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Dietary prebiotic-stevioside modulates the growth, antioxidant enzymes, and immune response in thinlip mullets (*Liza ramada*) subjected to chronic cold stress

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Abstract

Plant active ingredients, including stevioside, with their prebiotic properties and antioxidant potential, hold promise as innovative components in aquaculture. This study assessed the multi-level impacts of stevioside (0, 100, 200, 300, 400, or 500 mg/kg) on thinlip mullet (N = 540 juveniles with an initial weight 3.50 ± 0.07 g) exposed to a long-term low temperature regime (18 °C) for 60 days. Growth, blood parameters, enzymes, immune genes, and histological structure of intestine and liver were analyzed. Moderate stevioside doses (300–500 mg/kg) significantly boosted growth, with 400 and 500 mg/kg groups showing the highest final and daily weight gain. These doses also increased total protein in blood serum. Interestingly, stevioside did not affect key blood markers like albumin, triglycerides, liver enzymes (ALT and AST), urea, or creatinine. However, it markedly enhanced ($P < 0.05$) antioxidant enzyme activity in liver (SOD, CAT, GPx) of experimental groups compared to the control group. Gene expression analysis in liver tissue revealed notable effects. Lower stevioside doses (100–300 mg/kg) downregulated genes linked to inflammation (*IL-1 β*) and iron regulation (*hepcidin*), while higher doses (400–500 mg/kg) showed dose-dependent upregulation ($P < 0.05$). Histological studies confirmed improved intestinal villi structure and absorptive area in the 300–500 mg/kg groups. This valuable information paves the way for sustainable aquaculture practices utilizing stevioside as a functional ingredient. This study provides key insights into the multi-level responses of juvenile mullets to dietary stevioside, with potential implications for aquatic animals exposed to similar circumstances.

Highlights

- ☒ Stevioside enhances growth and health of *Liza ramada* juveniles under cold stress.
- ☒ Stevioside boosts antioxidant activity and immunity in *L. ramada* juveniles.
- ☒ Dietary stevioside promotes gut and liver health under chronic cold stress.

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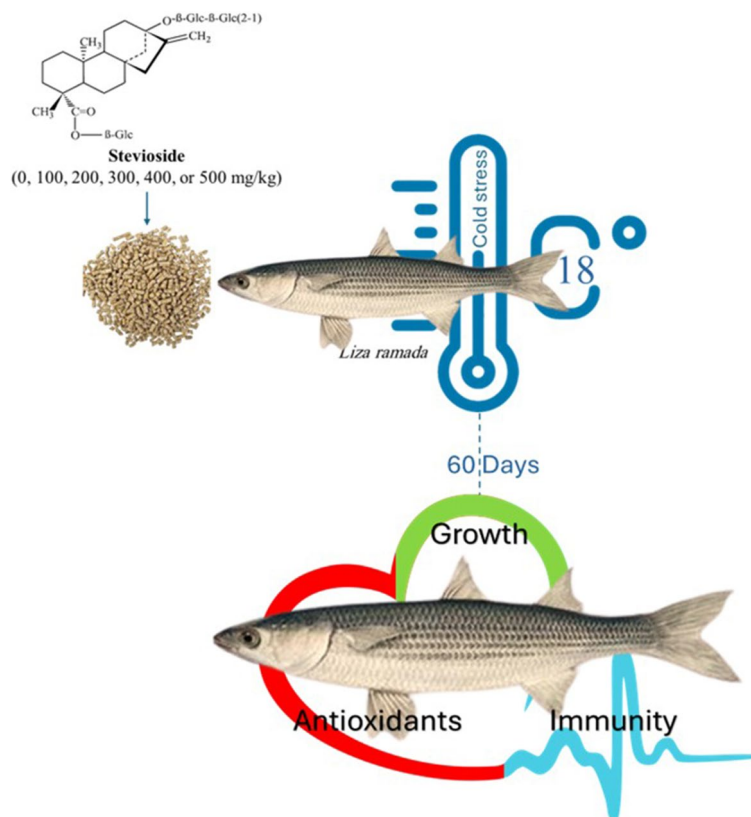


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- ☒ Stevioside modulates immune-related gene expression in *L. ramada* under cold stress.
- ☒ Optimal stevioside level for mullet under long-term low temperature regime is 300–500 mg Stev/kg in the diet.

