Fish and Shellfish Immunology 92 (2019) 612–620

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Effects of bioactive substance from turmeric on growth, skin mucosal immunity and antioxidant factors in common carp, *Cyprinus carpio*

Sib Sankar Giri[a], V. Sukumaran[b], Se Chang Park[a,*]

[a] Laboratory of Aquatic Biomedicine, College of Veterinary Medicine and Research Institute for Veterinary Science, Seoul National University, Seoul, 08826, South Korea
[b] Dept. of Zoology, Kundavai Nachiyar Government Arts College for Women (Autonomous), Thanjavur, 613007, Tamil Nadu, India

ARTICLE INFO

Keywords:
Curcumin
Carp
Innate immunity
Pathogen resistance
Antioxidant
Gene expression

ABSTRACT

Present study evaluated the effects of curcumin, the principal curcuminoid of turmeric, on *Cyprinus carpio* growth, skin mucosal immune parameters, immune-related gene expression, and susceptibility to pathogen challenge. Diets containing four various concentrations of curcumin (g Kg^{-1}): 0 g [basal diet], 5 g [T5], 10 g [T10], and 15 g [T15] were fed to the carp (average weight: 16.37 g) for 8 weeks. Growth parameters were analysed at 4 and 8 weeks post-feeding. Skin mucosal immune responses and expression were examined in 8 weeks post-feeding. Growth performance was significantly higher in T10 and T15, with final weight gain of 102.26 ± 2.31 g and specific growth rate of 3.24 ± 0.37, respectively. The lowest feed conversion ratio (2.35 ± 0.16) was recorded in T15 than in the control ($P < 0.05$). Among the skin mucosal immune parameters examined, lysozyme (36.8 ± 4.03 U mL^{-1}), total immunoglobulin (6.74 ± 0.5 mg mL^{-1}), protein level (18.7 ± 1.62 mg mL^{-1}), alkaline phosphatase (96.37 ± 6.3 IU L^{-1}), and protease activity (9.47 ± 0.82%) were significantly higher in T15, while the peroxidase activity was higher in T10 (10.24 ± 0.9 U mg^{-1} protein). Further, lysozyme, superoxide dismutase (SOD) and catalase (CAT) activities were measured in serum and found to be higher in T10 or T15 than in the control ($P < 0.05$). However, malondialdehyde level decreased significantly in T10 and T15. Furthermore, antioxidant genes (SOD, CAT, nuclear factor erythroid 2-related factor 2) and anti-inflammatory cytokine Interleukin-10 were upregulated in the head kidney, intestine, and hepatopancreas of fish in T10 and T15. Conversely, expression of pro-inflammatory cytokines (IL-1β, tumour necrosis factor-alpha), signalling molecule NF-κBp65 were down-regulated in the tested tissues of T10 and T15. Expression of Toll-like receptor 22 (TLR22) was down regulated in head-kidney and intestine of T15. Fish from T15 exhibited significantly higher relative post-challenge survival (69.70%) against *Aeromonas hydrophila* challenge. Results of the present study suggest that dietary supplements of curcumin at 15 g Kg^{-1}can significantly improve the growth performance, skin mucosal and serum antioxidant parameters, and strengthen the immunity of *C. carpio*. Therefore, curcumin represents a promising food additive for carps in aquaculture.

1. Introduction

Aquaculture continues to be the fastest growing animal food-producing sector worldwide. The rising human population requires an additional 23 million tons of aquatic foods to maintain the current level of per capita consumption. To meet the future needs, aquaculture will depend largely on the availability of quality feeds at cheaper prices [1]. Carp, the major cultured fish, contributes to 72% of total freshwater fish production [2]. The intensive farming conditions increase the risk of disease outbreaks, which leads to huge economic loss due to mortality or reduces the profit margins [3]. Commonly, antibiotics and chemotherapeutics are applied to control disease outbreaks. Application of these substances has led to the development of drug-resistant bacteria, increased environmental hazards, and suppression of immune systems [4,5]. As an alternative approach, the use of probiotics, prebiotics, and natural immunostimulants, including specific dietary manipulation have recently received greater attention. These ingredients confer protection against various pathogens, enhancing the immune response of fish and minimizing the risk associated with the use of chemotherapeutics [6].

Various plant derived additives or plant extracts can be considered as a novel trend to control fish diseases. Immunostimulant plants or their by-products contain various phenolic, alkaloid, polyphenolic, quinine, terpenoid, lectin, and polypeptide compounds, many of which are effective alternatives to antibiotics, chemicals, vaccines, and other synthetic compounds [7,8]. Use of herbal immunostimulants in

* Corresponding author.
E-mail addresses: giribiotek@gmail.com (S.S. Giri), drvsukumar@gmail.com (V. Sukumaran), parksec@snu.ac.kr (S.C. Park).

https://doi.org/10.1016/j.fsi.2019.06.053
Received 2 April 2019; Received in revised form 14 June 2019; Accepted 28 June 2019
Available online 29 June 2019
1050-4648/

aquaculture has increased globally because they are cheaper, easy to prepare, and non-toxic to aquatic life or human health [9]. Whole plants or their parts (leaf, root, or seed) or extracts have been used as feed additive in fish aquaculture. Turmeric (*Curcuma longa* L) is a plant known for its medicinal use, dating back to 4000 years ago in the Vedic culture in India. In herbal and traditional medicine, turmeric is used for treatment of rheumatoid arthritis, chronic anterior uveitis, conjunctivitis, skin cancer, small pox, chicken pox, wound healing, urinary tract infections, and liver ailments, strengthening the overall energy of the body, dispelling worms, regulating menstruation, dissolving gallstones, cleansing wounds, and even for various digestive disorders [10]. The curcumin, the principal curcuminoid of turmeric, has been studied extensively due to its wide range of medicinal properties [11]. Plant derived bioactive components have several potent activities including antioxidant, anti-diabetic, anti-cancer, antimicrobial, anti-inflammatory, and immunostimulatory effects in fish [3,5,12–14]. Only few studies have been conducted regarding the use of curcumin in aquaculture. Akdemir et al. [15] studied the effect of 200 mg kg^{-1} and 400 mg kg^{-1} of curcumin on the growth performance and antioxidant status of rainbow trout. Dietary supplementation of curcumin (0.5–1.0%) influenced leptin, a growth hormone and hepatic growth factor in tilapia, *Oreochromis mossambicus* [13].

The skin of vertebrates serves as the first line of defence against pathogens. The teleost skin is a mucosal surface that harbours abundant mucus-producing cells, lacks keratinization, and possesses living epithelial cells that make direct contact with the water medium [16]. The skin is one of the important immune barriers in teleost fish, which represent the most ancient bony vertebrates that contain skin-associated lymphoid tissue (SALT) and elicit gut-like immune responses [17]. Recently, few studies demonstrated that herbal administration could alter the skin mucosal immune responses in fish. Garlic supplementation improved the skin mucosal immune parameters and growth performance of Caspian roach fry [18]. Dietary myrtle (*Myrtus communis*) at 20 g kg^{-1} improved immune responses, and upregulated the expression of antioxidants and growth related genes in zebrafish [19]. Further, dietary white-button mushroom powder administration positively influenced the skin mucosal immunity, antioxidant defence, and growth responses of *Cyprinus carpio* [5]. In our previous study, we demonstrated that dietary ginger increased the skin mucosal immune parameters such as lysozyme, immunoglobulin, protein level, and alkaline phosphatase activities in *Labeo rohita* [12]. Further, ginger supplementation altered the expression of various immune and antioxidant genes in head kidney, intestine, and hepatopancreas of fish [12]. However, till date, no study has examined the effect of curcumin on skin mucosal immune responses in fish. Further, curcumin is well known for its anti-inflammatory potential, and many clinical investigations have been done to evaluate its bioactive effects in various inflammatory conditions [10]. Thus, the present study aimed to investigate the effects of dietary curcumin on the skin mucosal immune responses, and cytokine gene expression of *Cyprinus carpio*, and disease resistance against pathogen infection.

2. Materials and methods

2.1. Diet preparation

The basal diet containing 29.1% protein, 7.24% lipid, and 11.83% ash was prepared as described in our previous study [12]. The basal diet was supplemented with curcumin powder (Synthite Industrial Chemicals Limited, Kochi, India) at four different concentrations (g kg^{-1}): 0 g (Control), 5.0 g (T5), 10.0 g (T10), and 15.0 g (T15). The ingredients were blended thoroughly and pelleted, air-dried, ground, and sieved into appropriate pellet sizes. The experimental feed was prepared weekly and stored at 4 °C.

2.2. Fish maintenance and experimental design

Healthy *C. carpio* (average weight: 16.37 ± 0.79 g) obtained from a local fish farm were acclimatized to laboratory conditions for 2 weeks at 24 ± 1.8 °C and fed a basal diet [12]. Approximately, 20% of the water in all the tanks was replaced daily, and 100% of the water was replaced weekly. Basic physicochemical parameters of the water were monitored weekly; oxygen and ammonia concentrations were 6.2–7.3 mg L^{-1} and 0.03–0.06 mg L^{-1}, respectively, and pH ranged from 7.0 to 7.6.

