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Research Article

Dietary effect of chitosan on immunity, antioxidant status, and immune-related gene expression in juvenile starry sturgeon (*Acipenser stellatus*)

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Abstract

This study investigated the effects of the dietary supplement chitosan (CH) on various parameters including hematological indices, immune response, liver enzyme levels, antioxidant capacity, and the expression of immune-related genes in juvenile starry sturgeon (*Acipenser stellatus*). 180 juvenile starry sturgeons with an average weight of 31.90 ± 0.73 g were divided into five feeding groups for 60 days includes: control (CTR), 1.5 g ($CH_{1.5}$), 3.0 g ($CH_{3.0}$), 4.5 g ($CH_{4.5}$), and 6.0 g ($CH_{6.0}$) of CH powder per kg of a basal diet. Blood analyses conducted after the dietary trial demonstrated that CH significantly increased total red blood cells, white blood cells, hematocrit, and hemoglobin levels, with the highest values recorded in the $CH_{6.0}$ group. CH enhanced immune responses as evidenced by elevated levels of total protein, albumin, globulin, lysozyme activity, and immunoglobulin, particularly in the $CH_{6.0}$ group. Additionally, dietary CH reduced serum lipid levels, and the lowest liver enzyme activities were observed in the $CH_{4.5}$ and $CH_{6.0}$ groups. CH also stimulated the activity of antioxidant enzymes, with the most pronounced antioxidant activity noted in the $CH_{6.0}$ group. Moreover, CH improved the regulation of the relative expression of interleukin-1 beta (*IL-1β*) and tumor necrosis factor alpha (*TNF-α*) genes in liver, intestine, spleen, and head kidney tissues, with the highest expression levels for both genes recorded in the $CH_{6.0}$ group. These findings suggest that the inclusion of 6 g kg^{-1} CH in the diet of starry sturgeon enhances hematological and biochemical parameters, boosts antioxidant capacity and immune response, and supports immune-related gene expression.

Introduction

The aquaculture industry is undergoing rapid development and has increasingly shifted toward intensive farming practices. A significant challenge in fish farming is the declining survival rates against disease and contamination, particularly during the early life stages, where stress from high fish density can negatively impact growth performance, immune response, and increase susceptibility to diseases. The use of antibiotics to treat diseases poses further issues, including environmental risks, the emergence of bacterial resistance, the accumulation of these substances in farmed fish, and potential transmission to humans, the ultimate consumers (Harikrishnan *et al.*, 2012). Consequently, the use of antibiotics in aquaculture is prohibited in many countries (Yu *et al.*, 2022). To address these concerns, it is crucial to enhance the immune systems of fish, especially in economically important species. Research has increasingly focused on environmentally friendly alternatives, such as dietary supplements that can bolster the aquatic immune system.

Recently, the use of immunostimulants in farmed fish has risen, positioning them as viable alternatives to antibiotics. Immunostimulants, either natural or synthetic substances, serve to stimulate and regulate the immune system, thereby enhancing immune responses in the body. They can increase phagocytic activity, antibody production, lysozyme activity, and antioxidant enzyme activity, among other effects (Anderson, 1992). Among these immunostimulants, biological materials, particularly by-products from other industries, are recognized for their environmental friendliness.

Chitosan (CH), β-(1-4)-N-acetyl-D-glucosamine, a biopolymer derived from the deacetylation of chitin, is a biological immunostimulant. It is found in the exoskeletons of insects, the shells of crustaceans, and the cell walls of fungi (Perini *et al.*, 2020). CH is characterized by its non-toxic, biodegradable, and biocompatible properties, along with numerous physiological functions, which include antibacterial (Kong *et al.*, 2010), antioxidant (Chang *et al*., 2018), antitumor (Qin *et al.*, 2002), hypolipidemic (Zhang *et al.*, 2012), growth-promoting (Imefon Udo, 2018), and immune-stimulating effects (Fadl *et al.*, 2020). Recently, CH has garnered considerable attention in aquaculture, with studies highlighting its positive impacts on growth performance, intestinal morphology, non-specific immunity, pathogen resistance, antioxidant activity, and lipid metabolism in various fish species (Chen and Chen, 2019; Dawood *et al.*, 2020; Younus *et al.*, 2020; Stanek *et al.*, 2023).

The antioxidant potential of CH has also been increasingly recognized. During metabolic processes, reactive oxygen species (ROS) are generated, capable of interacting with crucial biological macromolecules such as lipids, proteins, and nucleic acids. Under normal circumstances, a delicate balance exists between ROS production and elimination. However, any disruption of this balance can lead to oxidative stress (Yan *et al.*, 2017). The antioxidant role of CH has been established across various fish species (Dawood *et al.*, 2020; Abdel-Tawwab *et*

al., 2021), with antioxidant status serving as an indicator of an organism's ability to effectively remove ROS (Enomoto *et al.*, 2001).

Sturgeons are among the most valuable aquatic species, yet their populations face threats of extinction due to overfishing, habitat loss, and various forms of pollution (Cvijanović *et al.*, 2024). Accordingly, artificial breeding for these fish presents a promising approach to conservation and sustainability. The starry sturgeon (*Acipenser stellatus*) is one of the most valuable sturgeon, which is valuable for caviar production due to its early sexual maturity and is considered one of the most profitable aquaculture species. However, farmed sturgeon suffer from infectious diseases, with the development of sturgeon farming, it is necessary to use immunostimulants to strengthen the immune system of these fish.

Since chitosan (CH) exerts multifaceted effects on immune gene regulation, antioxidant activity, and lipid metabolism, its therapeutic efficacy in enhancing physiological functions is contingent upon the specific requirements of different fish species. Therefore, studying individual species is crucial for determining the most effective use of CH. While recent advances have illuminated the efficacy of CH across various fish species, there remains limited information regarding its effects in sturgeon. This study aimed to investigate the impact of dietary CH on the immune response, antioxidant status, liver enzymes, and the expression of immune-related genes, specifically interleukin-1 beta (*IL-1β*) and tumor necrosis factor alpha (*TNF-α*), in starry sturgeon.