Keywords Gene expression, Low temperature stress, Stevioside, Thinlip mullet

Graphical Abstract



Introduction

Aquaculture is at the forefront of global food production, driven by the increasing human population and the depletion of natural aquatic resources, necessitating sustainable and efficient farming practices that prioritize optimal fish health and growth [1, 2]. In this context, a comprehensive assessment of fish health and performance is essential and must integrate multiple complementary parameters to provide a holistic understanding of physiological and immunological status. This multidimensional approach includes evaluations of growth performance, whole-body proximate composition, serum biochemical markers, and antioxidant enzyme activities to assess production efficiency and metabolic balance. Additionally, it involves the analysis of immune responses, gene expression profiling through high-throughput molecular techniques, and histopathological

examinations, particularly of the intestine, to elucidate the impacts of dietary and environmental factors on immune modulation, nutrient assimilation, and overall fish welfare [3, 4].

To optimize fish health and growth, researchers are investigating natural additives and dietary supplements as potential solutions [5–7]. In particular, various bioactive ingredients have been employed in aquaculture to enhance meat quality, bolster immune responses, and improve antioxidant capacity, contributing to overall fish welfare and product value [8, 9]. Prebiotics, non-digestible dietary components that support gut bacteria and host health, are promising [10]. Their use in fish diets has grown to improve development and feed utilization across varied species [11–14]. Plant-derived components such as polysaccharides and oligosaccharides, in particular, have demonstrated positive effects on fish health

and performance [15, 16]. Their benefits are attributed to several mechanisms, including pathogen trapping, gut health enhancement, and microbiota modulation [12, 17]. Research indicates that prebiotics boost fish immune responses, including lysozyme and immunoglobulin synthesis [13, 18, 19]. Immune boost enhances fish health and resilience, contributing to more sustainable and efficient aquaculture [20]. However, further research on specific prebiotics like stevioside may uncover additional benefits for various fish species.

Stevioside, derived from *Stevia rebaudiana*, is being investigated as a prebiotic in aquaculture. It is a popular sugar replacement due to its sweetness intensity several hundred times that of sucrose and low calorie content [21, 22]. Its potential health benefits make it a promising fish prebiotic, surpassing artificial sweeteners in this regard. Stevioside may benefit gastrointestinal and systemic health due to its immunomodulatory and antioxidant properties, as demonstrated in various species and at different doses: in vitro studies on porcine intestinal epithelial cells (IPEC-J2) have shown protective effects against oxidative stress [23], while in vivo studies on broiler embryos have established safety at doses up to 5 mg per egg [24]. Furthermore, stevioside has been extensively studied in rodents and humans, showing safety even at high doses (up to 1500 mg/kg body weight/day in rats for 2 years) and extended periods of consumption [25].

In aquaculture specifically, Wang, et al. [19] found substantial improvements in liver antioxidant capacity, growth performance, and immunological function in juvenile mirror carp (*Cyprinus carpio*). Their broken-line regression analysis found that 217.68 mg/kg and 215.21 mg/kg stevioside concentrations maximized weight gain ratio (WGR) and SOD activity. Stevioside has demonstrated immunomodulatory effects in various species, including aquatic organisms, by influencing cytokine production (e.g., IL-1 β , IL-8, TNF- α) and regulating immune cell functions. This enhances host defenses and reduces inflammation, suggesting its potential to improve immune function in aquaculture [26–28]. Further study is needed to understand its impact on other fish species, especially under stressful situations including poor water quality, low or high temperatures, and crowding.