In total, 240 fish were randomly assigned into the four experimental groups. In each independent experimental group, 60 fish were divided into three tanks (20 fish per tank). The following groups were established: (I) Control, basal diet; (II) diet supplemented with 5 g kg^{-1} curcumin (T5); (III) diet supplemented with 10 g kg^{-1} curcumin (T10); and (IV) diet supplemented with 15 g kg^{-1} curcumin (T15). Fish were fed with 2–4% of body weight per day up to eight weeks. The amount of feed consumed was determined by daily recovery of excess feed (in any), which was then adjusted every 2 weeks by batch weighing after 24 h of starvation.

2.3. Growth performances

Thirty-six fish were collected from each group (i.e. 12 fish per tank) at the end of 4 and 8 weeks of trial for evaluating the following growth parameters:

weight gain rate (WGR; %) = $[(W_t–W_0)/W_0] \times 100$; specific growth rate (SGR; % day^{-1}) = $[(\ln W_t–\ln W_0)/t] \times 100$; feed conversion ratio (FCR) = [total dry feed intake (g)/wet weight gain (g)]; survival rate (%) = 100 × (final number of fish/initial number of fish); where, Wt and W_0 were the final and initial weights (g) of the fish, respectively; 't' is the duration of feeding (in days).

2.4. Challenge test

After 8 weeks of experimental feeding, 12 fish from each tank (i.e. 3 × 12 = 36 fish per group) were selected for the challenge test. All the fish were intraperitoneally (i.p.) injected with 100 μL of phosphate buffered saline (PBS) containing 1×10^7 live *A. hydrophila* cells. Another group of 10 fish (fed basal diet) was i.p. injected with 100 μL of PBS to test if there is any adverse effect of PBS on fish. The challenged fish were kept under observation for one month and fed a basal diet. The mortality of fish in each group was recorded daily over a course of 4 weeks. The relative per cent survival (RPS %) was calculated by the following formula:

$$\text{RPS}(\%) = \left[1 - \left(\frac{\text{mortality in treated group}(\%)}{\text{mortality in control group}(\%)}\right)\right] \times 100.$$

2.5. Sample collection

At the end of 8 weeks of feeding trial, 15 fish from each group were anesthetized with MS222. Skin mucous was collected according to the procedure described earlier [12]. Each fish were transferred into separate polyethylene bags containing 10 mL of 50 mM NaCl. The bags were gently shaken for 2 min and thereafter mucus was collected (pooled), transferred to centrifuge tubes, and centrifuged at 1500 *g* for 10 min at 4 °C. Supernatant was filtered through 0.45-μm Millipore filter and stored at– 80 °C for further use.

For serum collection, six fish from each group were randomly sampled and blood samples were collected from the caudal vein using a 2-mL syringe following anaesthetisation with MS222 (Sigma-Aldrich, St. Louis, MO, USA). The blood samples were transferred to centrifuge tubes (Eppendorf, Germany). Serum was collected by centrifugation (4000 × *g*, 10 min, 4 °C) and stored at −20 °C until use.

Table 1
Real-time primer sequences and thermocycling conditions.

Target Gene	Primer sequence (5′ to 3′)	Thermocycling conditions	Reference/Accession no.
IL-1β	CTCTACCTTGCTTGTACCCAG AGCTGTGCTAATAAACCATCCAG	94 °C 5 min, 45 cycles of 94 °C 15 s, 60 °C 15 s and 72 °C 20 s	KC008576.1
IL-10	AACCATTACTGGACGAA CGAACTCAAAGGGATT	94 °C 5 min, 45 cycles of 94 °C 15 s, 60 °C 15 s and 72 °C 20 s	JX524550.1
TNF-α	TGGCTTGAAATTAGTGGACAG TAGATGCCGAAGAAATCAGAG	94 °C 5 min, 45 cycles of 94 °C 15 s, 60 °C 15 s and 72 °C 20 s	AB112424.1
HSP70	GGCAGAAAGTTTGATGACCCA GCAATCTCCTTCATCTTCACC	95 °C 3 min, 40 cycles of 95 °C 15 s, 61 °C 30 s and 85 °C 30 s	AY120894
SOD	TGGCGAAGAAGGCTGTTTGT TTCACTGGAGACCCGTCACT	95 °C 30 s, 40 cycles of 95 °C 5 s, 61 °C 30 s and 72 °C 30 s	JF342355
CAT	CTGGAAGTGGAATCCGTTTG CGACCTCAGCGAAATAGTTG	95 °C 30 s, 40 cycles of 95 °C 5 s, 61 °C 30 s and 72 °C 30 s	JF411604
Nrf2	TTCCCGCTGGTTTACCTTAC CGTTTCTTCTGCTTGTCTTT	95 °C 30 s, 40 cycles of 95 °C 5 s, 61 °C 30 s and 72 °C 30 s	JX462955
NF-κB p65	GGCAGGTGGCGATAGTGTT CATTCCTTCAGTTCTCTTGCG	94 °C 5 min, 45 cycles of 94 °C 15 s, 60 °C 15 s and 72 °C 20 s	KJ526214
TLR22	TCAAGGTTTGTCCTCCTTGG TTTGGTTAGCCTCGAAATGG	94 °C 1 min, 40 cycles of 94 °C 20 s, 59 °C 20 s and 72 °C 50 s	[27]
β-actin	GAAGTGTGGTGTGGACATCCGTAA AGACTCATCGTACTCCTGCTTGCTTGCT	94 °C 5 min, 45 cycles of 94 °C 15 s, 60 °C 15 s and 72 °C 20 s	JQ619774.1

2.6. Skin mucosal immune parameters

2.6.1. Lysozyme activity

Lysozyme activity (LA) of skin mucus was determined using a turbidimetric method based on the lysis of the lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus* (Sigma) [20]. A unit of LA was defined as the amount of mucus that caused a decrease in absorbance at 450 nm of 0.001 min^{-1}.

2.6.2. Total immunoglobulin

Skin mucus total immunoglobulin (Ig) levels were measured using the method described by Hoseinifar et al. [21]. The difference in protein contents prior to and after immunoglobulin molecule precipitation was considered as the Ig content.

2.6.3. Protein level

The mucus protein level was determined according to Lowry et al. [22] using bovine serum albumin as standard. The absorbance was measured using a spectrophotometer at 750 nm.

2.6.4. Alkaline phosphatase (ALP) activity

The activity of alkaline phosphatase (ALP) in the mucus was measured using a commercial kit (Sigma). Samples were prepared according to the manufacturer's protocol, and the absorbance was measured at 405 nm.

2.6.5. Protease activity

Protease activity was measured according to the method described by Guardiola et al. [23]. Briefly, equal volume of skin mucus was incubated with 100 mM ammonium bi-carbonate buffer containing 0.7% azocasein (Sigma-Aldrich) for 19 h at 30 °C. Trichloroacetic acid (4.6%) was added to stop the reaction and the mixture was centrifuged for 10 min at 6000 *g*. The supernatant was transferred to a 96-well plate in triplicate containing 100 μL 0.5 N NaOH per well; the OD was measured at 450 nm using a plate reader. Skin mucus was replaced by trypsin solution (5 mg mL^{-1}) as positive control (100% of protease activity), or by buffer as negative control (0% activity).

2.6.6. Peroxidase activity

Skin mucosal peroxidase activity was measured according to the method described by Guardiola et al. [23]. Mucus sample (30 μL) was diluted with 120 μL of Hank's buffer (HBSS) without Ca^{++} or Mg^{++} in 96-well microtitre plates. Then, 50 μL of 20 mM TMB and 5 mM H_2O_2 were added as substrates. After 2 min, 50 μL of 2 M sulphuric acid was added to stop the colour change reaction and OD was measured at 450 nm using a microplate reader. One unit was defined as the amount producing an absorbance change of 1 and the activity was expressed as units mL^{-1}.

2.7. Serum enzymatic or antioxidant parameters

Lysozyme activity was measured according to the method described previously [24]. One unit of lysozyme activity was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min^{-1} mL^{-1} serum.

Serum superoxide dismutase (SOD) activity was determined with an enzymatic assay method using a reagent kit (Randox, Crumlin, U.K.), as described previously [25]. One unit of SOD activity was defined as the amount of enzyme necessary to produce a 50% inhibition of the NBT reduction rate measured at 550 nm.

CAT activity was measured by the rate of decrease in H_2O_2 absorbance at 240 nm by using a commercially available kit (Sigma-Aldrich, USA).

Malondialdehyde (MDA) content in serum was measured using a colorimetric methods based on thiobarbituric acid reaction [26].

