International Immunopharmacology 73 (2019) 236–245

Contents lists available at ScienceDirect

International Immunopharmacology

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

Immunomodulatory effects of orally administrated florfenicol in rainbow trout (*Oncorhynchus mykiss*) following experimental challenge with streptococcosis/lactococcosis

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ARTICLE INFO

Keywords:
Rainbow trout (*Oncorhynchus mykiss*)
Florfenicol
Serum immune response
Immune-related genes expression
Lactococcus garvieae
Streptococcus iniae

ABSTRACT

The purpose of the present work was to determine whether florfenicol (FFC) as the prominent broad-spectrum antibiotic could affect serum biochemical and immunological parameters, as well as immune-related genes expression in rainbow trout (55 ± 7.6 g) challenged with the *Lactococcus garvieae* and *Streptococcus iniae*. In the first trial, the doses of the pathogens for challenge test were determined based on LD50. The therapeutic dosage of the drug (15 mg.kg^{-1} BW for 10 consecutive days) was administrated as medicated feed. After anesthesia, blood and kidney samples were collected from individual fish and were kept in deep freezing mode until the beginning of the measurements. Serum biochemical and enzymatic indices were measured using commercial kits. Immune parameters including total immunoglobulin level, lysozyme, ACH50, respiratory burst (RB), and phagocytic activities (PA) and the expression of immune genes namely TNF-α, IL-1β, IL-8, and IgM was evaluated. The levels of lysozyme and RB activities, as well as the expression of TNF-α and IL-1β genes, showed a significant increase in the FFC treated/infected fish compared to untreated diseased fish ($P < 0.05$). In contrast, serum total immunoglobulin and IgM-related genes expression were suppressed following drug administration represented by a significant reduction in untreated streptococcal infected fish compared to other treatments ($P < 0.05$). However, no significant effect of FFC was observed on serum ACH50 activity, PA values and IL-8-related gene expression ($P > 0.05$). These results demonstrated that FFC treatment could improve some physiological status including stress resistance and some liver function parameters, and much innate immunity was invigorated, but at the same time, the suppressive effects of FFC on acquainted immunity cannot be ignored.

1. Introduction

Streptococcosis/lactococcosis is a fatal and extremely prevalent disease of fish with complex etiological factors such as several species of *Streptococcus* [1,2], *Lactococcus garvieae* [3] and *Vagococcus salmoninarum* [4] which are frequently reported around the world [5–7]. Although this systemic infection is not included in the OIE notable list, the disease may lead to extensive losses mostly in farmed fish [5,8].

Rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792) is the most sensitive species to streptococcosis, and unfortunately, there is no effective vaccine against this disease [8]. Hence, using an antibiotic is a practical therapeutic strategy that is currently available [9]. However, the emergence of antimicrobial resistance [10,11] and immunosuppression may accompany antibiotic therapy [12]. Nevertheless, some reports show that antibiotic combinations can stimulate the antibody response in vaccinated fish [13].

Florfenicol (FFC) is recognized as one of the most prominent broad-spectrum antibiotics in aquaculture. The sensitivity of Gram-positive bacteria (such as streptococcus) to FFC is at least four times higher than Gram-negatives [14]. There are some reports about the suppressive effects of this FDA-approved drug [15] on the humoral immune parameters in mammals [16–18], birds [19,20], and fish [21,22]. Those studies pointed out that the cellular immunity could be altered in a dose-dependent manner following administration of FFC. In mice, sub-therapeutic doses of FFC could improve the phagocytic index and proliferation of thymocytes [16,17], though the presence of this

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https://doi.org/10.1016/j.intimp.2019.05.007
Received 16 March 2019; Received in revised form 5 May 2019; Accepted 6 May 2019
Available online 17 May 2019
1567-5769/

antibiotic in the plasma reduces the antibody titer [16,19]. Formerly, the suppression of respiratory burst activity of phagocytic cells of rainbow trout after receiving FFC was shown, despite a significant enhancement on antibody production [23,24]. Reda et al. (2013) observed no unfavorable impact on immunoglobulin M (IgM) levels and phagocytic activity after administration 5 mg.kg^{-1} FFC, even beyond this dose, serum lysozyme activity was increased [21]. Moreover, in brown trout (*Salmo trutta*), no changes were observed the levels of tumor necrosis factor alpha (TNFα) following the application of FFC at 40 mg.kg^{-1} BW, in [22].

Similar to other infectious pathogens, *S. iniae* and *L. garvie* can trigger immune responses such as enhancement in lysozyme activity, IgM level, respiratory burst, and phagocytic activities in the challenged fish [25,26]. Even, immunization through cohabitation and weakened pathogens can lead to the appearance of infection symptoms due to chemotaxis of phagocytes and emerging inflammation [27]. Previous studies illustrate that bacterial challenge generally induces significant changes in some of the humoral immune parameters such as the level of IL-8 and TNF-α in internal organs or integument of rainbow trout [28]. Also, there is a positive correlation between changes in transcript abundance of some of the immune genes (IL-8, INF-γ, TNF-α, and IgM) and the magnitude of infection [29].

Due to inconsistent findings related to the effects of FFC on cellular [21,23,24] and humoral [22,23] immune parameters in fish, it seems evaluation of gene expression level could be informative but, unfortunately, such studies are quite scarce or absent. The present study aimed to assess the impacts of the administration of FFC, as well as, investigate the induction of the experimental infections with some pathogenic agents (*S. iniae* and *L. garvie*), and the interaction of them on selected serum biochemical and immunological parameters, and immune-related genes expression in the head kidney (HK) of rainbow trout. For this purpose, after determination of the median lethal dose of these intrusive bacteria, several fish groups were exposed with a single dose of FFC (T+), infectious challenge (C+), and without or with both of them. Our findings would presumably affect the decision to use a conventional dosage of FFC in the health management of rainbow trout farms.