Materials and methods

Ethical statement

All animal experiments performed in this study were ethically approved by the Shahid Beheshti University Ethics Committee for the Use of Animals (IR.SBU.REC.1402.171).

Chitosan extraction

Shrimp shells (*Penaeus semisulcatus*) were washed to remove residual tissues and impurities, then dried at 60°C for 24 h and ground to obtain a homogeneous powder. The extraction of chitosan followed the method outlined by Fernandez-Kim (2004). To eliminate CaCO3, 7% hydrochloric acid was added to the shrimp shell powder at a ratio of 1:20 (w v^{-1}) for 4 h at 65°C. Following this, 9% sodium hydroxide (1:20 w v^{-1}) was introduced for an additional 4 h at 65°C. Decolorization was performed using 0.32% sodium hypochlorite (1:15 w v^{-1}) for 5 min at room temperature to yield chitin. Subsequently, deacetylation was executed by adding 55% NaOH for 1 h at 75°C at a 1:20 (w v^{-1}) ratio to extract chitosan. The degree of deacetylation was assessed using Fourier-transform infrared spectroscopy (FTIR) (TENSOR 27, Bruker Optics Inc, Billerica, MA, USA) through the KBr tablet method over a wavelength range of 400-4000 cm^{-1}, revealing a deacetylation degree of 80%.

Preparation of diets and study design

Five specific diets were formulated, as detailed in Table 1. A basal diet without CH served as the control (CTR), while four additional diets incorporated 1.5 g ($CH_{1.5}$), 3.0 g ($CH_{3.0}$), 4.5 g ($CH_{4.5}$), and 6.0 g ($CH_{6.0}$) of chitosan powder per kg of the

basal diet. The diets were prepared via the extrusion method, whereby all dry ingredients were ground and mixed thoroughly using an electric mixer (Raybony, Garma Electric, Amol, Iran) for 10 min. Subsequently, the mixture was combined with fish oil and water to achieve a paste-like consistency. This dough was then processed through an extruder system (Polylab, Thermo Fisher Scientific Company, USA) and dried at 40°C for 24 h. The dried feed was stored at -20°C until required.

Table 1: Formulation and proximate composition of the experimental diets.

Ingredient (g kg^{-1})	CTR	$CH_{1.5}$	$CH_{3.0}$	$CH_{4.5}$	$CH_{6.0}$
Fish meal	500	500	500	500	500
Corn meal	140	138.5	137	135.5	134
Soybean meal	210	210	210	210	210
Soybean oil	60	60	60	60	60
Fish oil	60	60	60	60	60
Vitamin premix †	20	20	20	20	20
Mineral premix ‡	10	10	10	10	10
Chitosan	0.0	1.5	3.0	4.5	6.0
Proximate analysis (g kg^{-1} as fed basis)					
Moisture	90	91	91	90	90
Crude protein	489	491	488	490	490
Crude lipid	136	135	135	136	137
Ash	70.1	70.5	70.7	70.4	70.5
Fiber	25.8	25.8	25.5	25.6	25.5
Gross energy (kJ g^{-1})	18.2	18.1	18.5	18.1	18.3

† Vitamin premix (as m kg^{-1}): Alpha tocopherol 60 IU; Calciferol 3000 IU; Thiamine 15; Riboflavin 30; Pyridoxine 15; B12 0.05; Nicotinic acid 175; Folic acid 5; Ascorbic acid 500; Inositol 1000; Biotin 2.5; Calcium pantothenate 50. ‡ Mineral premix (Unit kg^{-1}) (Science Laboratory): Cobalt sulphate 100 mg; Zinc sulphate 10 g; Iron sulphate 6 g; Choline chloride 6 g; Selenium 20 mg; Manganese sulphate 5 g; Copper sulphate 600 mg; Iodine 600 mg

Fish and rearing conditions

A 60-day feeding trial was conducted at the International Sturgeon Research Institute (Rasht, Gilan, Iran). Initial acclimatization for the fish lasted two weeks during which they were fed a basic diet. Following acclimatization, 180 starry sturgeon juveniles with a mean initial weight of 31.90±0.73 g were randomly distributed into fifteen fiberglass tanks (1000 L, 12 fish per tank) and fed the five formulated diets, with three replicates for each treatment. The water source consisted of a mixture of aerated groundwater and Sefidrud River water at a flow rate of 2 L min^{-1}. Water quality parameters were monitored daily (temperature 17.51±0.2°C, dissolved oxygen 9.25±0.22 mg L^{-1}, and pH 7.50±0.10). Throughout the feeding trial, fish were fed manually four times daily (08:00, 12:00, 16:00, and 20:00) until visual satiation. Any uneaten food and waste were siphoned out before each feeding to maintain optimal water quality.

Sampling

At the end of the feeding trial, feeding was ceased for 24 h. Three fish from each replicate were then anesthetized using clove powder (300 mg L^{-1}). Blood samples

were collected from the caudal vessels using heparinized syringes for hematological tests. For serological tests, additional blood samples were collected without heparin, centrifuged (3000 rpm, 10 min, 4°C), and stored at -20°C until further analysis. For the gene expression study, spleen, head kidney, liver, and intestinal tissues were harvested from three anesthetized fish per tank, immediately frozen in liquid nitrogen, and subsequently stored at -80°C.

Hematological parameters measurements

The total counts of red blood cells (RBCs), white blood cells (WBCs), hematocrit (Hct), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and differential leukocyte cells (diff) were assessed following the method described by Feldman *et al*. (2000).