2.8. Antioxidant and immune-related gene expression

At the end of feeding, head kidney, intestine, and hepatopancreas were dissected from nine fish per group. The total RNA was extracted from the tissues (kidney, intestine, and hepatopancreas) using TRIZOL reagent (Invitrogen). The RNA concentration and purity was quantified by spectrophotometer, and the quality was checked by 1% agarose gel containing 0.5 μg mL^{-1} ethidium bromide. Complementary DNA (cDNA) was synthesized using SuperScript® cDNA synthesis kit (Life Technologies) by following the manufacturer's instructions. Real-time PCR analyses of SOD1, GPx, Nrf2, IL-10, IL-1β, TNF-α, NF-κB p65, TLR22, and β-actin (housekeeping gene) were carried out with a CFX96™ Real-Time PCR system (Bio-Rad, Laboratories, Inc.) following standard protocol with the primers and thermo cycling conditions as indicated in Table 1. To verify the accuracy of each amplicon, melt curve analysis was performed after amplification. All samples were run in parallel with the housekeeping gene to normalize cDNA loading. Gene expression results were analysed using the $2^{-\Delta\Delta CT}$ method after verification that the primers amplified with an efficiency of approximately 100% [28]. Data for all treatment groups were compared with

Table 2
Effects of curcumin powder on the growth performance of *Cyprinus carpio*.

Parameters	Control	T5	T10	T15
0–28 days				
Initial weight (g)	16.81 ± 0.93	16.46 ± 0.62	17.14 ± 1.03	16.39 ± 0.47
FW (g)	39.46 ± 1.82^{a}	39.81 ± 1.07^{a}	43.01 ± 1.72^{a}	43.79 ± 2.24^{a}
WGR (%)	133.73 ± 2.4^{a}	139.42 ± 3.94^{b}	148.7 ± 2.51^{c}	165.73 ± 2.1^{d}
SGR	2.97 ± 0.36^{a}	3.08 ± 0.18^{ab}	3.26 ± 0.41^{ba}	3.41 ± 0.29^{b}
FCR	3.26 ± 0.14^{a}	3.22 ± 0.17^{a}	3.11 ± 0.22^{a}	3.06 ± 0.12^{a}
Survival rate (%)	100	100	100	100
0–56 days				
Initial weight (g)	16.81 ± 0.93	16.46 ± 0.62	17.14 ± 1.03	16.39 ± 0.47
FW (g)	87.94 ± 2.83^{a}	93.14 ± 3.16^{b}	102.26 ± 2.31^{c}	99.97 ± 1.74^{c}
WGR (%)	421.96 ± 6.38^{a}	465.3 ± 4.95^{b}	494.76 ± 7.1^{c}	508.5 ± 6.53^{d}
SGR	2.94 ± 0.52^{a}	3.06 ± 0.22^{ab}	3.20 ± 0.19^{b}	3.24 ± 0.37^{b}
FCR	2.62 ± 0.11^{a}	2.59 ± 0.23^{ab}	2.38 ± 0.14^{b}	2.35 ± 0.16^{b}
Survival rate (%)	100	100	100	100

Note: FW = final weight; WGR = weight gain rate; PWG = percent weight gain; SGR = specific growth rate; FCR = feed conversion ratioValues in the same column with different superscripts letters are significantly different ($P < 0.05$). Values are presented as mean ± SEM (*n* = 36 fish in each group).

that of the control group.

2.9. Statistical analysis

The obtained data were analysed by one-way analysis of variance, and Tukey's test was employed to assess differences between treatments. All statistical analyses were performed using OriginPro software (version 8; OriginLab Corporation, Northampton, MA, USA). $p < 0.05$ was considered statistically significant. The results are expressed as mean values ± standard error of mean (mean ± SEM).

3. Results

3.1. Growth performance

The effects of curcumin on the growth performance of *Cyprinus carpio* are presented in Table 2. Curcumin supplementation had no significant effect on WG after 4 weeks of feeding. Although, SGR was higher ($P < 0.05$) in the T15, curcumin supplementation had no significant effect on FCR level. Interestingly, WGR was higher ($p < 0.05$) in the curcumin fed groups.

Eight weeks of curcumin feeding registered significant effects on the growth performance of *C. carpio* (Table 2). The FWG was significantly higher in all the treatment groups (T5, T10, and T15, with the highest FWG (102.26 ± 2.31 g) was recorded in T10. The WGR was higher ($p < 0.05$) in curcumin fed groups, with highest in T15 (compared with the control). Significantly highest SGR (3.24 ± 0.37) was recorded T15, compared with the control. FCR was significantly lowered in both T10 and T15 (compared with control), with lowest FCR recorded in the T15. No mortality was noticed in control or treatment groups during the entire trial period.

3.2. Skin mucosal immune responses

Dietary supplementation of curcumin increased the LA, total Ig, and ALP levels in skin mucus (Table 3). The LA was significantly higher in the treatment groups compared with control, with T15 showing the highest levels (36.8 ± 4.03 U mL^{-1}). Although IgM levels were higher in the curcumin supplementation groups, differences were significant only in T15 when compared with the control group. The ALP activity was higher ($P < 0.05$) in the T10 and T15 compared with the control, with T15 showing the highest activity (96.37 ± 6.3 IU L^{-1}).

Dietary intake of curcumin significantly augmented the protein level and protease activity in the mucus of carps (Table 4). Highest supplementation level of curcumin resulted in the highest augmentation of protein level (18.7 ± 1.62 mg mL^{-1}) and protease activity (9.47 ± 0.82%) in T15. Peroxidase activity was higher ($P < 0.05$) in T10 and T15 compared with the control, with T10 showing the highest activity (10.24 ± 0.6 U mg^{-1} protein). Furthermore, very slight reduction in peroxidase activity was observed in T15 than in T10.

Table 3
The lysozyme activity (LA), immunoglobulin (Ig) level, and alkaline phosphatase (ALP) activities in the skin mucus of *Cyprinus carpio* fed diets **supplemented** with graded level of curcumin for 8 weeks.

Group	LA (U mL^{-1})	Total Ig (unit-mg mL^{-1})	ALP (IU L^{-1})
Control	16.4 ± 1.7^{a}	4.83 ± 0.4^{ab}	83.6 ± 5.8^{a}
T5	21.1 ± 2.6^{b}	4.91 ± 0.4^{a}	86.32 ± 4.3^{a}
T10	33.7 ± 2.9^{c}	5.3 ± 0.3^{ab}	94.8 ± 6.02^{b}
T15	36.8 ± 4.03^{c}	6.74 ± 0.5^{b}	96.37 ± 6.3^{b}

Values in the same column with different superscripts letters are significantly different ($P < 0.05$).
Values are presented as mean ± SEM (*n* = 15).

Table 4
The skin mucosal protein level, peroxidase and protease activities in *Cyprinus carpio* fed diets supplemented with graded level of curcumin for 8 weeks.

Group	Protein level	Peroxidase activity	Protease activity
	(mg mL^{-1})	(U mg^{-1} protein)	(%)
Control	09.06 ± 0.9^{a}	7.8 ± 0.8^{a}	3.71 ± 0.48^{a}
T5	11.2 ± 0.8^{b}	7.6 ± 0.9^{a}	5.88 ± 0.72^{b}
T10	16.8 ± 1.7^{c}	10.24 ± 0.6^{b}	8.13 ± 0.68^{c}
T15	18.7 ± 1.62^{c}	10.08 ± 0.9^{b}	9.47 ± 0.82^{c}

Values in the same column with different superscripts letters are significantly different ($P < 0.05$).
Values are presented as mean ± SEM (*n* = 15).

3.3. Serum antioxidant or enzymatic parameters

As shown in Table 5, lysozyme activity was significantly higher in the treatment groups than in the control, with highest activity recorded in T15 (51.06 ± 3.73 U mL^{-1}). Similarly, catalase activity was higher in the treatment groups, although the difference from the control was significant only in case of T15 (20.02 ± 1.38 IU L^{-1}). SOD activity was significantly higher in T10 and T15 compared with the control, with T15 showing the highest activity (35.72 ± 2.26 U mL^{-1}). Conversely, MDA level was significantly lower in T10 and T15, with lowest activity was recorded in T15 (32.21 ± 2.4 nmol mL^{-1}).

Table 5
The LA, SOD, CAT, and MDA activity in the serum of *Cyprinus carpio* fed diets supplemented with graded level of curcumin for 8 weeks.

Group	LA (U mL^{-1})	CAT (IU L^{-1})	SOD (unit mL^{-1})	MDA (nmol mL^{-1})
Control	43.27 ± 2.81^{a}	16.11 ± 1.42^{a}	29.47 ± 2.14^{a}	48.26 ± 2.7^{a}
T5	46.53 ± 3.7^{ab}	18.04 ± 1.26^{a}	29.86 ± 1.84^{a}	45.72 ± 3.02^{ba}
T10	47.88 ± 3.26^{bc}	18.63 ± 2.03^{a}	34.18 ± 2.62^{b}	40.38 ± 2.18^{c}
T15	51.06 ± 3.73^{c}	20.02 ± 1.38^{b}	35.72 ± 2.26^{b}	32.21 ± 2.4^{d}

Values in the same column with different superscripts letters are significantly different ($P < 0.05$).
Values are presented as mean ± SEM ($n = 6$).