2. Materials and methods

2.1. Animals and experimental design

Rainbow trout with an average weight of 55 ± 7.6 g was purchased from a private sector in Fars province (Iran). Fish were kept in 500 l tanks for two weeks as acclimatization period with daily water renewing equal to 20% of tank volume. Feeding was performed with commercial pellets (BioMar, Denmark) twice daily at 2% of body weight. The water temperature and dissolved oxygen were set on 16.5 ± 0.6 °C and 5.57 mg/l, respectively. Total hardness of water supply was 344.55 mg $CaCO_3$/l. The experiment was conducted using a generalized randomized block design that six treatments were considered including 1) no bacterial challenge, without antibiotic treatment (C−/T−), 2) no bacterial challenge, under antibiotic treatment (C−/T+), 3) *L. garvieae* challenged without antibiotic treatment (*L. g* C+/T−), 4) *S. iniae* challenged without antibiotic treatment (*S. i* C+/T−), 5) *L. garvieae* challenged under antibiotic treatment (*L. g* C+/T+), and 6) *S. iniae* challenged under antibiotic treatment (*S. i* C+/T+). All experimental groups were triplicated simultaneously (15 fish/tank). The used drug was FFC with a commercial name Aquaflor® 50% (Rooyan Darou, Iran) which was administrated in the form of the medicated feed (drug-dipped food) for the positive treatment groups (T+), after 12 h of starvation. The therapeutic dosage was 15 mg.kg^{-1} BW for 10 consecutive days which is in the permitted range of USFDA [15]. That way, the amount of drug needed to achieve this dose was added to the soaked commercial food (BioMar, Denmark) 2.5% body weight for once on a day, and handed over to the tanks.

2.2. Pathogens and determination of LD50

The doses of the pathogens and the exact time to launch the oral antibiotic treatment in experimental groups were determined experimentally. In the first trial in order to find the median lethal dose (LD50), freeze-dried *S. iniae* and *L. garvieae* bacteria _ previously isolated from diseased rainbow trout from commercial farms of Fars Province_ were taken and cultivated on brain heart agar (BHA) at 25 °C for 48 h. After the bacterial count, serial dilutions were prepared (10^2 to 10^9 CFU/ml). Inoculation of the pathogens was accomplished through peritoneal injection of 200 μl of bacterial suspension in sterile PBS per each specimen, after MS-222 anesthesia (30 ppm) by the immersion method. During the trial period, fish losses were recorded in 4 consecutive days (96 h LD50). The bacterial concentrations which were used for the main experiment were calculated as 30% of LD50. Therapeutic period with FFC was launched 72 h after bacterial inoculation when the clinical signs were observed in most fish.

2.3. Sampling

Ten days after starting feeding on medicated feed, the fish were fasted for 24 h and then anesthetized. Blood sampling was carried out from the caudal vein of three fish per replicate using 2 ml syringe with and/or without anticoagulant. Sera samples were obtained following clot at 4 °C for 4 h and instant centrifugation at 11000*g* for 10 min (4 °C) and kept at −80 °C until use [30,31]. For isolation of leukocytes as phagocytic agents, the method described by Van Doan et al. [32] with some modifications was used. Briefly, 0.5 ml of obtained blood from each fish was diluted with 1 ml of RPMI 1640 (Biomark labs, India), mixed with 1.5 ml of Histopaque (HPq, Sigma-Aldrich) in a 10 ml tube, and the resulting mixture was centrifuged at 400*g* for 30 min. Afterward, a white buffy coat of WBCs which was suspended on the top of HPq was transferred to a clean 10 ml tube. As a washing step, 4 ml PBS (pH = 7.4) was added and gently mixed. It was centrifuged at 250*g*, 10 min. These stages were triply repeated to remove any residual HPq. For determining of phagocytic activity, the isolated leukocytes were re-suspended in the PBS (as solvent) and adjusted to the required cell numbers.

Moreover, HK of fish that were euthanized under overdose anesthesia (by MS-222) and were necropsied was immediately removed and frozen by liquid nitrogen [30] for gene expression assay.

All procedures were according to Shiraz University institutional ethical guidelines for care and use of vertebrate animals in experimental studies (IACUC. No. 4687/63).

2.4. Measurements of serum biochemical indices

Some biochemical parameters in the serum of fish including total protein, albumin, globulin, glucose, and cortisol were assayed. Serum total protein was measured based on the method suggested by Lowry et al. [33]. The determination of albumin values was done using a commercial kit (Pars Azmun, Iran). The globulin amount was calculated as the difference between the two former parameters [34]. Glucose levels were obtained using the standard procedure of glucose oxidase kit (Pars Azmun, Iran) with a colorimetric assay. Determination of serum cortisol levels was carried out by a competitive ELISA kit (MultiSciences, China) according to the protocol suggested by manufacturer.

Additionally, the activity of enzymes such as creatine kinase (CK), lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) was assayed. Measurement of CK activity was done based on the NADPH generating as an enzyme reaction product [35]. LDH activity assessed by determination of the pyruvate to L-lactate conversion and monitoring of NADH oxidation [36]. Aspartate aminotransferase and ALT were estimated based on Reitman & Frankel's method at 340 nm [37]. The basis of a method used to measure ALP was the conversion of p-

Table 1
Primers used for qPCR.

Primer	FWD or REV	Sequence	Length (bp)	Accession[a]
TNF-α	Forward	TGGAGGGGTATGCGATGACACCTG	116	AJ249755.1
	Reverse	TGAGGCCTTTCTCTCAGCGACAGC		
IL-1β	Forward	ACATTGCCAACCTCATCATCG	91	AJ223954
	Reverse	TTGAGCAGGTCCTTGTCCTTG		
IL-8	Forward	AGAATGTCAGCCAGCCTTGT	69	AJ279069
	Reverse	TCTCAGACTCATCCCCTCAGT		
IgM	Forward	CCAAACCGGTGGAAGCTACATG	150	TC138653
	Reverse	GAGACGGCTGCTGCAGATATTC		
β actin	Forward	TCACCCACACTGTGCCCATCTACGA	295	AC006483.3
	Reverse	CAGCGGAACCGCTCATTGCCAATGG		

[a] Derived from: http://www.ncbi.nlm.nih.gov.

nitrophenol phosphate to nitrophenol in an alkaline buffer at 405 nm [36]. All of the enzymatic assays were performed spectrophotometrically (UV/VIS HITACHI 704, Japan) via the standard biochemical kits (Pars Azmun, Iran) in accordance with the short described above for each factor.