Blood serum immunological and biochemical parameters measurements

Serum lysozyme (LYZ) activity was measured using a turbidometric assay in accordance with the methodology outlined by Ellis (1990). The determination of total immunoglobulin (IgM) was executed using Siwicki and Anderson (1993) method. The total protein content was assessed both before and after IgM precipitation with a 12% polyethylene glycol solution (Sigma, USA). Respiratory burst activity was evaluated using the nitro blue tetrazolium (NBT) dye method based on the protocol established by Anderson and Zeeman (2020). The levels of total protein, albumin, triglycerides, cholesterol, glucose, calcium, alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were quantified using commercial kits (Pars Azmun, Karaj, Iran) in accordance with the manufacturer's instructions and analyzed using an automated analyzer (Fuji Photo Film Ltd., Tokyo, Japan). Additionally, the serum globulin concentration was calculated by subtracting albumin from the total protein content (Abdel-Wahab *et al.*, 2021).

The activity of serum antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx), was evaluated spectrophotometrically using commercial kits (ZellBio GmbH, Lonsee, Germany).

Real-time quantitative PCR (RT-qPCR)

RNA extraction was conducted using an RNA extraction kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. The quantity and quality of RNA were assessed by measuring absorbance at 260/280 nm using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, USA) and through electrophoresis on a 1% agarose gel. RNA samples were treated with a DNase I enzyme kit (Sinaclone, Tehran, Iran) in accordance with the manufacturer's instructions. cDNA synthesis was performed using a cDNA synthesis kit (Thermo Fisher Scientific, Massachusetts, USA). Primers for this study were designed based on sequences available for the sturgeon species in the NCBI database, with their specific sequences provided in Table 2. Beta-actin (*β-actin*) served as the reference gene for data normalization. Relative expression levels in tissue samples

were analyzed via RT-qPCR using the CFX-96 Real-Time PCR Thermocycler (Bio-Rad, Hercules, CA, USA) in a reaction volume of 20 µL, which comprised 10 µL of 2X SYBER Green (Ampliqon, Copenhagen, Denmark) and 100 nM of each primer. The amplification was carried out under the following conditions: initial cooling of the products at 55°C for 90 s, followed by gradual heating from 55°C to 95°C at a rate of 0.5°C every 5 s. The $2^{-\Delta\Delta Ct}$ method for relative gene expression analysis, as described by Livak and Schmittgen (2001), was employed to calculate gene expression levels.

Table 2: Primer pair sequences of the genes used for quantitative real-time PCR.

Gene name	Gene abbreviation	Sequences of primers	Accession number
Interleukin-1 beta	*IL-1β*	F: TGGTGGAGAAGAGAACGCAAGATG R: GCGTCACTGCCTGTCTCATGG	MF818014.1
Tumor necrosis factor alpha	*TNF-α*	F: AAAATGTCCAGCCATCAAAGCA R: GTGAGGAATGATCCCGTTGGT	XM_034003960.3
Beta-actin	*β-actin*	F: TTGCCATCCAGGCTGTGCT R: TCTCGGCTGTGGTGAA	AY878120.1

Statistical analysis

All data are expressed as mean ± standard error (SE). SPSS software (Version 26) was utilized for all statistical analyses. The normality of the data distribution was verified using the Kolmogorov-Smirnov test, after which One-way ANOVA was performed to analyze the collected data. Significant differences between mean values were identified using Duncan's multiple range test at a *p*-value of 0.05.

Results

Hematological parameters

The results regarding hematological parameters are presented in Table 3. Significant increases in RBCs, Hb, and Hct were observed in the $CH_{6.0}$ group compared to other groups ($p<0.05$). While MCV increased in the CH-treated groups, no significant differences were noted among the $CH_{3.0}$, $CH_{4.5}$, and $CH_{6.0}$ groups. MCH values in the treated groups did not show significant differences when compared to the CTR group ($p>0.05$). Additionally, there was no significant difference in MCHC among the groups ($p>0.05$). WBC counts displayed significant differences between the groups ($p<0.05$), with increases noted in the $CH_{4.5}$ (6.03) and $CH_{6.0}$ (6.11) groups. The diff results indicated that monocytes and eosinophils did not exhibit significant differences across the treated and CTR groups ($p>0.05$). However, neutrophil levels rose in the CH-treated groups, and the $CH_{6.0}$ group had the highest percentage of lymphocytes ($p<0.05$).

Blood serum immunological and biochemical parameters

The results detailing blood serum immunological and biochemical parameters of juvenile starry sturgeon are shown in Table 4. The highest total protein levels were recorded in the $CH_{6.0}$ group

(3.12 g dL^{-1}), while no significant differences were seen between the CTR, $CH_{1.5}$, and $CH_{3.0}$ groups (p>0.05). In groups with higher CH levels ($CH_{4.5}$ and $CH_{6.0}$), serum albumin levels increased, but no significant differences were observed between the CTR group and the $CH_{1.5}$ and $CH_{3.0}$ groups (p>0.05). Globulin levels were highest in the $CH_{6.0}$ group (p<0.05).

Table 3: Hematological parameters of juvenile starry sturgeon fed with experimental diets for 60 days.