3.4. Gene expression

The expression of immune related genes in the head kidney, hepatopancreas, and intestine of fish was examined at the end of the feeding trial (Figs. 1–3). Transcription levels of SOD were significantly higher in all three organs in T15 (Fig. 1). T15 exhibited highest SOD expression in head kidney and intestinal tissues, whereas SOD mRNA expression was highest in the hepatopancreas from T10 (Fig. 1A). Curcumin administration had no significant effect on the treatment groups, although CAT mRNA expression was slightly increased in T10 and T15. The Nrf2 mRNA expression was significantly higher in head kidney and hepatopancreas from both T10 and T15, whereas it was higher ($P < 0.05$) in the intestine from T5-T15. Highest expression of Nrf2 mRNA was registered in head-kidney from T10 (Fig. 1).

The effects of curcumin on pro-inflammatory cytokines (TNF-α and IL-1β) and anti-inflammatory cytokine (IL-10) are shown in Fig. 2. TNF-α expression was down-regulated ($P < 0.05$) in T15 compared with the control. However, in case of T10, TNF-α expression was significantly lower only in the head kidney. IL-1β expression was significantly down-regulated in head kidney and intestine of fish in T10 compared with the control. Further, IL-1β was significantly downregulated in the intestine and hepatopancreas of fish in T15. Overall, lowest expression of IL-1β and TNF-α was noticed in T15.

IL-10 mRNA expression was upregulated in all treatment groups (Fig. 2C). IL-10 expression was significantly higher in the intestine and hepatopancreas, with the highest expression noticed in the intestine from the T10. In case of head kidney, significantly higher IL-10 expression was observed only in T15 ($P < 0.05$).

Notably, curcumin supplementation did not affect the expression of heat-shock protein 70 (HSP70) (Fig. 3).

NF-κBp65 expression was down-regulated in the experimental groups (Fig. 3), particularly in the head kidney and hepatopancreas of carp in T102 and T15, with lowest expression recorded in hepatopancreas from the T10. TLR22 expression was significantly down-regulated in the head-kidney and intestine of fish in T10 and T15, and in intestine of fish in T15 (Fig. 3).

3.5. Pathogen resistance

Dietary administration of curcumin enhanced the resistance of carps to pathogen infection. After 28 days post-challenge, the highest RPS (69.70%) was recorded in T15, followed by T10 (60.6%) and T5 (36.3%). Highest mortality (91.66%) was recorded in the control group fish. In negative control group, no mortality was recorded.

4. Discussion

Natural immunostimulants are receiving extensive attention due to their potential beneficial effects like protection against various pathogens, enhanced immune response of fish, reduced risk associated with the use of chemical agents and preventing the damage caused by toxic compounds in fish [6,9,29]. Plant based immunostimulants possess a number of admissible characteristics such as natural origin, high beneficial effect on organism, and less toxicity. Results of the present study show that curcumin at 10 or 15 g kg^{-1} doses for 8 weeks can significantly improve the growth performance of *C. carpio*. Further, these two doses significantly reduced the FCR values (Table 2), suggesting that the fish utilised dietary nutrients more efficiently when feed was supplemented with curcumin. FCR affects the amount of food waste produced, and its physical and chemical composition [30]. Higher SGR and lower FCR may be attributed to better nutritional status offered by curcumin. The better immunologic and enzymatic activities in T10 and T15 (described later) may have contributed to better growth performance. The growth regulating effects of herbs or its active component on fish have been extensively investigated [5,21,31–33]. Recently, Akdemir et al. [15] demonstrated that dietary curcumin at 200 mg kg^{-1} induces remarkable improvement of growth performance of high-stocking density in rainbow trout.

Immunological parameters have been considered as important tools to assess the health status of fish. Numerous studies have been conducted on the non-specific immune system of fish at cellular and humoral level. However, very limited work has been conducted on the interaction between immunostimulants and fish mucus [34]. Fish are in close contact with the aquatic environment, which is rich in a wide range of non-pathogenic and pathogenic microorganisms. The skin mucosal immune system comprises immune related enzymes that help in the control of pathogens [35]. Lysozyme, an important component in the immune system of fish, is bactericidal due to its ability to hydrolyse bacterial cell wall peptidoglycans [29]. In the present study, lysozyme activity in the skin mucus of carps fed diet supplemented with 10 or 15 g kg^{-1} of curcumin was significantly higher. Similarly, elevated mucus lysozyme level has been reported in *Labeo rohita* fed with ginger-supplemented diet [12]. On the other hand, predominant antibody type in fish is the high molecular weight Ig referred to as IgM or IgM-like because this molecule has a heavy chain isotype similar to the mammalian μ chain [36]. Further, distributions of these antigens are not uniform. For example, Ig levels in channel catfish were found to be highest in lateral skin, lower between the pectoral and anal fins, and lowest in the caudal fin and ventral skin [37]. Results of the present study revealed significantly higher IgM level in mucus of carp fed 15 g kg^{-1} of curcumin. IgM participates in the opsonisation of pathogens by facilitating their phagocytosis [38] and presence of high level of IgM in the skin mucus of carp fed curcumin may provide significant protection by reacting specifically or non-specifically with pathogens [39]. Earlier studies have demonstrated that fish fed herbal immunostimulant-supplemented diets (e.g: fenugreek seeds and ginger) had higher IgM level than those in fish fed non-supplemented diets [12,14,39,40]. More recently, gilthead sea bream fed with yeast *Sterigmatomyces halophilus* for 30 days exhibited higher level of IgM in skin mucus at 7 days post-challenge with *Vibrio parahaemolyticus* [41]. However, mechanisms involved in IgM elevation are unknown and need further investigation.

Alkaline protease plays potential protective role in teleosts during the initial stage of stress or pathogenic infections [42]. It is an indication of osteoblast function and indirectly reflects the physical condition of fish [43]. In this study, we found that that dietary curcumin at 10 or 15 g kg^{-1} elevated the skin mucosal ALP activity of carp. Similar results were observed in previous studies when ginger (at 0.8% or 1.0% level)-supplemented diet was given to *Labeo rohita* for 60 days [12] or administration of 0.5% Hilyses in rainbow trout for 50 days [44].

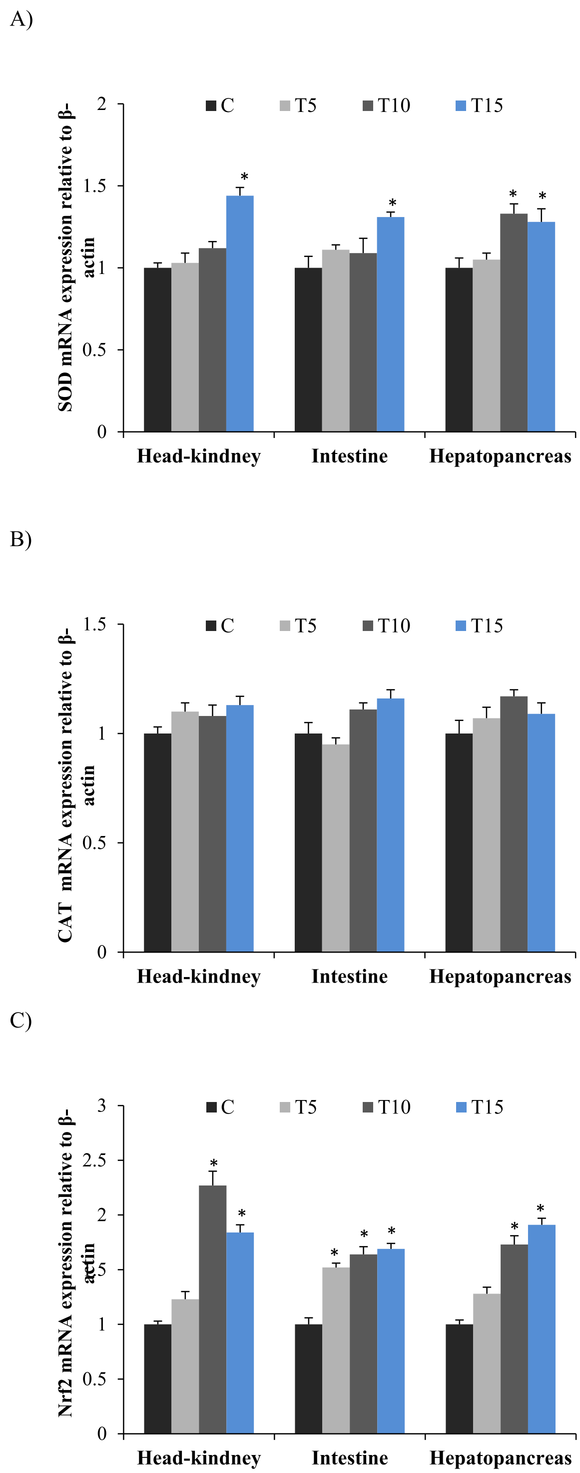

Fig. 1. The relative mRNA expressions of antioxidant-related genes (SOD, CAT, and Nrf2) in the head kidney, intestine, and hepatopancreas of *C. carpio* fed curcumin-supplemented diets. A significant difference from the control is denoted by asterisk ($P < 0.05$). Each bar represents the mean ± SEM ($n = 9$).