Thinlip mullet (*Liza ramada*), an important aquaculture fish, thrives above 20 °C. However, its cultivation in places with seasonal temperature changes exposes it to inadequate winter temperatures [29, 30]. This cold stress significantly impacts warm-water fish, hindering their health, growth, and economic viability. Reduced feeding and metabolic rate below 18 °C lead to stunted growth, and lower productive and reproductive processes in warm-water fish [31, 32]. These negative impacts

necessitate sustainable aquaculture mitigation measures in temperature-sensitive locations. Using antioxidant and immunomodulatory dietary supplements like stevioside seems promising.

Thus, this study aims to elucidate the potential modulatory effects of stevioside, administered at different dosages, on the development, biochemical composition, metabolic performance, antioxidant enzyme activity, and immunological response of juvenile *Liza ramada* subjected to chronic low-temperature stress.

Materials and methods

Ethical approval

This research has received ethical approval from the College of Agriculture Committee for Animal Care at Alexandria University, Egypt, with reference number AU: 19/23/07/24/3/33. All study techniques were conducted in strict adherence to the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines v2.0 [33].

Juvenile fish acquisition and acclimatization

Juvenile thinlip mullets (*Liza ramada*) used in the experiment, with an initial body weight of 3.50 ± 0.07 g, were sourced from a private farm in Kafr Elsheikh City, Egypt. After acquisition, the fish were transported in a healthy state to the Baltim Research Station of the National Institute of Oceanography and Fisheries in Egypt, where the feeding trials were conducted. Upon arrival, all mullets underwent a 15-day acclimatization period. The mullet were acclimatized to 18 °C, with this temperature chosen based on the median value of mullet occurrence [34]. During this period, they were fed the basal diet (Table 1). After acclimatization, 540 fish were randomly distributed among 18 tanks (100 L polycarbonate) and fed the experimental diets for 60 days.

Experimental design and setup

The experiment was conducted at the research station of the National Institute of Oceanography and Fisheries in Baltim, Egypt. Employing a completely randomized design, each treatment was replicated three times. Juvenile thinlip mullets (*Liza ramada*) were stocked in tanks maintained at a constant temperature of 18.2 ± 0.21 °C, pH of 7.22 ± 0.27 , dissolved oxygen concentration of 6.68 ± 0.34 mg/L, and total ammonia concentration of 0.05 ± 0.03 mg/L. Fish were reared in a controlled environment with natural light condition and 20% of the culture water was replaced daily with dechlorinated water. To manage metabolic waste, including ammonia, nitrite, and carbon dioxide and uneaten food, a siphoning system was employed. This involved using a vacuum siphon to carefully remove settled waste and uneaten food from the bottom of the tanks without disturbing the fish.

Table 1 Feed composition (g/kg) and nutrient levels (% dry weight; n = 3) of basal diet

Feed composition ¹	(g/kg)	Nutrient levels (%)	
		Dry matter	89.01 ± 0.35
Fish meal (65% CP)	50		
Soybean meal (48% CP)	365	Crude protein	30.87 ± 0.11
Meat meal (55% CP)	100	Crude lipid	5.94 ± 0.15
DDGS ²	65	Ash	8.14 ± 0.11
Wheat bran	55	Crude fiber	7.20 ± 0.11
Rice bran	200	NFE ⁵	47.84 ± 0.38
Wheat flour	40		
Sunflower oil	11		
Broken rice	75		
Mineral premix ³	1.5		
Vitamin premix ⁴	1.5		
Dicalcium phosphate	10		
Methionine	6		
Limestone	10		
Salt	10		
Total	1000		

¹ The ingredients are supplied by Feed Control Co., Ltd., which is located in Damro, Sidi Salem, Kafrelsheikh, Egypt

² DDGS = distiller's dried grains with solubles

³ Per kilogram of premix, the mineral mixture contains: manganese (325 mg), iron (200 mg), copper (25 mg), iodine, and cobalt (5 mg)

⁴ The mineral mixture includes (per kilogram of premix): vitamin A (3300 IU), vitamin D3 (410 IU), vitamin E (2660 mg), vitamin B1 (133 mg), vitamin B2 (580 mg), vitamin B6 (410 mg), vitamin B12 (50 mg), biotin (9330 mg), choline chloride (4000 mg), inositol (330 mg), para-aminobenzoic acid (9330 mg), niacin (26.60 mg), and pantothenic acid (2000 mg)

⁵ NFE nitrogen-free extract (by difference)

Continuous aeration was provided through a compressed air pump across all experimental tanks. To ensure a constant temperature of 18 °C, each tank was outfitted with an adjustable heater and a digital thermometer for continuous monitoring.