2.5. Measurements of serum immunological parameters

Innate and acquired immune indices that were assayed consisted of total Ig level, lysozyme activity, alternative complement pathway hemolytic activity (ACH50), respiratory burst activity (RBA), and phagocytic activity (PA). Total Ig titers were estimated according to the difference between total protein levels of serum (explained above), before and after addition of 12%-diluted polyethylene glycol which precipitates Ig molecules [30,38]. For measurement of lysozyme activity, the turbidometric assay was used based on lysis of lyophilized *Micrococcus luteus* (Sigma, USA) [39]. The activity of ACH50 was determined according to hemolysis of rabbit red blood cells (RaRBC), and calculated as the volume of serum needed to cause 50% hemolysis [40,41].

For evaluating RBA, superoxide anion (O_2^-) production by immune cells was determined using the reduction of nitro blue tetrazolium chloride (NBT, Sigma). Briefly, 0.1 ml of the heparinized blood with the same amount of 0.2% NBT solution were added to a microplate well and then incubated at room temperature for 30 min. Before centrifugation (at 2000*g* for 10 min), 0.05 ml of the mixture was removed and added to 1.0 ml of N, N dimethylformamide. Afterward, supernatant optical absorption was read by an ELISA microplate reader (Bio Tek ELx 808, U.S.A) at 620 nm. N, N dimethylformamide blanks were set as the positive control [42]. PA was measured using the modified method of Kim and Austin [43] that evaluates adherence of phagocytic cells to the surface of methanol-cleaned glass slide in a humid chamber (at 18 °C for 1 h). The rinsing step was done by Hank's balanced salt solution (HBSS) for removing the non-adherent cells. Thereafter, 1 ml of Congo red dyed-yeast cells (10^8 CFU/ml) (C.I. 22120, Merck, Germany) was added, and the process of phagocytosis lasted for 1 h, then absolute methanol and Giemsa solution were applied to fix and stain the slides, respectively. The phagocytic rate was expressed as the percentage of phagocytized cells per total phagocytic cells under a light microscope [25].

2.6. Measurement of immune-related genes expression

At the end of the experiment, relative expression of immune-relevant genes (TNF-α, IL-1β, IL-8, and IgM) was measured in kidney samples obtained from fish using quantitative real-time PCR (qPCR) as described by Hoseinifar et al. [44] with some modifications. Briefly, after pooling the replicates of each treatment and exposing them with liquid nitrogen, samples were poured into separate three vials and kept at −80 °C. The total RNA was extracted according to Wizol™ Reagent protocol (Wizibiosolutions, South Korea). The sufficient concentration and integrity of the purified nucleic acid molecules were ensured through Nanodrop spectrophotometry (Wilmington, DE, USA) at 260 nm, and electrophoresis on 1% dyed-agarose gel (by ethidium bromide), respectively. After removing the probable contamination of genomic DNA with DNase I (Fermentas, France), 1 mg of treated RNA was used for synthesis of complementary DNA by a synthesis kit (GeNet Bio, South Korea) according to the manufacturer's instruction and by applying thermal cycling of PCR using MJ mini™ (Bio-RAD Laboratories, USA). The expression of the selected genes was determined with an iCycler iQ™ real-time PCR detection system (Bio-RAD Laboratories, USA) using Wizpure™ qPCR master mix (Wizibiosolutions, South Korea) as an SYBR green reagent, and the Beta-actin as a housekeeping gene. For preparing samples and dilutions, 2 µl of each sample was mixed with 18 µl of the master mix containing 6.4 µl injectable water, 1 µl DSMO, 0.2 µl forward primer, 0.2 µl reverse primer, 0.2 µl Taq polymerase, and 10 µl SYBR green, while each qPCR reaction was triplicated. Thermal programs or TM optimization conditions were adjusted according to the suggestion of primers' manufacturer and previous works [29,45]. The primers sequences which presented in Table 1, were designed by Primer3 online software and made by BioNeer corporation (Daejeon, South Korea). Finally, the relative gene expressions were calculated by the ΔΔCt method [45] using IQ5 software (Bio-RAD).

2.7. Statistical analysis

LD50 values were computed according to the Reed and Muench equation [46]. Comparison among different groups was performed using one way-ANOVA, then Duncan's test as post-hoc, after assessing the normality of data by Kolmogorov–Smirnov test. $P < 0.05$ was considered as the significance level.

3. Results

3.1. Bacterial challenge and LD50

Challenging fish with increasing doses of pathogens led to an exponential increase in mortality. Based on the recorded casualties, the 96-h median lethal dose of *L. garvieae* and *S. iniae* for rainbow trout were 2.72×10^6 and 1.15×10^8 CFU/ml, respectively (Table 2). Part of the population of inoculated fish with *L. garvieae*, especially those exposed to higher doses of the pathogen, showed complete or partial darkening of the body peculiarly the base of the pectoral and dorsal fins, and the operculum, about 48 h after injection. The emergence of the fulminant disease was extremely acute, and lesions which were observed in the gross pathological examination of the survived fish were limited to darkening, slight ascites, petechial lesions in internal organs such as pyloric caeca and peritoneum. In the case of inoculated fish with *S. iniae*, the clinical signs initiated later and generally culminated at the beginning of the fifth day. The infectious symptoms extended throughout the body like partial darkening and petechial lesions on anterior parts. Severe dropsy and/or exophthalmia was quite

Table 2
Different lethal doses of the bacterial pathogens for rainbow trout.

	L. garvieae			*S. iniae*		
Lethal dose	LD10	LD50	LD90	LD10	LD50	LD90
(CFU/ml)	3.1×10^3	2.72×10^6	2.37×10^9	9.55×10^3	1.15×10^8	1.38×10^{12}
Equation	Y = 1.36 × -3.75			Y = 0.98 × − 2.9		
Intercept	−2.9			−3.75		
X-variable	0.98			1.26		