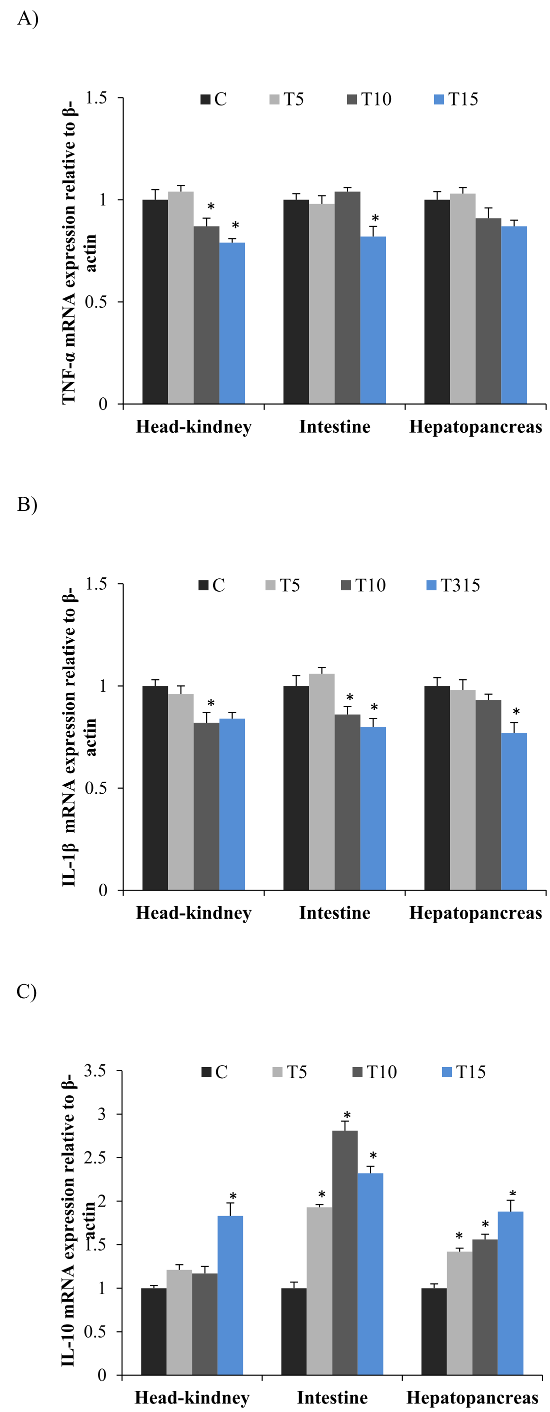

Fig. 2. The relative mRNA expressions of up-regulated anti-inflammatory cytokine (IL-10) and pro-inflammatory cytokines (IL-1β, TNF-α) in the head kidney, intestine, and hepatopancreas of *C. carpio* fed curcumin-supplemented diets. A significant difference from the control is denoted by asterisk ($P < 0.05$). Each bar represents the mean ± SEM ($n = 9$).

Ghehdarijani et al. [18] also reported that garlic supplementation increased the ALP in Caspian roach fry. The increase in ALP may be associated with improved mucosal immune responses in fish. Elevation of mucus protein contents is an indicator of the amount of skin mucus secretion [45]. In the present study, protein level in skin mucus of fish fed curcumin-supplemented diets was significantly higher and was highest in the T15. In agreement with the result of this study, Hoseinifar et al. [5] and Ghehdarijani et al. [18] reported an increase in the skin mucus protein level in *Cyprinus carpio* and Caspian roach (*Rutilus rutilus*) fry following administration of white-button mushroom powder or garlic supplementation for 8 weeks.

Immunoglobulin M as well as enzymes such as protease, anti-protease, and peroxidase are major components of the fish immune system, and play an important role in protecting fish against pathogens. In skin mucus, proteases may play a protective role against pathogens and also activate and enhance the production of other innate immune components such as complement, Igs, or antibacterial peptides [46]. For

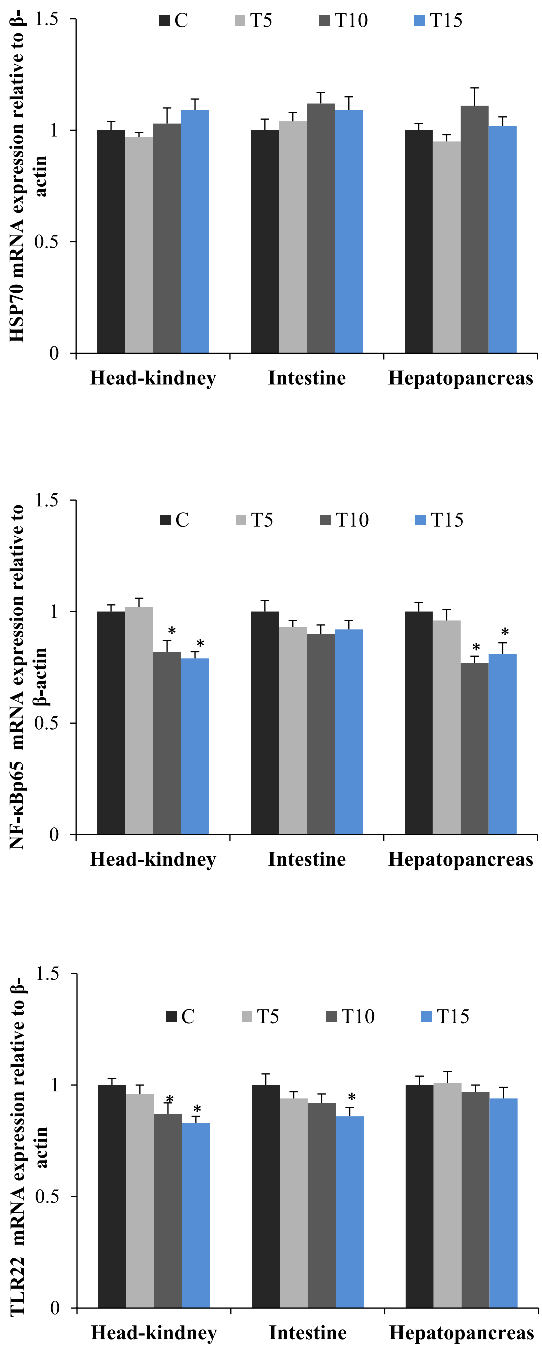

Fig. 3. The relative mRNA expressions of HSP70, TLR22, and NF-κB p65 in the head kidney, intestine and hepatopancreas of *C. carpio* fed curcumin-supplemented diets. A significant difference from the control is denoted by asterisk ($P < 0.05$). Each bar represents the mean ± SEM ($n = 9$).

example, proteases in skin mucus of fish have the ability to degrade bacterial pathogens such as *Vibrio anguillaram* [46]. In the present study, the protease activity in the skin mucus of *C. carpio* fed curcumin-supplemented diets was significantly higher, with the highest activity observed in the 15 g kg^{-1} supplemented group (T15). Safari et al. [19] demonstrated that zebra fish fed different levels of Myrtle had significantly higher level of protease activity. Similarly, our results are in agreement with previous reports showed that dietary supplementation of herbal immunostimulants could increase protease activity in skin mucus of fish [12,14,39]. It has been suggested that relative importance of protease enzymes is higher in fish mucus than that of other enzymes like lysozyme, ALP, or esterases [47]. In healthy status, anti-protease maintains a homeostatic balance with proteases, preventing the associated inflammatory damage from excess protease activity [39]. In order to get positive effect with an immunostimulant, both protease and anti-protease activity should increase and an imbalance could be dangerous. In the present study, beside the significant increase in protease activity in skin mucus, anti-protease activity increased non-significantly in the treated groups. Our results are in contrast with that of a previous study [39], which showed opposite trends in protease and anti-protease activity in skin of fish fed palm fruit extracts and probiotics. However, protease and anti-protease was higher in skin mucus of sea bream fed *Saccharomyces halophillus* (0.55% or 1.1%) [41].