Stevioside source and feeding regime

Stevioside of 98.5% purity was procured from Shana Natural House in El Asafra Bahary, Alexandria, Egypt. Six dietary treatments were used in this experiment. The first group received a control diet without stevioside (Control, Stev 0). The remaining five groups received the same basal diet supplemented with increasing levels of stevioside: 100 mg/kg (Stev 100), 200 mg/kg (Stev 200), 300 mg/kg (Stev 300), 400 mg/kg (Stev 400), and 500 mg/kg (Stev 500). The specific stevioside concentrations were chosen based on a previous study by Shehata, et al. [28]. The experimental diets were formulated to include high-quality protein sources, such as Fish meal, Soybean meal, Meat meal, and distiller's dried grains with soluble (DDGS), to achieve a crude protein content of 30%. Sunflower oil provided the lipid source (5.94% crude lipid).

A vitamin and mineral premix following Shehata, et al. [7] ensured a complete nutritional profile. All ingredients were carefully blended before processing into 1–2 mm pellets using a laboratory pelletizer with oils and water. The final pellets were air-dried at room temperature and stored at 4 °C. Juvenile thinlip mullets received the formulated diets (Table 1) three times daily for 60 days at a feeding rate corresponding to 3% of their body weight.

Samples collection and measurements

Following a 60-day feeding experiment, fish in each replicate (tank) were weighed and individually counted approximately 24 h after the final feeding. The thinlip mullets were then carefully anesthetized using a 50 µl/L clove oil solution as an anticoagulant for further blood and tissue parameters analysis. Blood specimens were collected through caudal puncture into centrifuge tubes and allowed to clot at room temperature. Serum was subsequently separated by centrifugation at 3000 rpm for 10 min. Various tissues, including the liver, were collected for further analysis, which included assessments of antioxidant enzymes and gene expression. Additionally, the intestine and liver were used for histological evaluations.

Growth and feed utilization measurements

Following the experiment, various growth and feed utilization parameters were calculated for each treatment group [35]. These included:

$$WG, g = FW, g - IW, g$$

$$ADG, g = \frac{WG, g}{T, \text{day}}$$

$$SGR\%/day = \frac{LnFW - LnIW}{T} \times 100$$

$$SR, \% = \frac{FN}{IN} \times 100$$

$$FI, g/fish/Tdays = \frac{\text{Drydietprovided, g} - \text{Uneatendietrecovered, g}}{\text{FishNo.}}$$

$$FCR = \frac{FI, g}{WG, g}$$

where:

WG: weight gain, g; FW: final weight, g; IW: initial weight, g; ADG: average weight gain, g; T: trial period, day; SGR: specific growth rate; SR: survival rate; FN: final number; IN: initial number; FI: feed intake, g; FCR: feed conversion ratio.

Chemical composition analysis

Utilizing established protocols from the Association of Official Analytical Chemists [36], the nutrient analysis of the basal diet and whole-body fish samples involved different methodologies tailored to each sample type. The moisture content of samples was evaluated by exposing them to a steady temperature of 105 °C until they reached a stable weight. To determine ash content, samples were incinerated at 550 °C for 36 h in a muffle furnace. Fat extraction, performed over the 6-h, utilized an ether extractor (SoxROC, OPSIS, Sweden). Protein content quantification followed the Kjeldahl method, where samples underwent digestion with concentrated sulfuric acid, and measurements were conducted using an automatic Kjeldahl apparatus (KD210, OPSIS, Sweden). Fiber content in diet samples was determined by following the procedure outlined by Van Soest, et al. [37].