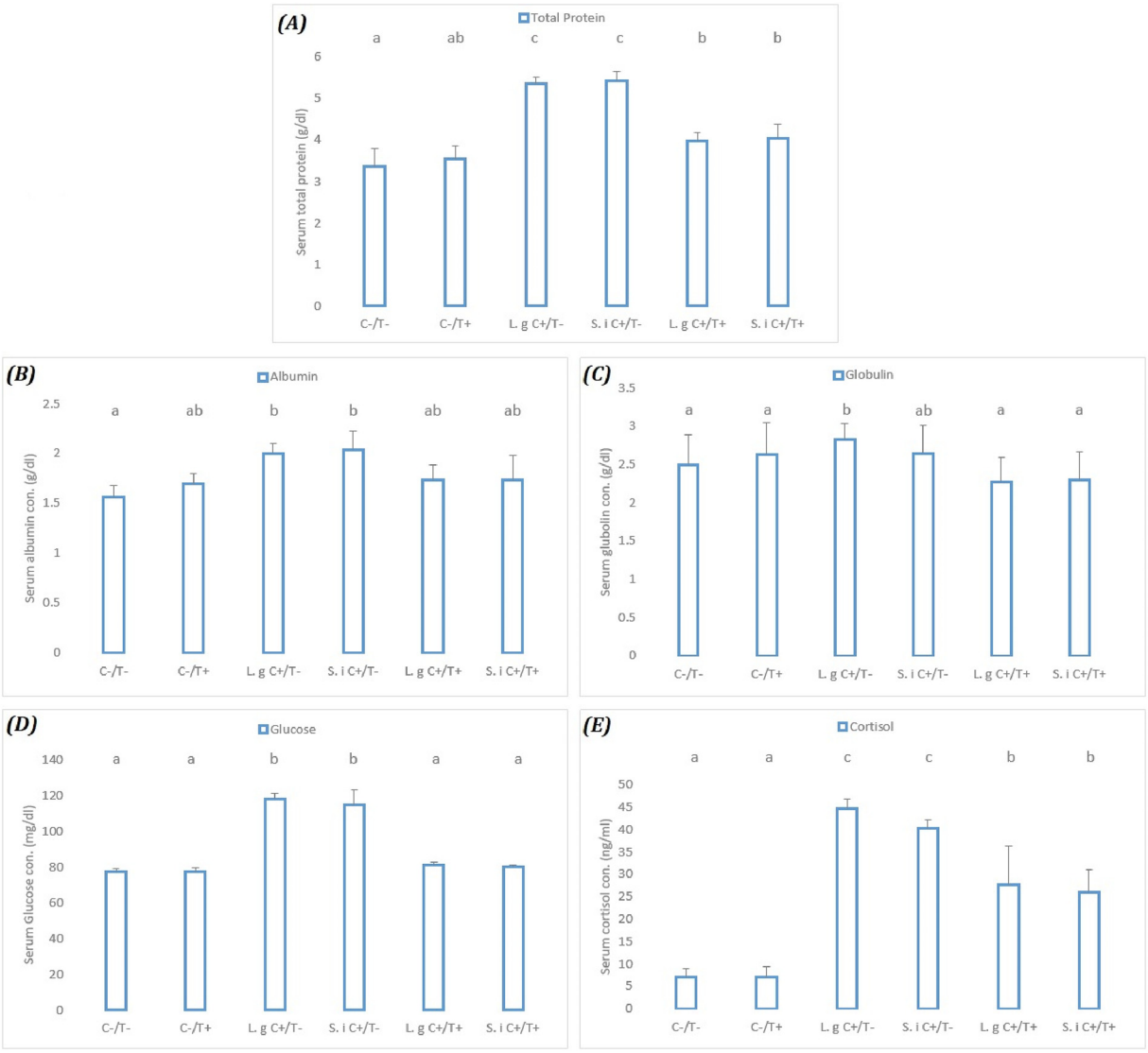

Fig. 1. Serum biochemical parameters (Mean ± SD) of rainbow trout after 10-day experimental period with 15 mg.kg BW FFC treatment and infectious challenge (mean ± S.E): C−/T− (control), C−/T+ (No challenge, under antibiotic treatment), *L. g* C+/T− and *S. i* C+/T− (*L. garvieae*/*S. iniae* challenged without antibiotic treatment), *L. g* C+/T+ and *S. i* C+/T+ (bacteria challenged under antibiotic treatment); Statistical differences ($P < 0.05$) between trial groups are shown with different letters (a, b, c, etc.).

evident, in fish infected with higher doses. Gross examination of dead fish exhibited hemorrhagic lesions on the liver and digestive tract. The common behavioral disorders of the challenged fish with both bacteria were slow swimming, lethargy, and unbalanced motions. By considering these findings, the start of the treatment for the main experiment was set 3 days after injection of both the pathogenic agents.

3.2. Serum biochemical indices

Effects of oral administration of the antibiotic on some serum biochemical metabolites of diseased rainbow trout are presented in Fig. 1. In this regard, there was a significant difference between experimental groups for all of the studied indices. In the case of total protein, albumin, and globulin no statistical difference was observed between control (C−/T−) and FFC receiving groups without challenge (C−/T+) ($P > 0.05$), though these parameters were significantly different