Parameter	CH level (g kg^{-1})				
	CTR	$CH_{1.5}$	$CH_{3.0}$	$CH_{4.5}$	$CH_{6.0}$
RBC ($\times10^6$ mm^{-3})	0.53 ± 0.01^a	0.53 ± 0.00^a	0.53 ± 0.00^a	0.54 ± 0.01^a	0.59 ± 0.01^b
Hb (g dL^{-1})	5.18 ± 0.10^a	5.12 ± 0.03^a	5.19 ± 0.14^a	5.14 ± 0.06^a	5.74 ± 0.09^b
Hct (%)	22.80 ± 0.20^a	22.60 ± 0.11^a	22.86 ± 0.49^a	23.20 ± 0.68^a	24.67 ± 0.32^b
MCV (fL)	415.00 ± 0.57^a	421.00 ± 0.57^b	422.66 ± 1.76^{bc}	424.00 ± 1.53^{bc}	426.33 ± 1.20^c
MCH (Pg)	97.13 ± 0.37	95.96 ± 0.28	95.73 ± 0.89	96.63 ± 0.26	95.93 ± 0.18
MCHC (g dL^{-1})	22.70 ± 0.25	22.70 ± 0.57	22.70 ± 0.20	23.17 ± 0.03	22.10 ± 0.15
WBC ($\times10^3$ mm^{-3})	4.00 ± 0.05^a	4.43 ± 0.18^a	5.26 ± 0.39^b	6.03 ± 0.09^c	6.11 ± 0.05^c
Neutrophil (%)	9.33 ± 0.88^a	12.00 ± 1.52^{ab}	13.66 ± 0.33^b	13.33 ± 0.88^b	14.67 ± 0.33^b
Lymphocyte (%)	79.33 ± 0.33^a	79.66 ± 0.33^a	81.66 ± 1.33^a	80.67 ± 1.20^a	88.33 ± 0.67^b
Monocyte (%)	3.33 ± 0.33	5.00 ± 0.57	5.33 ± 0.66	3.67 ± 0.33	4.33 ± 0.33
Eosinophil (%)	0.66 ± 0.33	0.33 ± 0.33	0.00 ± 0.00	0.33 ± 0.33	0.00 ± 0.00

Mean±SE (n = 3); Different letters in horizontal rows indicate a significant difference between treatments (p<0.05). RBC: Red blood cells, Hb: Hemoglobin, Hct: Hematocrit, MCV: Mean corpuscular volume, MCH; Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, WBC: White blood cells

Table 4: Serum immunological and biochemical parameters of juvenile starry sturgeon fed with experimental diets for 60 days.

Parameter	CH level (g kg^{-1})				
	CTR	$CH_{1.5}$	$CH_{3.0}$	$CH_{4.5}$	$CH_{6.0}$
Lysozyme Activity (u/ml/min)	27.87 ± 0.48^a	30.20 ± 0.25^b	32.17 ± 0.38^c	32.17 ± 0.38^c	33.70 ± 0.06^d
Total Immunoglobulin (mg mL^{-1})	13.80 ± 0.21^a	14.83 ± 0.03^{ab}	14.02 ± 0.51^{ab}	15.61 ± 0.24^{ab}	16.13 ± 0.02^b
NBT (mg mL^{-1})	1.15 ± 0.01	1.21 ± 0.04	1.28 ± 0.01	1.31 ± 0.02	1.16 ± 0.01
Total protein (g dL^{-1})	2.34 ± 0.01^a	2.48 ± 0.02^a	2.36 ± 0.11^a	2.66 ± 0.04^b	3.12 ± 0.04^c
Albumin (g dL^{-1})	0.97 ± 0.02^a	0.95 ± 0.01^a	0.97 ± 0.01^a	1.15 ± 0.02^b	1.19 ± 0.01^b
Globulin (g dL^{-1})	1.37 ± 0.01^a	1.52 ± 0.02^a	1.39 ± 0.10^a	1.50 ± 0.05^a	1.93 ± 0.03^b
Triglycerides (mg dL^{-1})	510.67 ±10.81^a	452.00 ± 7.51^b	412.33 ± 8.37^c	332.33 ± 7.31^d	322.67 ± 15.50^d
Cholesterol (mg dL^{-1})	144.33 ± 3.71^a	123.00 ± 4.58^b	113.66 ±1.45^b	114.66 ± 1.20^b	91.67 ± 2.40^c
ALT (U L^{-1})	46.17 ± 1.96^a	39.47 ± 1.10^b	38.60 ± 0.35^b	31.10 ± 0.56^c	31.13 ± 0.34^c
AST (U L^{-1})	410.67 ± 6.57^a	400.00 ± 3.79^{ab}	392.33 ± 4.67^b	351.33 ± 4.10^c	342.00 ± 3.51^c
ALP (U L^{-1})	309.00 ± 5.86^a	299.67 ± 2.40^a	260.67 ± 2.33^b	243.67 ± 4.63^c	233.33 ± 2.40^c
Glucose (mg dL^{-1})	48.73 ± 1.33^a	56.83 ± 0.83^b	55.50 ± 0.55^b	59.73 ± 0.26^c	61.80 ± 0.45^c
Calcium (mg dL^{-1})	9.54 ± 0.25^a	9.92 ± 0.09^a	9.85 ± 0.17^a	10.67 ± 0.04^b	10.81 ± 0.11^b

Mean±SE (n=3); Different letters in horizontal rows indicate a significant difference between treatments (p<0.05). ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase

Serum LYZ activity showed significant differences across all groups (p<0.05), with the highest values in the $CH_{6.0}$ group (33.70) and the lowest in the CTR group (27.86). IgM levels significantly differed between the CTR group and $CH_{6.0}$ (p<0.05). No significant differences in the NBT parameter were detected between treated groups and the CTR group (p>0.05).

Triglyceride levels decreased with increasing CH doses in the diet, with the highest triglyceride level in the CTR group at 510.67 mg dL^{-1}, demonstrating significant differences in the treated groups (p<0.05). The lowest triglyceride levels were noted in the $CH_{4.5}$ and $CH_{6.0}$ groups (p>0.05). Significant differences in cholesterol levels were also observed (p<0.05), with the highest level in the CTR group (144.33 mg dL^{-1}) and the lowest in the $CH_{6.0}$ group (91.67 mg dL^{-1}). The lowest serum glucose level was recorded in the CTR group (48.73 mg dL^{-1}), with higher levels noted in the $CH_{4.5}$ and $CH_{6.0}$ groups. Calcium levels were highest in the $CH_{4.5}$ and $CH_{6.0}$ groups, significantly differing from the other groups (p<0.05).