Peroxidases are important microbicidal agents that effectively eliminate H_2O_2 and maintain the redox balance of the immune system [48]. The peroxidase in skin mucus is essential for mucosal immunity and skin defence [14]. In the present study, significantly higher peroxidase activity was recorded in fish fed higher doses (10 or 15 g kg^{-1}) of curcumin. Similarly, dietary administration of fenugreek seeds [14] or palm fruits with probiotics [39] to gilthead sea bream exhibited higher peroxidase activity in skin mucus. In another study, Nile tilapia fed spent mushroom substrate-supplemented diets with or without *Lactobacillus plantarum* significantly increased skin mucus peroxidase activity after 8 weeks of trial [49]. In the current study, SOD and CAT enzyme activities were increased in serum of fish fed curcumin-supplemented diets. SOD and CAT are key enzymes that convert the highly toxic reactive oxygen species (ROS) into less harmful oxygen species for the host [41]. Moreover, curcumin supplementation (10 or 15 g kg^{-1}) significantly reduced the serum MDA level in fish. MDA is the main product of lipid peroxidation. It has strong biotoxicity and it can damage the structure and function of cells [50]. The decreased MDA content indicated the enhancement of the endogenous antioxidant defence system in circumin fed fish. Loganathan et al. [47] hypothesized that the presence of lysozyme, protease, alkaline phosphatase and esterase may have direct effects on the skin innate immune responses against pathogenic microorganisms. Other studies have suggested that an increase in lysozyme, immunoglobulins, alkaline phosphatase, and protein level by herbal supplementation might improve skin mucosal immunity [12,18]. Together, these findings indicate that increment in the mucosal immune response obtained through the supplementation of diet with curcumin is important to promote fish immunity.

The expression of immune-related genes in head kidney, intestine, and hepatopancreas was evaluated to explore the influence of curcumin on immune function of carp. In the present study, expression of SOD gene was significantly upregulated in all tested tissues of the T15 and CAT gene expression was upregulated in T10 and T15, although differences were not significant, compared with the control. Consistent with our results, dietary administration of immunostimulants was reported to upregulate the antioxidant gene expression and activities; these immunostimulants included white button mushroom powder in common carp [5], Myrtle (*Myrtus communis*) in zebrafish [19], ferula (*Ferula assafoetida*) in common carp [51], palm fruit extracts and probiotics in common carp [52]. The alteration of antioxidant enzyme gene expression together with elevated levels of SOD and CAT may hint at the positive effects of dietary curcumin on antioxidant defence. The free radical scavenging activity of curcumin can arise either from the phenolic OH group or from the CH_2 group of the β-diketone moiety [53]. A previous study reported that compounds (C1 and C4) of curcumin, both of which contain a phenolic group at position-4 of the aromatic ring, were found to be highly potent antioxidants with higher antioxidant values than the synthetic antioxidant butylated hydroxytoluene [53].

The dietary supplementation of 10–15 g kg^{-1} of curcumin upregulated Nrf2 mRNA expression in all tested tissues. Nrf2 protects the cell from reactive oxygen species and other electrophilic components by generating phase II detoxifying antioxidant enzymes [54]. Akdemir et al. [15] showed that dietary curcumin at 200 mg kg^{-1} of diet increased the hepatic Nrf2 levels in rainbow trout under high stocking density. It has been postulated that curcumin may induce the Nrf2 expression in rat kidney and ischaemic brain through the protection of antioxidant enzymes and the inhibition of oxidative stress [54]. Further, dietary administration of optimum levels of phospholipids up-regulated the Nrf2, SOD1, and GPx mRNA expression in the intestine of grass carp [55]. Previous studies, including ours, showed that mRNA levels of SOD were positively related to Nrf2 levels [18,56].

The cytokines IL-1β and TNF-α are primarily produced by monocytes and macrophages and regulate multiple aspects of the immune response [57]. IL-1β is activated in response to microbial invasion and tissue injury, which can stimulate immune responses by activating lymphocytes or by inducing the release of other cytokines that activate macrophages, NK cells, and lymphocytes [58]. In the present study, dietary curcumin at 15 g kg^{-1} suppressed the mRNA abundance of TNF-α and IL-1β, and enhanced the mRNA abundance of anti-inflammatory cytokine IL-10. The inverse expression patterns of pro- and anti-inflammatory cytokines in fish following the dietary administration of herbal immunostimulants have been reported earlier [12,59]. In an *in vitro* study, curcumin was identified as a pro-drug that requires oxidative activation into reactive metabolites to exert anti-inflammatory activities [60]. The metabolites of curcumin, produced by oxidation reactions, covalently bind to and inhibit proteins in the inflammatory NF-κB signalling pathway. Further, the upregulated expression of IL-10 might have controlled or suppressed the expression of TNF-α and IL-1β [61] in the present study.

The NF-κB is a key signalling molecule in the inflammatory response. In the present study, dietary curcumin administration at 10 or 15 g kg^{-1} significantly down-regulated NF-κBp65 expression in head kidney and hepatopancreas of fish. Previous studies reported that curcumin supplementation at 200 and 400 mg kg^{-1} decreased ($P < 0.01$) NF-κB expression in hepatopancreas of rainbow trout [15]. Recently, an *in vitro* study demonstrated that synthetic curcumin analogues that undergo oxidative transformation potently inhibited the pro-inflammatory NF-κB [60]. Oxidative metabolites of curcumin adducted to and inhibited the inhibitor of NF-κB kinase subunit β (IKKβ), an activating kinase upstream of NF-κB. Further, Olivera et al. [62] demonstrated that curcumin or its analogue (EF31) acts as a NF-κB inhibitor and exhibits both anti-inflammatory and anti-cancer properties in RAW264.7 macrophages. In addition, EF31 exhibited greater inhibition of NF-κB nuclear translocation as well as the induction of downstream inflammatory mediators including pro-inflammatory cytokine mRNA and protein (TNF-α, IL-1β, and IL-6) [62]. Chen et al. [56] demonstrated that administration of exogenous phospholipids that down-regulated the expression of pro-inflammatory cytokines might inhibit NF-κB p65 translocation to the nucleus in fish. Present study suggests that dietary curcumin may be involved in inhibition of the NF-κB p65 translocation to the nucleus and thereby down-regulates the gene expression of pro-inflammatory cytokines. However, further investigation is warranted to explore the potential mechanisms. Moreover, curcumin supplementation (at 10 or 15 g kg^{-1}) significantly down-regulated the TLR22 expression in the head-kidney of fish. Among various TLRs discovered, TLR22 is unique to aquatic animals. It can detect infections with dsRNA and mediate antiviral protection in fish [63]. Therefore, role of curcumin on TLR signalling pathway warrants further study.

The present study has revealed that dietary administration of 15 g $Kg^{-1}$of curcumin resulted into highest post-challenge survival (69.7% RPS). The challenge test can evaluate the effectiveness of immunostimulant against pathogen infection. Earlier, dietary supplementation of herbal immunostimulants increased the disease resistance of various fish species, such as *Lates calcarifer* [3,64], *L. rohita* [12,59], *Huso huso* [65], *C. carpio* [66], and crucian carp [67] against pathogen infection. The enhanced skin mucosal immune parameters (LA, ACP, IgM), serum antioxidant enzymes (SOD, CAT), mucosal enzymatic activities (ALP, protease, peroxidase), and altered cytokine gene expression in tested tissues in fish fed diets supplemented with 15 g kg^{-1} of curcumin might be associated with the improved resistance of fish against pathogen infection, resulting in higher post-challenge survival rates.

In conclusion, the dietary administration of curcumin at 15 g kg^{-1} for 8 weeks can modulate growth performance, skin mucosal immune parameters, serum enzymatic parameters and expression of immune related genes. In addition, dietary administration of 15 g kg^{-1} of curcumin improved the resistance of fish against *Aeromonas hydrophila* infection. Moreover, curcumin supplementation might exhibit anti-inflammatory effects by down-regulating the expression of TNF-α and IL-1β, and up-regulating IL-10. However, further studies on the mode of action and growth promotion should be investigated to completely understand the effect of curcumin in fish.

Funding

Korea Research Fellowship Program of the National Research Foundation of Korea (NRF), Ministry of Science and ICT [grant number 2016H1D3A1909005], and Cooperative Research Program for Agriculture Science and Technology Development (Supportive managing project of Centre for Companion Animals Research) by Rural Development Administration [grant number PJ013985032018].

Acknowledgment

This research was supported by the 'Korea Research Fellowship Program' of the National Research Foundation of Korea (NRF), Ministry of Science and ICT (KRF: 2016H1D3A1909005), and Cooperative Research Program for Agriculture Science and Technology Development (Supportive managing project of Center for Companion Animals Research) by Rural Development Administration (PJ013985032018).