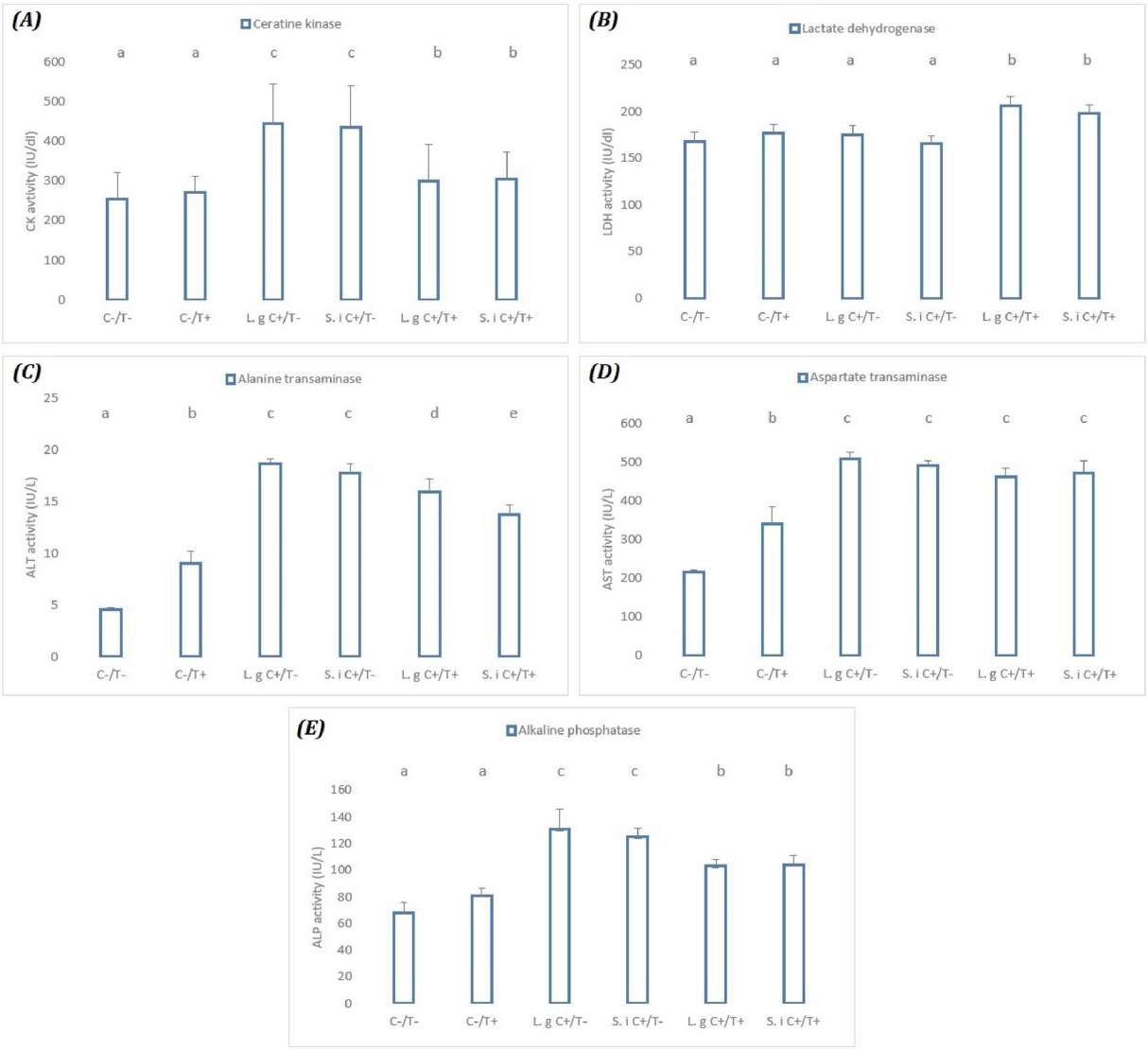

Fig. 2. Serum enzymatic parameters (Mean ± SD) of rainbow trout after 10-day experimental period with 15 mg.kg BW FFC treatment and infectious challenge (mean ± S.E): C−/T− (control), C−/T+ (No challenge, under antibiotic treatment), *L. g* C+/T− and *S. i* C+/T− (*L. garvieae*/*S. iniae* challenged without antibiotic treatment), *L. g* C+/T+ and *S. i* C+/T+ (bacteria challenged under antibiotic treatment); Statistical differences ($P < 0.05$) between trial groups are shown with different letters (a, b, c, etc.).

between streptococcal infected fish (C+/T−) and those infected fish that received FFC (C+/T+) ($P < 0.05$, Fig. 1A–C). A similar case was observed for stress factors so that the highest amounts of glucose and cortisol in challenged groups without treatment were significantly higher than groups that received the drug at the same time for both bacteria ($P < 0.05$). Likewise, there was no significant difference between control and unchallenged FFC-treated groups in those parameters ($P > 0.05$, Fig. 1D, E).

3.3. Liver and kidney function enzymes

Fig. 2 shows the level of some enzymatic markers of the liver and kidney in the experimental groups. Except for LDH and AST, other parameters in C+/T− groups showed higher values for both bacteria ($P < 0.05$). Lactate dehydrogenase levels showed a completely different pattern as compared to challenge alone, the interaction of antibiotic and pathogenic agents exacerbated LDH levels, significantly ($P < 0.05$, Fig. 2B). Levels of AST were statistically similar between only challenged fish and those challenged and received FFC for both pathogenic bacteria ($P < 0.05$, Fig. 2D).

3.4. Humoral and cellular immunity parameters

Serum total Ig of fish was suppressed following drug administration as shown by a significant difference between challenged untreated groups and challenged fish with both bacteria and then treated with FFC ($P < 0.05$, Fig. 3A). Daily administration of the antibiotic for 10 days in uninfected fish increased lysozyme activity ($P < 0.05$). Whereas, the infectious status did not change the activity of this antiseptic enzyme ($P > 0.05$, Fig. 3B). The medication showed a neutral effect on the alternative complement system, and no significant difference in ACH50 activity was observed ($P > 0.05$, Fig. 3C). On the other hand, RBA of phagocytic cells was significantly enhanced by drug therapy in challenged and unchallenged fish as compared to control ($P < 0.05$, Fig. 3D). Nevertheless, the PA of circulating leukocytes did not show any significant difference among groups ($P > 0.05$, Fig. 3E).

3.5. Immune-relevant genes expression

At the level of immunity gene expression, FFC administration resulted in a significant change in TNF-α and IL-1β as the major cytokines