Serum liver enzymes

Table 4 illustrates that the levels of liver enzymes ALT, AST, and ALP decreased with increasing CH levels in the diet. The highest ALT level was observed in the CTR group, showing significant differences from the treated groups (p<0.05). The lowest AST levels were recorded in the $CH_{4.5}$ and $CH_{6.0}$ groups, while the highest AST levels were found in the CTR group. Both the $CH_{4.5}$ and $CH_{6.0}$ groups displayed the lowest ALP levels, whereas the highest ALP levels were seen in the CTR and $CH_{1.5}$ groups.

Antioxidant enzyme activity

Table 5 presents the results of antioxidant enzyme activity. Dietary CH increased CAT activity, with the highest CAT activity found in the $CH_{6.0}$ group and the lowest in the CTR group. SOD activity was not significantly different in the treated groups compared to the CTR group. GPx activity also increased in the $CH_{6.0}$ group relative to the other groups.

Table 5: Antioxidant activity of juvenile starry sturgeon fed with experimental diets for 60 days.

CH level (g kg^{-1})	CAT (u mL^{-1})	SOD (u mL^{-1})	GPx (u mL^{-1})
CTR	111.00 ± 1.53^{a}	52.03 ± 0.95^{a}	204.00 ± 2.08^{a}
$CH_{1.5}$	125.33 ± 0.88^{b}	54.17 ± 0.91^{a}	207.67 ± 2.40^{a}
$CH_{3.0}$	127.00 ± 1.53^{bc}	54.53 ± 0.75^{a}	254.67 ± 1.76^{b}
$CH_{4.5}$	130.33 ± 0.88^{c}	52.43 ± 0.91^{a}	253.67 ± 2.03^{b}
$CH_{6.0}$	136.33 ± 0.88^{d}	58.32 ± 0.62^{c}	269.00 ± 2.31^{c}

Mean±SE (n = 3); Different letters in vertical rows indicate a significant difference between treatments (p<0.05).
CAT: Catalase, SOD: Superoxide dismutase, GPX: Glutathione peroxidase.

Immune-related gene expression (IL-1β and TNF-α)

The *IL-1β* gene expression results are illustrated in Figure 1. The highest levels of *IL-1β* gene expression in the liver and intestine were observed in the $CH_{6.0}$ group, demonstrating significant differences from the other groups (p<0.05).

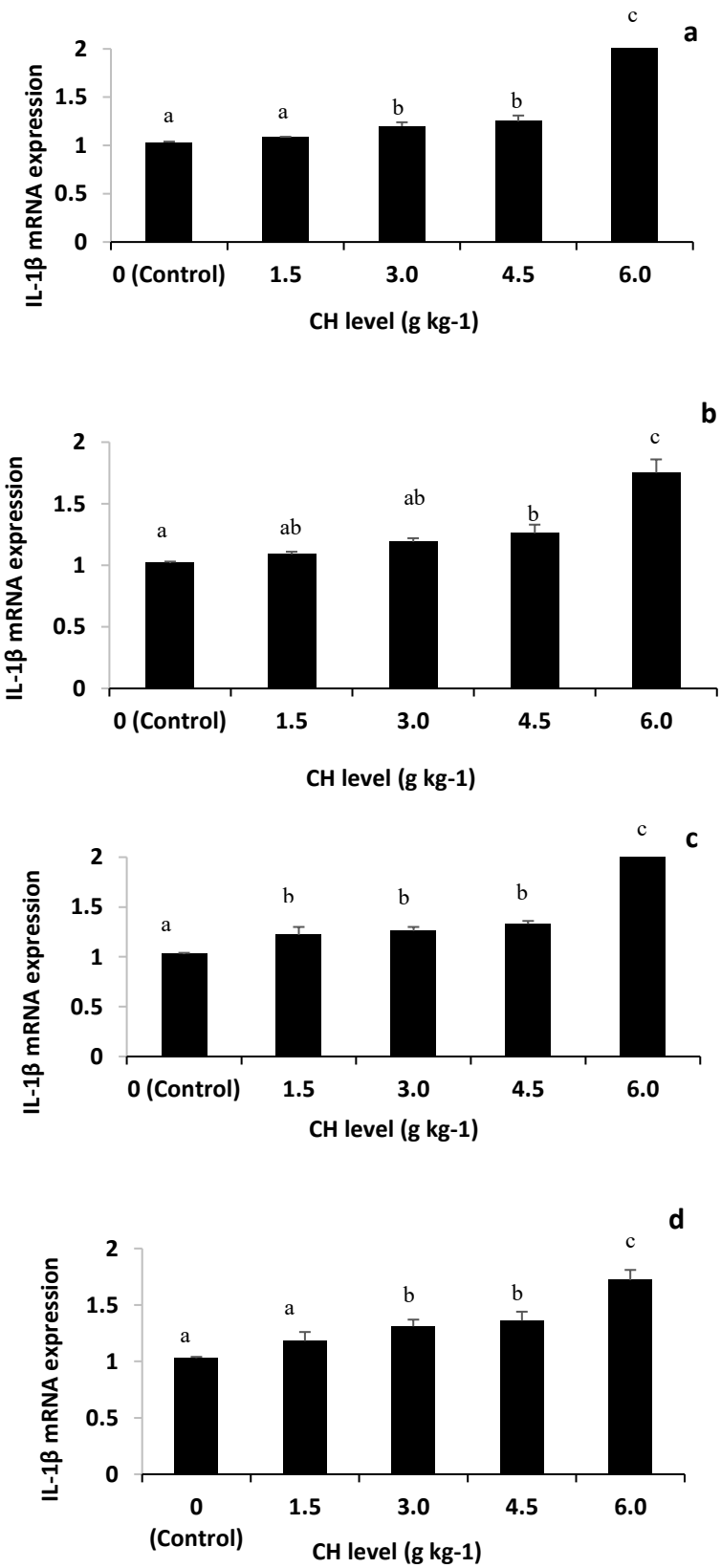
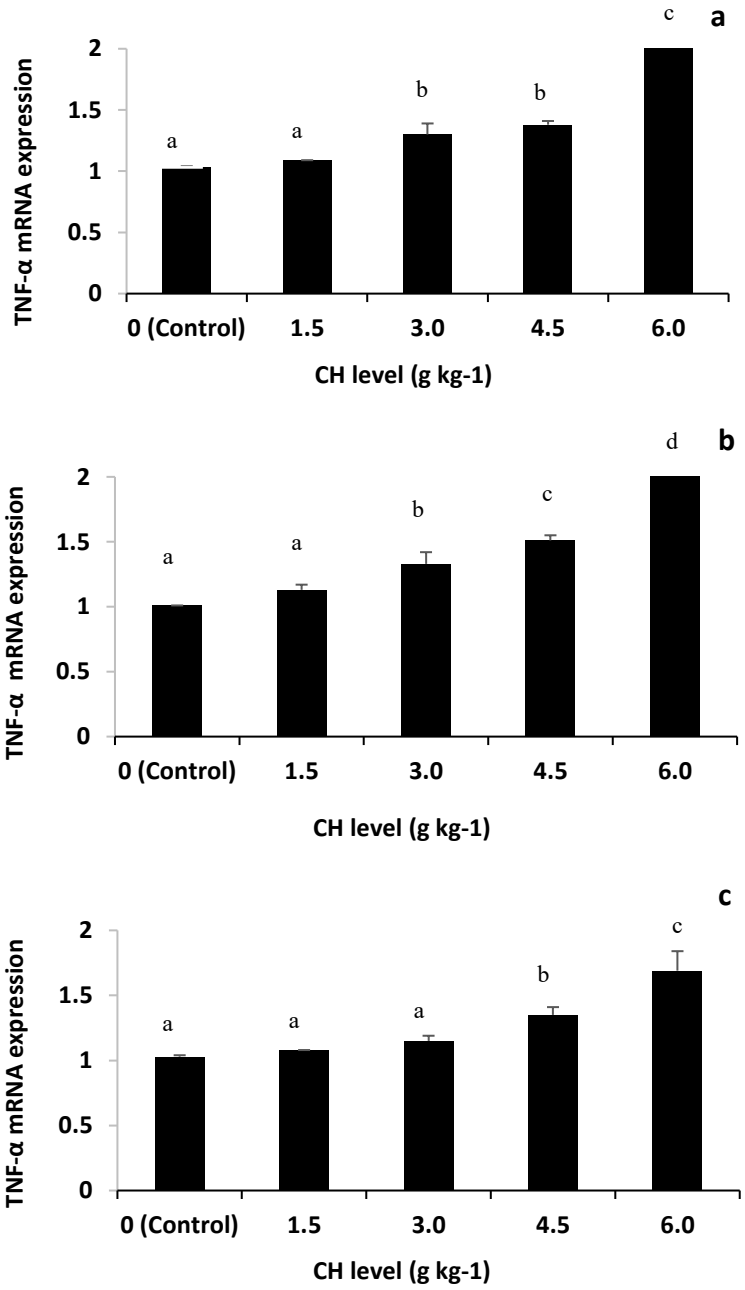