Biochemical parameters analysis

Five fish per replicate were allocated for serum collection. The biochemical parameter in serum was examined using a biochemical kit obtained from Bio-Diagnostic Co. in Cairo, Egypt. The measured biochemical parameters are Glucose (Glu, mmol/L, Cat. No. GL 13 20), Total protein (TP, g/dL, Cat. No. TP 20 20), Albumin (Alb, g/dL, Cat. No. AB 10 10), Globulin (Glob, by differences), Total cholesterol (T-CHOL, mg/dL, Cat. No. TC 20 10), Triglyceride (TG, mg/dL, Cat. No. TG 20 11), Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) (U/L, Cat. Nos. AT 10 34 and AT 10 45), Urea (mg/dL, Cat. No. UR 21 10), and Creatinine (mg/dL, Cat. No. CR 12 50). Adhering to the instructions outlined in the respective kit packages, the analysis of biochemical blood profiles was conducted to ensure the accuracy and consistency of serum parameter assessments. The Model CBC Micros ABX, France automatic analyzer for clinical chemistry and hematology assays was used, along with Diatron Q.C Reagents strips [P500 kinetic & Quality control].

Assessment of antioxidant enzyme activities

Livers from three fish per tank (9 fish/treatment) were carefully excised, immediately placed on ice, and then stored at −20 °C for antioxidative enzyme analysis. Liver samples were homogenized in cold 0.86% NaCl solution using a VEVOR FSH-2 A homogenizer according to the manufacturer's instructions. The homogenate was centrifuged at 12,000 rpm for 10 min at 4 °C. The resulting supernatant was used for both protein content determination and antioxidative enzyme activity assays. Total protein content was determined using the Bradford method [38]. Briefly, the supernatant was diluted

appropriately, and 10 µL of diluted sample or bovine serum albumin (BSA) standards (0–2000 µg/mL) were added to 200 µL of Bradford reagent in a 96-well microplate. After 5 min of incubation at room temperature, the absorbance was measured at 595 nm using a microplate spectrophotometer. Protein concentrations were calculated using a BSA standard curve. Antioxidative enzyme activities were measured using colorimetric methods. Superoxide dismutase (SOD) activity was measured by the inhibition rate of autocatalytic adrenochrome synthesis at 550 nm, following Misra and Fridovich [39]. Catalase (CAT) activity was assessed by tracking the degradation of hydrogen peroxide at 280 nm, according to Góth [40]. Glutathione peroxidase (GPx) activity was determined by monitoring the oxidation of NADPH with absorbance readings at 412 nm, following Arun, et al. [41]. All of these studies were carried out using a microplate spectrophotometer, ensuring accurate and repeatable detection of enzyme activity.

Evaluation of immune response

We assessed the serum activities using a 96-well microplate turbidimetric assay, following the method described by Lygren, et al. [42]. The test used lyophilized *Micrococcus lysodeikticus* cells (Sigma-Aldrich, India) as the substrate. We inserted 10 µL of serum samples into 96 microplate tubes throughout this process. The substrate combination of 190 µL of 0.2 mg/mL *M. lysodeikticus* in PSB (pH = 7.4) was then added. We measured changes in cloudiness at 450 nm after 1 and 5 min of gently stirring the solution at room temperature. We assessed enzyme activity by calculating the quantity of enzyme required to generate a 0.001 unit per minute drop in absorbance.