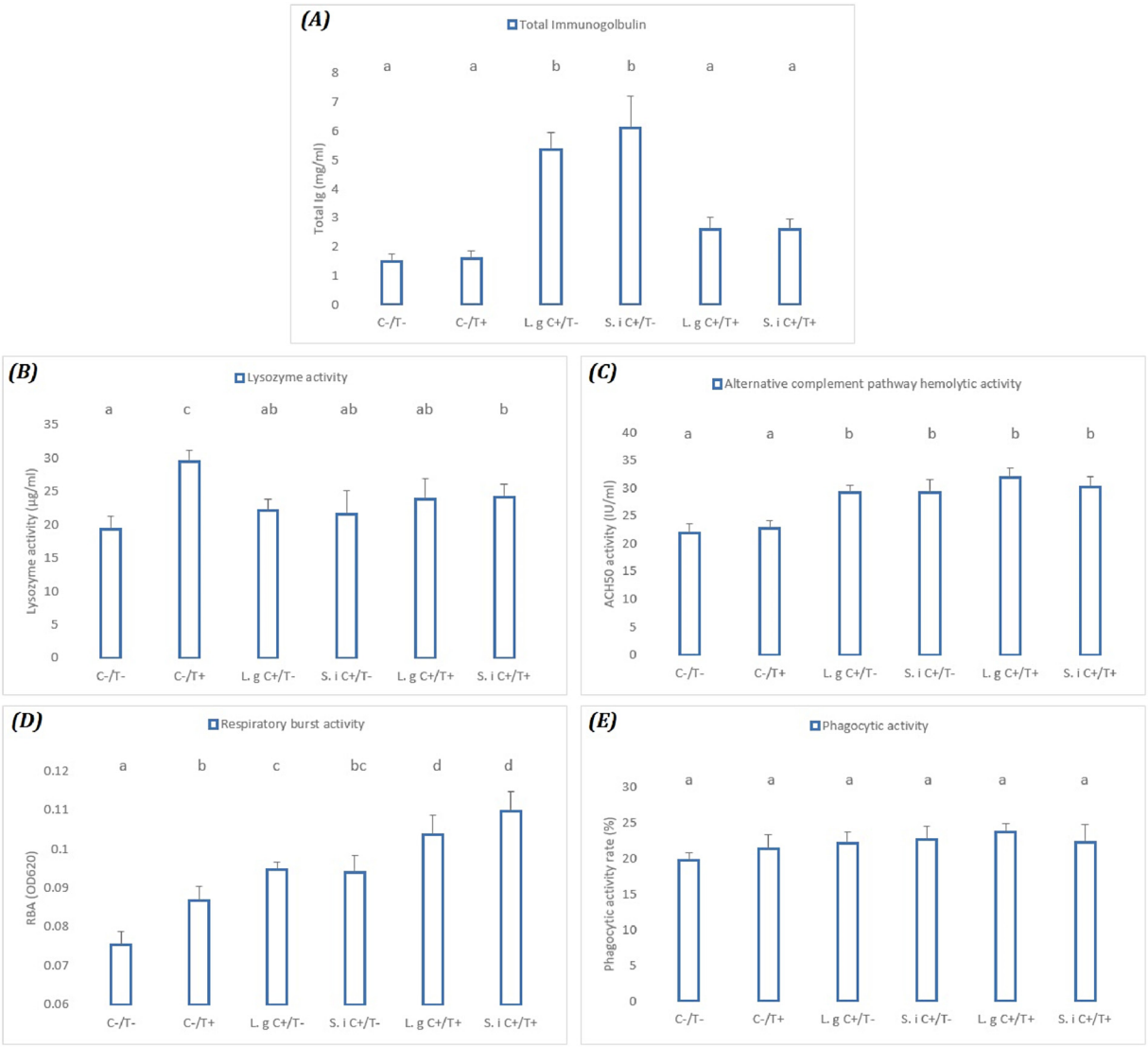

Fig. 3. Serum Immunological parameters (Mean ± SD) of rainbow trout after 10-day experimental period with 15 mg.kg BW FFC treatment and infectious challenge (mean ± S.E): C−/T− (control), C−/T+ (No challenge, under antibiotic treatment), *L. g* C+/T− and *S. i* C+/T− (*L. garvieae*/*S. iniae* challenged without antibiotic treatment), *L. g* C+/T+ and *S. i* C+/T+ (bacteria challenged under antibiotic treatment); Statistical differences ($P < 0.05$) between trial groups are shown with different letters (a, b, c, etc.).

where the level of these indices in C−/T− group was significantly different from FFC-treated groups ($P < 0.05$, Fig. 4A, B). In the case of IL-8, only bacterial infection could increase this cytokine, and inducing effects of FFC was not remarkable ($P < 0.05$, Fig. 4C). IgM gene expression levels showed notable suppression following FFC administration in challenged groups as compared to untreated challenged fish ($P > 0.05$, Fig. 4C).

4. Discussion

In the current study, the immunomodulatory effects of FFC in rainbow trout experimentally infected with streptococcosis/lactococcosis agents was investigated by evaluating the levels of serum biochemical, enzymatic, humoral and cellular immunity indices as well as immune-relevant genes mRNA expression. Experimental infection with *L. garvieae* and *S. iniae* in the present study resulted in severe disease with obvious clinical signs. Generally, the difference between LD50 values of these bacteria for rainbow trout demonstrated that *L. garvieae* (2.72×10^6) had higher pathogenicity and fatality than *S. iniae* (1.15×10^8). Moreover, infectious symptoms in *L. garvieae*-challenged fish appeared earlier (started at the end of the second-day post-challenge) than the *S. iniae*-related signs (started on the fifth-day post-challenge). However, *S. iniae* made more prominent lesions on the surface of the body and on internal organs. These findings are in agreement with other studies in terms of the casualty trend. While *L. garvieae*-caused infection in rainbow trout is a hyperacute disease with somewhat rapid mortality and usually without clinical signs, the disease due to *S. iniae* is usually associated with seizure and hemorrhagic septic symptoms during the more elongated period [3,5,47].

Our results indicated that oral treatment of FFC modulated the serum biochemical and stress-related indices of the diseased rainbow trout. Alternation of serum total protein in challenged fish could be representative of enzymes and hormones production rate for body protection against pathogenic agents [48]. The FFC-induced decrease of protein synthesis may show that the presence of a sufficient dose of antibiotic in plasma (like 15 $mg.kg^{-1}$ BW) could be contributory in protection against the pathogens. As a result, it has reduced the pathogen load and subsequent lower need for a synthesis of protective proteins such as lysozyme, complement and, immunoglobulin. As it was expected and was shown previously [49], plasma cortisol values