References

[1] FAO, FAO Aquaculture Newsletter vol. 56, (2017) (April), Rome).
[2] H. Kühlwein, D.L. Merrifield, M.D. Rawling, A.D. Foey, S.J. Davies, Effects of dietary β-(1,3)(1,6)-D-glucan supplementation on growth performance, intestinal morphology and haemato-immunological profile of mirror carp (*Cyprinus carpio* L.), J. Anim. Physiol. Anim. Nutr (Berl). 98 (2014) 279–289.
[3] A.D. Talpur, M. Ikhwanuddin, A. Bolong, M. Abol-Munafi, A.D. Alpur, Nutritional effects of ginger (*Zingiber officinale* Roscoe) on the immune response of Asian sea bass, *Lates calcarifer* (Bloch) and disease resistance against *Vibrio harveyi*, Aquaculture 400–401 (2013) 46–52.
[4] G. Brogden, T. Krimmling, M. Adamek, H.Y. Naim, D. Steinhagen, M. Von Köckritz-Blickwede, The effect of β-glucan on formation and functionality of neutrophil extracellular traps in carp (*Cyprinus carpio* L.), Dev. Comp. Immunol. 44 (2014) 280–285.
[5] S.H. Hosseinifar, H.K. Zou, H. Paknejad, A. Hajimoradloo, H.V. Doan, Effects of dietary white-button mushroom powder on mucosal immunity, antioxidant defence, and growth of common carp (*Cyprinus carpio*), Aquaculture 501 (2019) 448–454.
[6] B. Magnadottir, Immunological control of fish diseases, Mar. Biotechnol. 12 (2010) 361–379.
[7] H.M.R. Abdel-Khalil, R.H. Khalil, Evaluation of two phytobiotics, *Spirulina platensis* and *Origanumn valgare* extract on growth, serum antioxidant activities and resistance of Nile tilapia (*Oreochromis niloticus*) to pathogenic *Vibrio alginolyticus*, Int. J. Fish. Aquatic Stud. 1 (5) (2014) 250–255.
[8] R. Harikrishnan, C. Balasundaram, M.S. Heo, Review: impact of plant products on innate and adaptive immune system of cultured finfish and shellfish, Aquaculture 317 (2011) 1–15.
[9] N. Van Hai, The use of medicinal plants as immunostimulants in aquaculture: a review, Aquaculture 446 (2015) 88–96.
[10] B. Salehi, Z. Stojanović-Radić, J. Matejić, M. Sharifi-Rad, N.V. Anil Kumar, N. Martins, J. Sharifi-Rad, The therapeutic potential of curcumin: a review of clinical trials, Eur. J. Med. Chem. 163 (2019) 527–545.
[11] S. Mishra, S. Patel, C.G. Halpani, Recent updates in curcumin pyrazole and isoxazole derivatives: synthesis and biological application, Chem. Biodivers. 16 (2019) e1800366https://doi.org/10.1002/cbdv.201800366.
[12] V. Sukumaran, S.C. Park, S.S. Giri, Role of dietary ginger *Zingiber officinale* in improving growth performances and immune functions of *Labeo rohita* fingerlings, Fish Shellfish Immunol. 57 (2016) 362–370.
[13] M.V. Sruthi, Akhila B. Nair, Damodaran Arun, V.V. Thushara, C.C. Sheeja, A.S. Vijayasree, Oommen V. Oommen, L. Divya, Dietary curcumin influences leptin, growth hormone and hepatic growth factors in Tilapia (*Oreochromis mossambicus*), Aquaculture 496 (2018) 105–111.
[14] F.A. Guardiola, A. Bahi, A. Bakhrouf, M.A. Esteeban, Effects of dietary supplementation with fenugreek seeds, alone or in combination with probiotics, on gilthead seabream (*Sparus aurata* L.) skin mucosal immunity, Fish Shellfish Immunol. 65 (2017) 169–178.

[15] F. Akdemir, C. Orhan, M. Tuzcu, N. Sahin, V. Juturu, K. Sahin, The efficacy of dietary curcumin on growth performance, lipid peroxidation and hepatic transcription factors in rainbow trout *Oncorhynchus mykis* (Walbaum) reared under different stocking densities, Aquacult. Res. 48 (2017) 4012–4021.

[16] X. Zhang, L. Ding, Y. Yu, W. Kong, Y. Yin, Z. Huang, Z. Zhang, Z. Xu, The change of teleost skin commensal microbiota is associated with skin mucosal transcriptomic responses during parasitic infection by *Ichthyophthirius multifillis*, Front. Immunol. 9 (2018) 2972, https://doi.org/10.3389/fimmu.2018.02972.

[17] Z. Xu, D. Parra, D. Gomez, I. Salinas, Y.A. Zhang, L. von Gersdorff Jorgensen, et al., Teleost skin, an ancient mucosal surface that elicits gut-like immune responses, Proc. Natl. Acad. Sci. U.S.A. 110 (2013) 13097–13102.

[18] M.S. Ghehdarijani, A. Hajimoradloo, R. Ghorbani, Z. Roohi, The effects of garlic-supplemented diets on skin mucosal immune responses, stress resistance and growth performance of the Caspian roach (*Rutilus rutilus*) fry, Fish Shellfish Immunol. 49 (2016) 79–83.

[19] R. Safari, S.H. Hoseinifar, H.V. Doan, M. Dadar, The effects of dietary Myrtle (*Myrtus communis*) on skin mucus immune parameters and mRNA levels of growth, antioxidant and immune related genes in zebrafish (*Danio rerio*), Fish Shellfish Immunol. 66 (2017) 264–269.

[20] S. Subramanian, S.L. MacKinnon, N.W. Ross, A comparative study on innate immune parameters in the epidermal mucus of various fish species, Comp. Biochem. Physiol. B 148 (2007) 256–263.

[21] S.H. Hoseinifar, M. Khalili, R. Rufchaei, M. Raeisi, M. Attar, H. Cordero, M. Esteban, Effects of date palm fruit extracts on skin mucosal immunity, immune related genes expression and growth performance of common carp (*Cyprinus carpio*) fry, Fish Shellfish Immunol. 47 (2015) 706–711.

[22] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[23] F.A. Guardiola, A. Cuesta, M.A. Esteban, Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata* L.), Fish Shellfish Immunol. 59 (2016) 323–330.

[24] A.E. Ellis, Lysozyme assay, in: J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Robertson, W.B. Van Muiswinkel (Eds.), Techniques in Fish Immunology, SOS Publications, Fair Haven, NJ, USA, 1990, pp. 101–103.

[25] Y.-Z. Sun, H.-L. Yang, R.-L. Ma, W.-Y. Lin, Probiotic applications of two dominant gut *Bacillus* strains with antagonistic activity improved the growth performance and immune responses of grouper *Epinephelus coioides*, Fish Shellfish Immunol. 29 (2010) 803–809.

[26] H.H. Drape, E.J. Squires, H. Mahmoodi, J. Wu, S. Agarwal, M. Hadley, A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials, Free Radic. Biol. Med. 15 (1993) 353–363.

[27] H. Li, G. Yang, F. Ma, T. Li, H. Yang, J.H. Rombout, L. An, Molecular characterization of a fish-specific toll-like receptor 22 (TLR22) gene from common carp (*Cyprinus carpio* L.): evolutionary relationship and induced expression upon immune stimulants, Fish Shellfish Immunol. 63 (2017 Apr) 74–86.

[28] K.J. Livak, T.D. Schmittgen, "Analysis of relative gene expression data using realtime Quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (2001) 402–408.

[29] B. Magnadóttir, Innate immunity of fish (overview), Fish Shellfish Immunol. 20 (2006) 137–151.

[30] S.M. Hixson, Fish nutrition and current issues in aquaculture: the balance in providing safe and nutritious seafood, in an environmentally sustainable manner, J. Aquac. Res. Development. 5 (2014) Article ID 3 https://doi.org/10.4172/2155-9546.1000234.

[31] J. Xie, B. Liu, Q. Zhou, Y. Su, Y. He, L. Pan, X. Ge, P. Xu, Effects of anthraquinone extract from rhubarb *Rheum officinale* Bail on the crowding stress response and growth of common carp *Cyprinus carpio var. Jian*, Aquaculture 281 (2008) 5–11.

[32] S.S. Giri, S. Jai Suda, V. Sukumaran, S.C. Park, Dietary emodin affects the growth performance, immune responses, and disease resistance of *Labeo rohita* against *Aeromonas hydrophila*, Aquacult. Int. 24 (2016) 85–99.

[33] H. Dadras, M.R. Hayatbakhsh, W.L. Shelton, A. Golpour, Effects of dietary administration of Rose hip and Safflower on growth performance, haematological, biochemical parameters and innate immune response of Beluga, *Huso huso* (Linnaeus, 1758), Fish Shellfish Immunol. 59 (2016) 109–114.

[34] D. Gomez, J.O. Sunyer, I. Salinas, The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens, Fish Shellfish Immunol. 35 (2013) 1729–1739.

[35] S. Subramanian, S. MacKinnon, N. Ross, A comparative study on innate immune parameters in the epidermal mucus of various fish species, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 148 (2007) 256–263.

[36] W.B. Van Muiswinkel, C.H.J. Lamers, J.H.W.M. Rombout, Structural and functional aspects of the spleen in bony fish, Res. Immunol. 142 (1991) 362–366.

[37] D. Ceballos-Francisco, H. Cordero, F.A. Guardiola, A. Cuesta, M.Á. Esteban, Healing and mucosal immunity in the skin of experimentally wounded gilthead seabream (*Sparus aurata* L), Fish Shellfish Immunol. 71 (2017) 210–219.