Figure 1: Relative mRNA expression of *IL-1β* gene in the liver (a), intestine (b) spleen (c) and head kidney (d) of juvenile starry sturgeon fed with experimental diets for 60 days. Mean±SE (n=3 repeat); Different letters indicate a significant difference between treatments (p<0.05).

The lowest expression was found in the CTR group for the spleen, while the $CH_{6.0}$ group had the highest. In the head kidney, *IL-1β* expression levels were highest in the $CH_{6.0}$ group, with no significant difference between the CTR and $CH_{1.5}$ groups (p>0.05).

Figure 2 presents the *TNF-α* gene expression results. Statistical analysis revealed that *TNF-α* gene expression in the liver and spleen increased with higher CH doses, peaking in the $CH_{6.0}$ group. In the intestine, the highest *TNF-α* expression was also seen in the $CH_{6.0}$ group. The head kidney exhibited the highest *TNF-α* expression in the $CH_{6.0}$ group and the lowest in the CTR group.



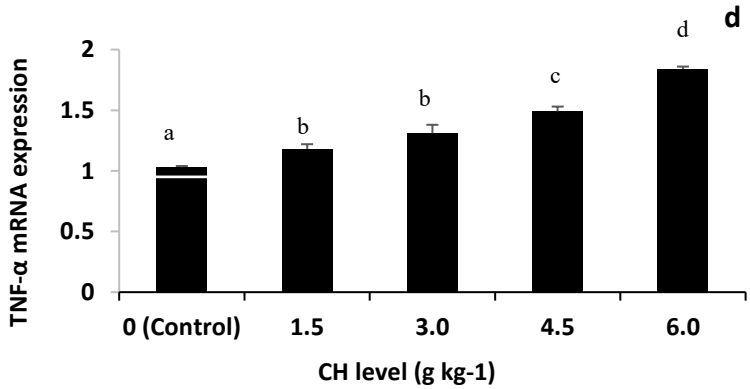


Figure 2: Relative mRNA expression of *TNF-α* gene in the liver (a), spleen (b), intestine (c) and head kidney (d) of juvenile starry sturgeon fed with experimental diets for 60 days. Mean±SE (n=3 repeat); Different letters indicate a significant difference between treatments (p<0.05).

Discussion

Strengthening the immune system is one of the most fundamental challenges in sturgeon farming, particularly during the early stages of life. Researchers are actively seeking compounds that can stimulate and modulate the immune systems of cultured animals. The present study investigates the impact of CH on the function of the immune system in juvenile starry sturgeon at a molecular level. The findings indicate that CH, functioning as an immunostimulant, enhances immune response and increases the expression of immune-related genes in juvenile starry sturgeon.