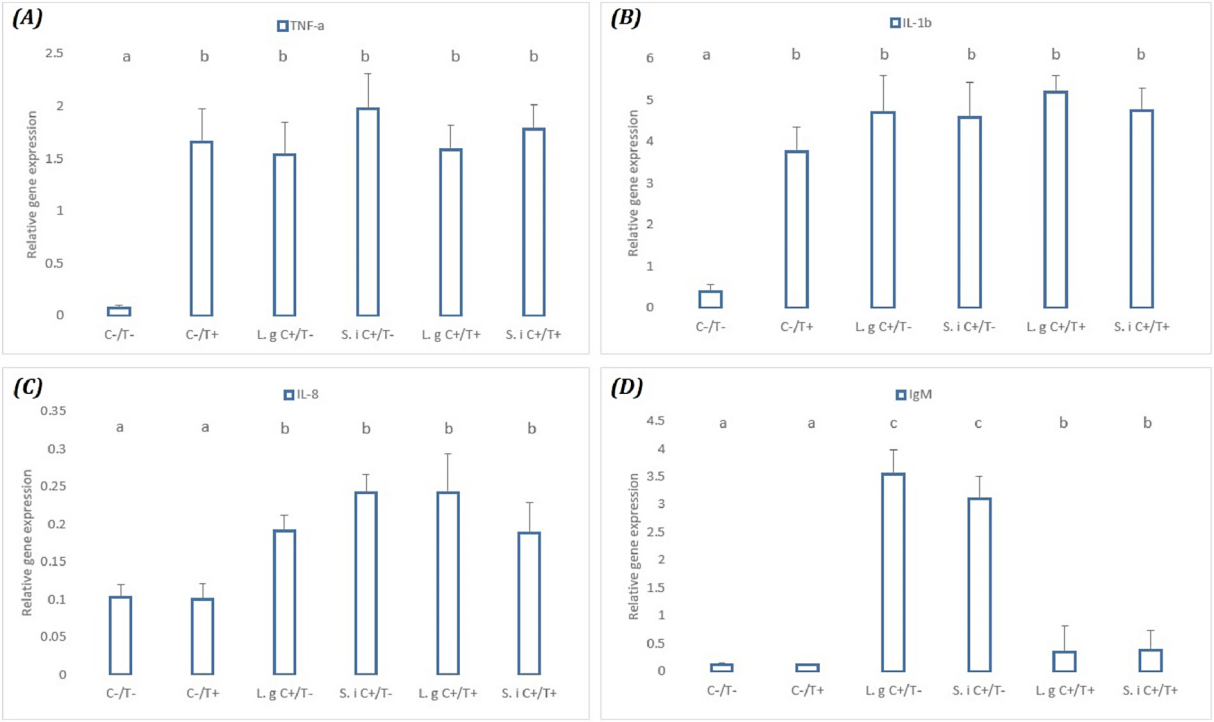

Fig. 4. Immune-related gene relative expression (Mean ± SD) of rainbow trout's head kindney after 10-day experimental period with 15 mg.kg BW FFC treatment and infectious challenge (mean ± S.E): C−/T− (control), C−/T+ (No challenge, under antibiotic treatment), *L. g* C+/T− and *S. i* C+/T− (*L. garvieae/S. iniae* challenged without antibiotic treatment), *L. g* C+/T+ and *S. i* C+/T+ (bacteria challenged under antibiotic treatment); Statistical differences ($P < 0.05$) between trial groups are shown with different letters (a, b, c, etc.).

increased after exposure to the stressful condition of the bacterial infection. Orally administrated FFC considerably reduced on levels of the glucocorticoid hormone in the infected animals, as well as, glucose as a principal product of cortisol's physiological process [38]. Cortisol is considered a suppressive agent for the immune system in vertebrates [36,50]. Therefore, its reduction by FFC can show relieved stress and maybe a booster for immune responses. Similarly, some researchers reported that the medicinal compounds like oxytetracycline hydrochloride, benzylpenicillin sodium, cefazolin, and neomycin sulfate [12], or the herbal alternatives such as *Rhodomyrtus tomentosa* [49], *Stachys lavandulifolia* [51], and *Nectandra grandiflora* [52] extracts show reducing effects on glucocorticoids in diseased rainbow trout, common carp and silver catfish, respectively.

In our study, measurement of the enzymatic markers of liver and kidney function including ALT, ALP, and CK pointed out a change in challenged groups which was ameliorated by FFC. Several factors cause ALT secretion to blood, and its blood increment acts as an alert [53]. As regards the increase in intracellular levels of ROS may lead to lipid peroxidation resulting in increased permeability of the liver cell membrane. So, these enzymes could release to plasma [36]. Although taking high doses of the drug and infectious agents can elevate the levels of these enzymes [22], however, their values were significantly reduced in diseased fish following the application of the recommended dosage of FFC. It seems that the inducing agents may affect the levels of ALT and AST differently because of their position in the hepatic cells. ALT exists only in the cytoplasm, but AST is mostly stored in mitochondria and then in the intracellular fluid [54]. Consequently, in acute infections like streptococcosis, ALT secretion to blood could be more increased after the destruction of the cell membrane as compared to AST [21]. Serum ALP usually has the ability to mark cholestatic damage besides hepatocellular disorder [53], and CK despite the representation of rhabdomyolysis, is applicable for the diagnosis of renal failure [35]. On the contrary, LDH increased in challenged fish that received FFC where AST showed no significant change. The synergistic effect of FFC administration and streptococcal infection on LDH illustrated that this enzymatic marker is more sensitive than other liver and renal damage indices for accurate recognition of infectious status [55], and hepatotoxicity derived from the chemotherapy.

Regarding the humoral defense system, parameters showed conflicting tendencies. For instance, Ig titer was decreased, and lysozyme activity was increased after the FFC administration. Previous studies clarified that one of the disadvantages of using antibiotics at their conventional dosage is suppression of acquired immunity [16,19], and perhaps using of moderate dosage (at least half of the dosage suggested by USFDA) diminish this adverse effect [21]. Florfenicol has suppressed humoral and cellular immune responses accompanied by destruction of lymphoid organs [12,19,23] and IgM immunoreactive cells of hematopoietic tissue [56]. In the present study, administration of FFC to healthy fish did not change Ig concentration compared to control, although increased lysozyme activity. The reducing effect of this antibiotic on Ig level might be related to the reduction in pathogen load caused by stay up the concentration of FFC in plasma to minimum inhibitory concentration (MIC) which can be considered as a proper response. Indeed, the descending load of bacteria in intercellular space, blood, and lymph leads to reduced exposure levels to lymphocytes and less acquired immune responses [8,26]. Some studies claimed that the progenitor cells of lymphocytes in HK or spleen of the teleosts were influenced indirectly by beta-lactams, chloramphenicol, and dapsone. Consequently, the production of lymphoblasts is limited, which reduces the generation of adult immunity cells [18,57]. In line with our findings and the same research [21], FFC had the potential to increase the lysozyme activity in serum. Lysozyme that involved in the hydrolysis of the β-(1, 4) linked glycoside bonds of bacterial cell wall peptidoglycans was increased in serum of diseased fish infected with the main bacterial pathogens [26,56]. This powerful bacteriolytic protein is secreted from neutrophilic granules of immune cells with other degradative enzymes could cleave directly the coverage of bacteria. It was reported that increasing the number of granulocyte cells, as well as monocytes, results

in elevated levels of serum lysozyme [58] which is in line with the finding of the present study indicating increased lysozyme activity following FFC administration. It is confirmed that phenicol type antibiotics not only do not inhibit the generation of polymorphonuclear neutrophils (PMN) in the blood unlike beta-lactam and tetracycline antibiotics, but also they enhance cellular innate immunity [59]. It is confirmed that phenicol type antibiotics not only do not inhibit the generation of polymorphonuclear neutrophils (PMN) in the blood unlike beta-lactam and tetracycline antibiotics but also they enhance cellular innate immunity [59].