According to Wang, et al. [43], the bactericidal activities of serum were measured spectrophotometrically at 570 nm using modified techniques based on Gallage, et al. [44]. In short, using a micro-tube rotator (Wavex-Tube Rotator E11270), serum samples were combined with a bacterial culture (*Streptococcus agalactiae*, 1.4×10^8) in a 1:1 ratio (50 µL sample: 50 µL bacterial suspension) and incubated at 25 °C for 2.5 h. After incubating for 15 min at 25 °C with moderate shaking, we transferred the mixtures to 96-microplate tubes and mixed them with 15 µL of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich, Egypt) solution (5 mg/mL). Dissolving the formazan required fifty microliters of dimethyl sulfoxide (DMSO). We used a bacterial suspension in PBS as a positive control, without serum samples. We made three measurements of the optical density (OD570) and expressed the antibacterial activities as a percentage of the growth of *S. agalactiae* being inhibited relative to the positive control.

$$S.agalactiae \text{ inhibition \%} = \frac{OD_{Control} - OD_{Sample}}{OD_{Control}} \times 100$$

The respiratory burst activities in whole blood were determined using a nitroblue tetrazolium (NBT) assay, following the modified protocol by Secombes [45] at 630 nm. Furthermore, we assessed the activities of the alternative complement pathway (ACP) using serum samples, following the methodologies described by El Basuini, et al. [31].

Gene expression analysis

The fish liver samples (5 fish/tank) were isolated and thereafter stored at a temperature of -80°C for analysis. The liver samples were partitioned into pieces weighing 500 mg each, then transferred to mortar and homogenized with lysis solution (Geneaid GenozolTri RNA Kit, Korea). Subsequently, the mixture was centrifuged to obtain the aqueous layer for separating the RNA by column according to manufacturer (Geneaid, Korea). After isolating RNA, The RNA yield integrity, quantities, and quality were checked by NanoDrop spectrophotometer (BioDrop, England). The normalization of RNA sample concentration was performed to be 50 ng for each sample.

We used the one-step RT q-PCR Syber Green Kit (enzymnomics, Korea) for real-time PCR analysis, following the primer recommendations in Table 2. The reactions were performed according to the manufacturer's procedure for expression analysis. The reaction conditions were as follows: We held the reaction at 50°C for 30 min (for cDNA synthesis), then started the PCR reaction at 95°C for 10 min, then shifted to 95°C for 5 s, and finally annealed at 60°C for 30 s using different primers for all the targeted genes. We repeated this process for 45 cycles. We conducted the amplification using the CFX-3110 2-step real-time PCR system (BIORAD, USA). Melting curves proved the specificity of real-time PCR amplification. This ensured the amplification of only one PCR product at the target size. We conducted the sample quantification in

triplicate for each treatment. We performed expression analysis using the $2^{-\Delta\Delta C_t}$ method [46]; where the fold change ($2^{-\Delta\Delta C_t}$) = 1 (control); < 1 = downregulated; > 1 = upregulated. The expression of the studied genes was normalized using the housekeeping gene β -actin gene expression.

Histological observation

The histological examination was conducted at the completion of the experiment. The intestine and liver specimens were promptly immersed in a 10% neutral buffered formalin solution for a duration of 48 h. Following fixation, the tissue specimens underwent processing. The tissue samples underwent dehydration using a series of increasing concentrations of ethyl alcohol. They were then treated with xylene to remove any remaining impurities. Next, the samples were embedded in paraffin wax and cut into several slices that were 5 μm thick using a rotary microtome (RM 20352035; Leica Microsystems, Wentzler, Germany). Finally, the sections were placed onto clean slides. The paraffin slices underwent rehydration and were stained with Hematoxylin and Eosin (H and E) using the methods outlined in Bancroft and Gamble [47] for overall histomorphology. Subsequently, a digital camera (Leica EC3, Leica, Germany) coupled with a microscope (Leica DM500) was used to record multiple typical photomicrographs from the stained sections. The morphometric analysis used three slides per treatment (one per replicate). Each slide was checked for three fields of view. The morphometric analysis was confirmed through two separate individuals. The morphometric analysis employed an automated image analysis system (Image); Bethesda, MD, USA) to evaluate villus height (μm ; from the tip to the base of the villus), villus width (μm ; at the midheight of villi), and muscularis thickness, following the methodology described by Schneider, et al. [48]. Measurements were taken in micrometers (μm) and the collected data were analyzed statistically.