Hematological indices are frequently employed as biological indicators to monitor the overall health of fish (Fazio, 2019). The efficacy of dietary supplements for fish can also be evaluated through these indices. Variations in these values may arise from changes in the health status and nutritional behavior of the fish (Dawood *et al.*, 2020). In our study, we observed significant positive changes in hematological indices in response to the CH diet. Specifically, the RBC count, Hb, and Hct levels increased in the $CH_{6.0}$ group. Additionally, while MCV levels rose in the treated groups, dietary CH did not significantly affect MCH or MCHC. The increase in MCV values likely indicates larger RBCs with a greater capacity for gas exchange (Snyder and Sheafor, 1999). In vertebrates, MCV and MCH values serve as indicators for assessing anemia and iron deficiency. WBCs play a crucial role in the immune system of fish, contributing to both the cellular component of innate immunity and the release of humoral substances such as cationic antimicrobial peptides, components of the complement system, lectins, and cytokines. In our study, the highest WBC counts were observed in $CH_{4.5}$ and $CH_{6.0}$ groups.

Elevated levels of hemoglobin and RBCs suggest an increased metabolic rate for nutrient processing in the fish body, while higher Hct and WBC counts are typically associated with improved immune function (Fazio, 2019). The increase in WBCs, particularly lymphocytes, in our study can be attributed to the positive immunostimulant effect of CH on the health of starry sturgeon, potentially

bolstering their immunity and preventing diseases. Previous studies by Abd El-Naby *et al*. (2020) and Younus *et al.* (2020) demonstrated similar increases in WBCs, RBCs, Hb, and Hct in *Oreochromis niloticus* and *Hypophthalmichthys molitrix* that were fed chitosan nanoparticles (ChNPs), attributing these effects to the immunostimulant potential of ChNPs. Kamali Najafabad *et al*. (2016) reported that WBCs increased in *Rutilus frisii* fed diets containing 1 and 2 g kg^{-1} CH.

Biochemical serum parameters serve as biomarkers for evaluating the health status of aquatic animals. Innate immunity is also linked to total protein, albumin, and globulin levels. Serum proteins, including albumin, are vital for maintaining osmotic blood pressure (Nya and Austin, 2009), while serum globulins, such as gamma globulins, serve as sources of immunoglobulins reflecting antibody concentration and immune status in fish (Goda, 2008). In our study, total protein and albumin levels increased in the $CH_{4.5}$ and $CH_{6.0}$ groups, with globulin also peaking in the $CH_{6.0}$ group, indicating enhanced immunity in starry sturgeon. Studies involving *Epinephelus bruneus* fed with 1% and 2% CH have shown similar increases in total protein, albumin, and globulin levels in the treated groups, which aligns with our findings (Harikrishnan *et al.*, 2012). Research by Dawood *et al.* (2020) on *Liza. ramada* also showed that total protein, albumin, and globulin levels rose in groups receiving 1.0 and 2.0 g kg^{-1} of ChNPs compared to controls and lower doses.

LYZ activity directly indicates aquatic animals' health and safety status (Wu, 2020). In our study, LYZ activity increased with the dietary CH dosage. IgM is a critical immune protein that plays roles in bactericidal activity, immune regulation, and agglutination (Krogdahl *et al.*, 2000). The $CH_{6.0}$ group exhibited significant differences in IgM levels compared to the control group, corroborating findings by Chen and Chen (2019), who reported increased LYZ and IgM in *Misgurnus anguillicadatus* when fed CH at doses of 1, 5, and 10 g kg^{-1}. Similarly, a study by Oushani *et al*. (2020) observed increases in LYZ, IgM, and total protein levels in *Oncorhynchus mykiss* fed with combinations of clinoptilolite and CH or ChNP-containing strains. Since monocytes and neutrophils are the primary sources of serum LYZ, the observed increases in serum LYZ in the CH-treated groups may be attributed to a higher percentage of neutrophils in the blood.

CH serves as a prebiotic that enhances the intestinal microbiome. Additionally, its role in immune responses may be linked to alterations in the intestinal microbiome and an increase in probiotics, which act as immunostimulants (Song *et al.*, 2014). Prebiotics can traverse the intestinal epithelium to stimulate leukocytes (Hoseinifar *et al.*, 2015). As a prebiotic, CH likely stimulates leukocytes, thereby contributing to enhanced immunity. Furthermore, it plays a significant role in the non-specific defense system of fish and other vertebrates, particularly within gut-associated lymphoid tissue (GALT), which is crucial for regulating the immune response to dietary supplements. The microbial flora of the digestive system may also influence this regulatory process

(Lazado and Caipang, 2014). The observed increase in IgM and total protein following CH administration may stem from changes in the gastrointestinal microbial flora, leading to enhancements in the non-specific immune system mediated by the GALT (Hoseinifar *et al.*, 2015). While the specific mechanisms by which CH stimulates immunity in fish are not yet fully understood, these findings suggest its potential as a modulator of the immune system, warranting further investigation.

CH has been noted to reduce cholesterol and triglyceride levels in species such as *Rutilus caspicus* (Zahmatkesh *et al*., 2020) and *Cyprinus carpio* (Stanek *et al.*, 2023), corroborating our findings. This reduction in serum cholesterol and triglycerides in our study can be attributed to the hypolipidemic effects of CH. CH interacts with cholesterol and other sterols through hydrophobic interactions, forming hydrophobic aggregates in the small intestine. At a pH level of 7-8, these CH-lipid complexes solidify and are subsequently excreted in feces (Wydro *et al*., 2007).