[38] E. Vallejos-Vidal, F. Reyes-Lopez, M. Teles, S. MacKenzie, The response of fish to immunostimulant diets, Fish Shellfish Immunol. 56 (2016) 34–69.

[39] R. Cerezuela, F.A. Guardiola, A. Cuesta, M.A. Esteban, Enrichment of gilthead seabream (*Sparus aurata* L.) diet with palm fruit extracts and probiotics: effects on skin mucosal immunity, Fish Shellfish Immunol. 49 (2016) 100–109.

[40] A. Cuesta, J. Meseguer, M.A. Esteban, Total serum immunoglobulin M levels are affected by immunomodulators in seabream (*Sparus aurata* L.), Vet. Immunol. Immunopathol. 101 (2004) 203–210.

[41] M. Reyes-Becerril, C. Guluarte, D. Ceballos-Francisco, C. Angulo, M. Angeles Esteban, Dietary yeast *Sterigmatomyces halophilus* enhances mucosal immunity of gilthead seabream (*Sparus aurata* L.), Fish Shellfish Immunol. 64 (2017) 165–175.

[42] M. Fast, D. Sims, J. Burka, A. Mustafa, N. Ross, Skin morphology and humoral non-specific defense parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon, Comp. Biochem. Physiol. A 132 (2002) 645–657.

[43] Q. Zhou, L. Wang, H. Wang, F. Xie, T. Wang, Effect of dietary vitamin C on the growth performance and innate immunity of juvenile cobia (*Rachycentron canadum*), Fish Shellfish Immunol. 32 (2012) 969–975.

[44] N. Sheikhzadeh, M. Heidarieh, A. Karimi Pashaki, K. Nofouzi, M. Ahrab Farshbafi, M. Akbari, Hilyses®, fermented *Saccharomyces cerevisiae*, enhances the growth performance and skin non-specific immune parameters in rainbow trout (*Oncorhynchus mykiss*), Fish Shellfish Immunol. 32 (2012) 1083–1087.

[45] Y. Taoka, H. Maeda, J.Y. Jo, M.J. Jeon, S.C. Bai, W.J. Lee, K. Yuge, S. Koshio, Growth, stress tolerance and non-specific immune response of Japanese flounder (*Paralichthys olivaceus*) to probiotics in a closed recirculating system, Fish. Sci. 72 (2006) 310–321.

[46] K. Hjelmeland, M. Christie, J. Raa, Skin mucus protease from rainbow-trout *Salmo gairdneri* Richardson and its biological significance, J. Fish Biol. (1983) 13–22.

[47] K. Loganathan, A. Arulprakash, M. Prakash, P. Senthilraja, Lysozyme, protease, alkaline phosphatase and esterase activity of epidermal skin mucus of freshwater snake head fish *Channa striatus*, Int. J. Res. Pharm. Biosci. 3 (2013) 17–20.

[48] R.A. Dalmo, K. Ingebrigtsen, J. Bogwald, Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES), J. Fish Dis. 20 (1997) 241–273.

[49] H. Van Doan, S.H. Hoseinifar, M.A.O. Dawood, C. Chitmanat, K. Tayyamath, Effects of *Cordyceps militaris* spent mushroom substrate and *Lactobacillus plantarum* on mucosal, serum immunology and growth performance of Nile tilapia (*Oreochromis niloticus*), Fish Shellfish Immunol. 70 (2017) 87–94.

[50] B.A. Freeman, J.D. Crapo, Biology of disease: free radicals and tissue injury, Lab. Investig. 47 (1982) 412–426.

[51] R. Safari, S.H. Hoseinifar, S. Nejadmoghadam, A. Jafar, Transciptomic study of mucosal immune, antioxidant and growth related genes and non-specific immune response of common carp (*Cyprinus carpio*) fed dietary Ferula (*Ferula assafoetida*), Fish Shellfish Immunol. 55 (2016) 242–248.

[52] S.H. Hoseinifar, M. Dadar, M. Khalili, R. Cerezuela, M.A. Esteban, Effect of dietary supplementation of palm fruit extracts on the transcriptomes of growth, antioxidant enzyme and immune-related genes in common carp (*Cyprinus carpio*) fingerlings, Aquacult. Res. 48 (2016) 3684–3692.

[53] M. Sökmen, M. Akram Khan, The antioxidant activity of some curcuminoids and chalcones, Inflammopharmacology 24 (2016) 81–86.

[54] M. Kobayashi, M. Yamamoto, Nrf2 –Keap1 regulation of cellular defense mechasims against electrophiles and reactive oxygen species, Adv. Enzym. Regul. 46 (2016) 113–140.

[55] V. Soetikno, F.R. Sari, A.P. Lakshmanan, S. Arumugam, M. Harima, K. Suzuki, H. Kawachi, K. Watanabe, Curcumin alleviates oxidative stress, inflammation, and renal fibrosis in remnant kidney through the Nrf2-keap1 pathway, Mol. Nutr. Food Res. 57 (2013) 1649–1659.

[56] Y.-P. Chen, W.-D. Jiang, Y. Liu, J. Jiang, P. Wu, et al., Exogenous phospholipids supplementation improves growth and modulates immune response and physical barrier referring to NF-κB, TOR, MLCK and Nrf2 signalling factors in the intestine of juvenile grass carp (*Ctenopharyngodon idella*), Fish Shellfish Immunol. 47 (2015) 46–62.

[57] F. Denis, D. Archambault, Molecular cloning and characterization of beluga whale (*Delphinapterus leucas*) interleukin-1β and tumor necrosis factor-α, Can. J. Vet. Res. 65 (2001) 233–240.

[58] C. Low, S. Wadsworth, C. Burrells, C.J. Secombes, Expression of immune genes in turbot (*Scophthalmus maximus*) fed a nucleotide-supplemented diet, Aquaculture 221 (2003) 23–40.

[59] S.S. Giri, S.S. Sen, C. Chi, H.J. Kim, S. Yun, S.C. Park, V. Sukumaran, *Chlorophytum borivilianum* polysaccharide fraction provokes the immune function and disease resistance of *Labeo rohita* against *Aeromonas hydrophila*, J. Immunol. Res. 2015 (2015) Article ID 256510.

[60] R.L. Edwards, P.B. Luis, P.V. Varuzza, A.I. Joseph, S.H. Presley, R. Chaturvedi, C. Schneider, The anti-inflammatory activity of curcumin is mediated by its oxidative metabolites, J Biol Chem. 2017 292 (52) (2017) 21243–21252.

[61] H. Wei, L. Yin, S. Feng, X. Wang, K. Yang, A. Zhang, H. Zhou, Dual-parallel inhibition of IL-10 and TGF-b1 controls LPS-induced inflammatory response via NF-κB signaling in grass carp monocytes/macrophages, Fish Shellfish Immunol. 44 (2015) 445–452.

[62] A. Olivera, T.W. Moore, F. Hu, A.P. Brown, A. Sun, D.C. Liotta, et al., Inhibition of the NF-κB signaling pathway by the curcumin analog, 3,5-Bis(2-pyridinylmethylidene)-4-piperidone (EF31): anti-inflammatory and anti-cancer properties, Int. Immunopharmacol. 12 (2) (2012) 368–377.

[63] J. Su, J. Heng, J. Huang, L. Peng, C. Yang, Q. Li, Identification, mRNA expression and genomic structure of TLR22 and its association with GCRV susceptibility/resistance in grass carp (*Ctenopharyngdon idella*), Dev. Comp. Immunol. 36 (2012) 450–462.

[64] Y.-L. Shiu, H.-L. Lin, C.-C. Chi, S.-P. Yeh, C.-H. Liu, Effects of hirami lemon, *Citrus depressa* Hayata, leaf meal in diets on the immune response and disease resistance of juvenile barramundi, *Lates calcarifer* (bloch), against *Aeromonas hydrophila*, Fish Shellfish Immunol. 55 (2016) 332–338.

[65] M. Adel, S. Yeganeh, M. Dadar, M. Sakai, M.A.O. Dawood, Effects of dietary *Spirulina platensis* on growth performance, humoral and mucosal immune responses and disease resistance in juvenile great sturgeon (*Huso huso* Linnaeus, 1754), Fish Shellfish Immunol. 56 (2016) 436–444.

[66] E. Baba, Ü. Acar, C. €Ontas, O.S. Kesbiç, S. Yilmaz, The use of *Avena sativa* extract against *Aeromonas hydrophila* and its effect on growth performance, hematological and immunological parameters in common carp (*Cyprinus carpio*), Ital. J. Anim. Sci. 15 (2) (2016) 325–333.

[67] E. Wang, X. Chen, K. Wang, J. Wang, D. Chen, Y. Geng, W. Lai, X. Wei, Plant polysaccharides used as immunostimulants enhance innate immune response and disease resistance against *Aeromonas hydrophila* infection in fish, Fish Shellfish Immunol. 59 (2016) 196–202.