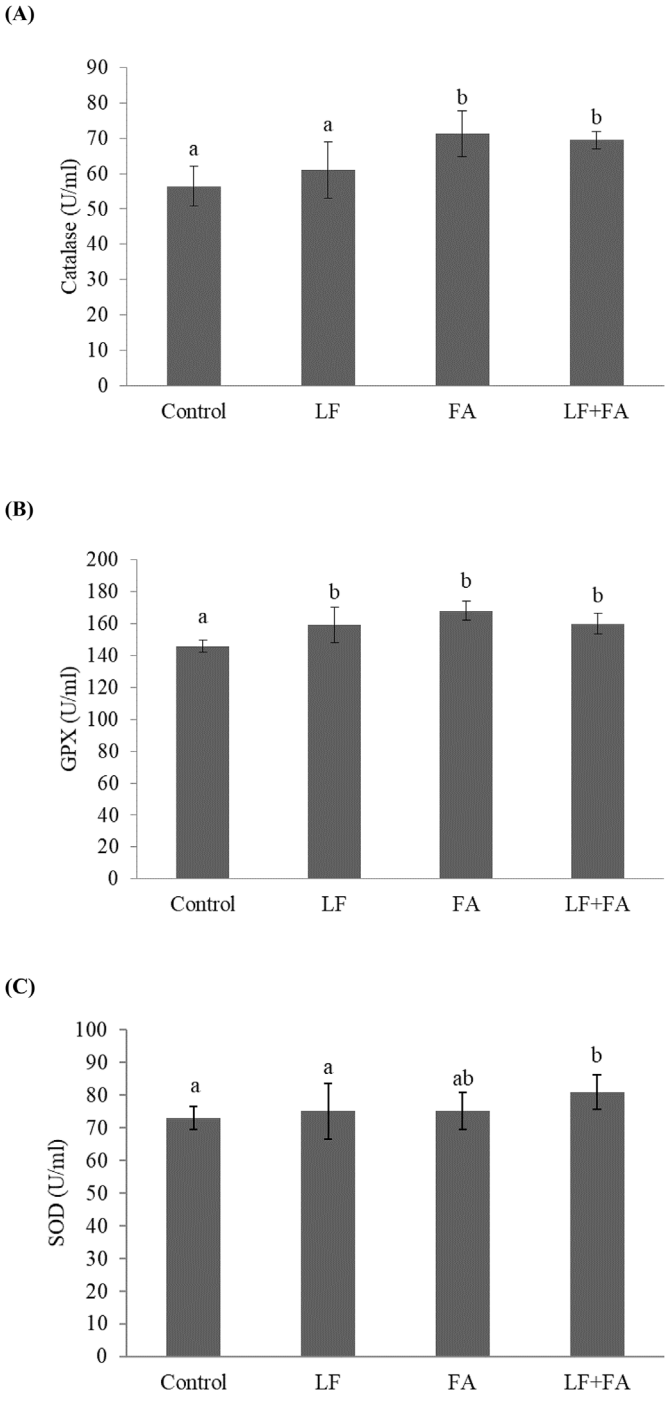

Fig. 2. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) of fish fed test diets for 8 weeks. Values are expressed as mean ± SE from triplicate groups. Bars with an asterisk are significantly different from those of control group ($P < 0.05$).

considered as aliens, provoking the defense system and multiplying WBCs and other immunizing compounds.

Immunoglobulins are among the blood proteins that can be considered as an indicator of the enhanced immune responses of fish [54,55]. The increased levels of total IgM and albumin in the current study is in line with the increased total protein, suggesting immunomodulatory effects of LF and FA on common carp. Further, globulin and albumin are the main resource for IgM production; hence its enhancement in serum affords immunostimulatory activity. The significantly higher total protein and albumin levels were observed in fish

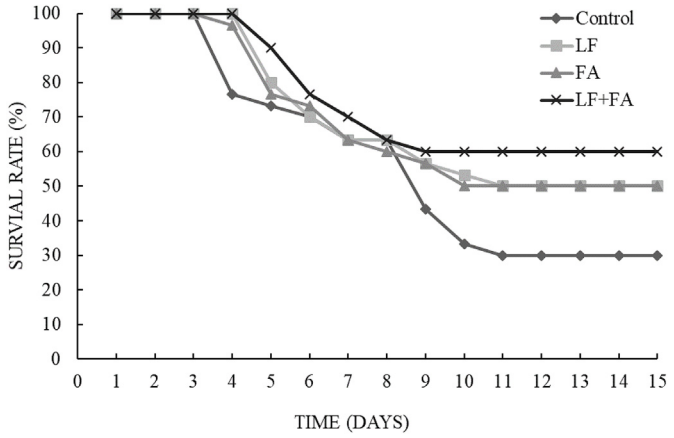

Fig. 3. Cumulative survival of common carp challenged with *A. hydrophila* after feeding the test diets for 8 weeks. The survival rate in the supplemented groups was significantly higher ($P < 0.05$) than that in the control group according to the Kaplan-Meier method.

fed with LF and FA. The present result is in line with the findings in Nile tilapia and *Labeo rohita* fed with probiotics [43,44,56].