The dietary supplementation of CH resulted in increased calcium levels in the $CH_{4.5}$ and $CH_{6.0}$ groups. Several factors influence mineral absorption via CH, including environmental pH, intestinal morphology, the water solubility of CH, molecular weight, and the degree of polymerization (Schmitz *et al.*, 2019). Research by Zhao *et al*. (2022) confirmed that CH can inhibit the digestion of peptide-calcium chelate in the stomach, allowing more calcium to enter the intestine and enhancing calcium absorption. Additionally, elevated calcium levels may be associated with increased albumin production, as calcium binds to albumin in the bloodstream (Stanek *et al.*, 2023).

In this study, we examined liver enzymes AST, ALT, and ALP, which are transaminase enzymes predominantly found in the mitochondria of liver cells. Any degeneration, inflammation, or necrosis of liver cells can lead to the release of these enzymes, resulting in elevated serum levels. ALP, ALT, and AST levels are generally considered indicators of liver damage influenced by nutritional status, stress, pollutants, or pathogenic infections (Taghavizadeh *et al.*, 2020).

Our findings indicated that serum levels of liver enzymes decreased with higher dietary CH levels, suggesting that CH does not adversely affect liver tissue. This protective effect may be due to CH's role in stabilizing cellular absorption and preventing the release of enzymes from liver cells into the bloodstream (Ramasamy *et al.*, 2014). Moreover, the liver-protective properties of CH might be linked to its stimulatory effects on the immune system in fish, given the liver's essential role in producing acute phase proteins and managing the destruction of pathogens, antigens, and inflammation-related molecules (Parker and Picut, 2012). The results of studies by Wu (2020) showed that diets containing 4, 6, and 8 g kg^{-1} CH decreased the liver enzymes of *O. niloticus*. In the study by Abd El-Naby *et al*. (2020), ALT and AST activities in *O. niloticus* were reduced by the addition of ChNPs in the diet.

The current study demonstrated that CH increased the activities of antioxidant enzymes CAT, SOD, and GPx in starry sturgeon, with the highest levels observed

in the $CH_{6.0}$ group. This suggests that CH can enhance the antioxidant capacity of sturgeon. Previous studies have also shown that CH increases antioxidant activity in various fish species, including *Trachinotus ovatus* (Yu *et al.*, 2022), *Dicentrarchus labrax* (Abdel-Tawwab *et al.*, 2021), *M. anguillicadatus* (Chen and Chen, 2019), and hybrid sturgeon (*A. baerii*♀ × *A. schrenckii*♂) (Xu *et al.*, 2023). The antioxidant properties of CH can be attributed to its ability to inhibit and chelate free radicals, such as hydroxyl radicals, by donating a hydrogen atom or an electron pair (Ngo and Kim, 2014). The structural basis for its antioxidant function lies in its amino groups and primary and secondary hydroxyl groups (Feng *et al.*, 2007).

Studying immune-related gene expression can provide insights into preventing, controlling, and treating diseases, particularly in protected species (Tian *et al.*, 2021). *IL-1β*, a pro-inflammatory cytokine, plays a crucial role in responding to microbial attacks and tissue damage, as it enhances phagocyte activity, macrophage proliferation, and leukocyte migration while triggering the secretion of various cytokines, including *TNF-α* from immune cells (Giri *et al.*, 2015). *TNF-α* is vital for immunity, secreted by leukocytes in response to immune system stimulation (Grayfer *et al.*, 2011).

In the current study, we evaluated the expression of *IL-1β* and *TNF-α* genes in the liver, intestine, spleen, and head kidney tissues, revealing that chitosan (CH) significantly increased the relative expression of these genes. Notably, the highest levels of *IL-1β* and *TNF-α* gene expression were observed in the $CH_{6.0}$ group. These findings align with previous research, including the study by Nikapitiya *et al.* (2018), which reported that dietary chitosan nanoparticles (ChNPs) enhanced *IL-1β* gene expression in zebrafish larvae. Similarly, Zou *et al*. (2016) found that feeding *C. carpio* with the prebiotic *Agaricus bisporus* led to an increased expression of *IL-1β* and *TNF-α* genes. Additionally, Oushani *et al*. (2020) reported that a combination of CH and clinoptilolite, as well as ChNP and clinoptilolite, could elevate *IL-1β* gene expression in the head kidney of *O. mykiss*. Moreover, Panase *et al*. (2023) demonstrated that the inclusion of *Bacillus subtilis* and fructooligosaccharides stimulated *IL-1β* and *TNF-α* gene expression in *O. niloticus*.

Dietary supplements, such as probiotics and prebiotics, can directly activate primary defense mechanisms by influencing receptors and associated genes, thereby enhancing immunity and resistance to pathogens (Bricknell *et al*., 2006). CH serves as an immune system stimulant, likely affecting these receptors and genes, and activating primary defense responses. As previously mentioned, the increase in *IL-1β* and *TNF-α* gene expression in response to CH is likely linked to alterations in the gastrointestinal bacterial flora, which impacts the gut-associated lymphoid tissue (GALT) system and strengthens the primary defense system. Overall, assessing the expression levels of cytokine system genes can serve as an indicator of immune system status (2007). Further research is warranted to elucidate

the mechanisms underlying CH stimulation.

The results indicate that CH can bolster the innate immunity of starry sturgeon. Variations in hematological and safety parameters across different studies are influenced by factors such as species, age, nutrition, and sex of the fish (Ahmed *et al.*, 2019).

Conclusion

Our study demonstrates that CH, as an immunostimulant, effectively stimulates and modulates the immunity of juvenile starry sturgeon. Based on these findings, the optimal dose of CH is determined to be 6 g kg^{-1}, which enhances immune response, elevates the expression of immune-related genes, reduces serum lipid levels, improves liver health, and boosts antioxidant capacity. CH presents a viable alternative to antibiotics in the rearing of starry sturgeon.

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Conflicts of interest

The authors declare no conflict of interest.

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