Cellular innate immunity in the present study was evaluated by measuring PA and RBA of white blood cells. We observed an enhancement in RBA of pathogen-infected fish following the drug consumption that was in accordance with Yilmaz's findings [60] about the same index in Nile tilapia (*Oreochromis niloticus*). Conversely, the suppressive effects of FFC on this parameter have been previously reported in rainbow trout [23]. We suppose that exerting different therapeutic dosage between our trial (15 $mg.kg^{-1}$ BW for 10 consecutive days) and Lundén et al.'s experiment (20 $mg.kg^{-1}$ BW for 10 consecutive days) may contribute to variations in the results. Different studies have shown a consensus about the stimulatory effect of the antibiotic on cellular defense mechanisms such as phagocytosis [16,19,21], and elevating of PA requires an increment in some reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide [25]. While, some beta-lactams inhibit RBA in PMNs, chloramphenicol could increase shed of ROS especially HOCl [59]. In fact, spreading of PMNs and other inflammatory mediators lead to mobilize of seric oxidants and increase the activity of ROS inside of phagosomes [55]. Mutually, increased ROS in blood and tissue fluids might affect the chemical structure of the antibiotics such as chloramphenicol, macrolides, and quinolones [59]. About phagocytosis, our findings illustrated only an insignificant incremental change in pathogen-challenged fish, as compared to the control group and FFC did not affect this parameter. Similarly, FFC did not affect the phagocytic activity of tilapia [60,61]. This is contrary to the results of some researches that claimed FFC intake had a modulatory or suppressive effect on the phagocytic index [17,23,25]. Such disagreement might be due to species variation, the difference in therapeutic dosage, type of pathogen, and methodology. Rationally, RBA and PA are related to each other in terms of performance and the source of action, and RBA can be considered as the indicator of phagocytosis activity of leukocytes [24].

An examination at the level of the gene expression can provide a relatively strict view into production level of inflammatory cytokines. Accordingly, three cytokines contributing to the immune system along with IgM were investigated in this way. Since TNF-α and IL-1β-related genes expression in FFC-treated healthy and infected fish significantly increased compared with the control group, the immunostimulatory effect of this antibiotic and the infectious agents is expected. In agreement with our results, Er & Dik [22] declared that bacterial pathogens could induce production of TNF-α, and the inflammatory/down-regulatory effects of this antibiotic on the interleukins were confirmed [20,62]. Furthermore, some research papers accentuated the inducing effects of FFC on IL-1β, IL-8, and TNF-α genes expression of Nile tilapia and Atlantic cod (*Gadus morhua*) [63,64]. Swain et al. reported that TNF-α has the ability to stimulate the transcription of nuclear factor kappa B (NF-??B) in cells [65]. There is relevant evidence that demonstrates macrolides have an inhibitory impact on a generation of inflammatory mediators such as ROS and pro-inflammatory cytokines, but quinolones especially ciprofloxacin strongly provoked anti-inflammatory cytokines (IL-2, IL-6, and IFNγ) genes expression [66,67]. Anyhow, both antimicrobial groups improve cellular immunity performance and facilitate to relieve prolonged inflammation [67,68]. Regards that spreading of neutrophils with other inflammatory mediators causes an enhancement in ROS products such as hydrogen peroxide [58], could be indirectly a representative of anti-inflammatory effects of FFC. Apparently, the applied dosage of the drug could not impress IL-8 in the low hierarchical level on our findings. Opsonization of bacterial agents by alternative pathway of complement components or immunoglobulin, then phagocytosis of the opsonized bacteria lead to RBA stimulation. Chemokines such as IL-8 toughen up this procedure and the generation of cytokines [68]. On the other hand, IgM-related gene expression was suppressed against FFC entry to the fish body that correlates with the result in its serum state.

In conclusion, 15 $mg.kg^{-1}$ BW for 10-day period FFC therapy improved physiological status in terms of relief of stress and some liver function indices (CK, ALP, and ALT) of streptococcosis/lactococcosis-infected fish. Lysozyme, RBA, and inflammatory cytokines (TNF-α and IL-1β at the level of gene expression) were invigorated after consumption of FFC that are important in accelerating the process of fighting the disease. Despite suppression in total serum immunoglobulin and IgM-related gene expression, our findings narrate enhancement and improvement of cellular immunity after receiving the used dosage. However, to prove the probable adverse effects, more studies are needed. As a suggestion, the problem can be solved through the determination of optimal clinical dosage via the median effective dose (ED50) of the antibiotic for the specified fish species. The obtained optimal dosage of FFC may be placed where has maximum efficacy in treating with at least side effects on immunity.

Conflicts of interest

None.

Authors' declaration

Authors of this article have seen and approved the final version of the submitted manuscript. We ensure the article is our original work and is not under review for publication elsewhere.

Funding source

More than 55% of the fund was supplied by Iran National Science Foundation (under the supervision of Vice Presidency of Science and Technology) and the rest was the passed grant of Shiraz University for Ph.D. dissertation (prepared by I.R. Ministry of Science, Research and Technology).

Acknowledgment

The authors gratefully acknowledge Marjan Hossieni (Gorgan University of Agricultural Sciences and Natural Resources), Mohammad Saeed Fereidouni, and Prof. Akhlaghi (Shiraz University) for their help with RT-PCR and infectious challenging, respectively. Also, we appreciate Iran National Science Foundation (INSF) and Shiraz University for providing financial support.

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