Phagocytosis, bactericidal, and respiratory burst activities are important cellular immune system component in fish [57,58]. Their role is to assist fish in avoiding pathogen attacks more efficiently by recognizing the existing pathogens, and to limit their spread and progress [59,60]. Respiratory burst activity, through stimulation by foreign agents, have been found to increase the oxidation levels in phagocytes, and are a crucial factor in the general defense mechanisms in fish [61]. The obtained results revealed that the administration of LF and/or FA appreciably enhanced the serum respiratory burst activity. The creation of respiratory burst activities and reactive oxygen metabolites (ROS) by phagocytes are vital factor in limiting the spread of diseases in fish [50]. Similarly, Nile tilapia and *Labeo rohita* fed probiotics showed enhanced phagocytic and respiratory burst activities [7,62].

Humoral immunity is managed by bacteriolytic or hemolytic enzymes engaged in direct lysis of foreign invaders. Among humoral parameters, lysozyme cleaves (1,4)-β-linked N-acetylglucosamine and N-acetylmuramic acid in bacterial cell walls as well as destroying pathogens engulfed by macrophages or neutrophils [63]. In this study, fish fed with LF and/or FA showed enhancement of the lysozyme activity. Similarly, the lysozyme activity was enhanced in Nile tilapia, European seabass, common carp, and red sea bream fed probiotic supplements [6,47,48,64–66]. Yu et al. [25] also reported that Nile tilapia fed FA showed improved lysozyme activity, indicating dietary FA stimulating stronger innate immune responses in tilapia. The increase in lysozyme activity in our study indicated that LF and FA might have stimulated the innate immune responses in common carp. This point was also confirmed by the enhanced phagocytosis, bactericidal, and respiratory burst activities, which are considered as the indicator of strong innate immune function.

The role of antioxidant enzymes is to remove damaging ROS from the cellular environment by catalyzing the dismutation of two superoxide radicals to hydrogen peroxide (H_2O_2) and oxygen (O_2) [67,68]. Consequently, evaluation of SOD, CAT, and GPX markers as the important antioxidant enzymes can be considered as the biomarkers of oxidative stress besides indicating antioxidant capacity of aquatic organisms [69–71]. Our results revealed higher SOD, CAT, and GPX activities in fish fed with the LF and/or FA indicated reduced cell damage compare to fish fed control diet. The previous studies also showed an enhanced SOD, CAT, and GPX levels by probiotics or FA feeding [11,12,25]. The authors attributed that to the presence of bioactive compounds, which might improve the antioxidant activity and regulate the ROS intermediate system. FA has previously been shown to have scavenging activity towards ROS in Nile tilapia [25]. The hydroxyl

group in the FA can readily form a resonance-stabilized phenoxy radical and is key to its antioxidant property [25]. FA can also protect biological membranes from lipid peroxidation and neutralized peroxyl and alkoxyl radicals [72]. Our results revealed that the LF and/or FA supplementation could significantly activate the antioxidant activity in common carp, which can efficiently eliminate excess free radicals and regulate the balance of ROS in the body, resulting in improved antioxidant potential.

The main difference between these natural additives and vaccines is that the former kill or eradicate nonspecific pathogens, whereas the latter kill or eradicate specific ones [56,73–76]. Protection against pathogen challenge is dependent on many factors, including injected pathogen concentration, fish immunological status with body weight, aquatic environmental parameters, and the types of additives ingested. Interestingly, our results showed an increased resistance against *A. hydrophila* infection, which might be related to the immunomodulatory role of LF and/or FA in common carp diets. Devi et al. [77] reported that *Labeo rohita* fed probiotics showed enhanced resistance protection against *A. hydrophila* infection. Kong et al. [78] also reported the effect of *Bacillus subtilis* on *A. hydrophila*-induced intestinal mucosal barrier function damage and inflammation in grass carp (*Ctenopharyngodon idella*). A detailed comparative study is required with various levels of LF and/or FA supplementation diets in other fish against pathogens.

5. Conclusion

To conclude, the application of functional feed additives continued to be an important choice for sustainable aquaculture. The present study revealed that LF and/or FA supplementation may potentially activate the oxidative and cellular immune mechanisms, which resulted in increasing the tolerance against *A. hydrophila* infection in common carp.

Acknowledgements

The current research was funded by the research affair of University of Zabol (Grant code: UOZ-GR-9618-104).

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