Table 2 RT-qPCR primer sequences used in this study

Gene	Sequences of forward and reverse primers (5'–3')	Annealing Temperature ($^{\circ}\text{C}$)	Amplification size (bp)	Accession numbers
Hep-F	ATGAAGGCATTCAGCATTGC	60	221	MH674371.1
Hep-R	TCAGAACTTGACAGAGAAGC			
il1 β -F	GAGGAGCTTGGTGCAGAACA	60	190	OY741297.1
il1 β -R	CTTTGTCGTACCTCTCTCCA			
β -actin-F	CCACGAGACCACCTACAACA	60	270	OY741309.1
β -actin-R	CTCTGGTGGGCAATGAT			

Hep Hepcidin, il1 β Interleukin 1- β

Statistical Analysis

We used SPSS Software V26 to calculate the mean and standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was applied to analyze the data. The Shapiro–Wilk test was used to assess normality, and the equality of variance was confirmed using Levene's test. A p -value threshold of 0.05 was employed to determine statistical significance.

Results

Growth performance, feed utilization, and survival rate

Table 3 presents the effects of varying dietary stevioside levels (Stev) on the growth variables, feed efficacy, and survival of juvenile thinlip mullet (*L. ramada*) under chronic low-temperature stress conditions. Supplementation with higher Stev doses (300–500 mg/kg diet) significantly improved FBW, WG, ADG, SGR, and FI compared to the control and lower Stev doses (100–200 mg/kg). Notably, the 400 and 500 mg/kg Stev groups exhibited the highest FBW, WG, ADG, and SGR values. Feed conversion ratio (FCR) was also lowest in the 400 and 500 mg/kg Stev groups. Survival (SR) remained unaffected across treatments.

Body chemical composition

The proximate composition analysis of whole-body juvenile thinlip mullet (*L. ramada*) following the 60-day feeding trial under cold stress conditions revealed no significant differences ($p > 0.05$) in moisture, protein, lipid, and ash content across the experimental groups fed varying dietary stevioside levels (0–500 mg/kg diet) (Table 4).

Biochemical Parameters

Table 5 reveals the significant impacts of dietary stevioside supplementation on the serum biochemical analysis of *L. ramada* following the 60-day cold stress feeding trial. The control group exhibited higher serum glucose and total cholesterol levels compared to fish-fed stevioside-supplemented diets. Serum glucose concentrations decreased significantly with stevioside inclusion, regardless of dose level. Total cholesterol levels gradually declined as dietary stevioside increased from 100 to 500 mg/kg. In contrast, total protein and globulin levels were elevated in the serum of fish receiving stevioside, with the highest total protein observed at doses over 100 mg/kg and peak globulin at 300 mg/kg. However, serum albumin, triglycerides, ALT, AST, urea, and creatinine remained unaffected ($p > 0.05$) across all dietary treatments.

Table 3 Impact of stevioside on Thinlip mullet (*Liza ramada*; IBW = 3.50 ± 0.07 g) growth and survival under chronic low temperature stress

Parameters	Dietary stevioside levels (mg/kg diet)					
	Cont 0 mg/kg	Stev 100 mg/kg	Stev 200 mg/kg	Stev 300 mg/kg	Stev 400 mg/kg	Stev 500 mg/kg
FBW (g)	9.50 ± 0.29^b	9.93 ± 0.07^b	9.87 ± 0.13^b	10.40 ± 0.10^a	10.77 ± 0.07^a	10.47 ± 0.03^a
WG (g)	6.00 ± 0.25^b	6.43 ± 0.05^b	6.37 ± 0.11^b	6.90 ± 0.09^a	7.27 ± 0.06^a	6.97 ± 0.04^a
ADG (g/fish/d)	0.10 ± 0.01^c	0.11 ± 0.01^{ab}	0.12 ± 0.01^{bc}	0.12 ± 0.01^a	0.12 ± 0.01^a	0.12 ± 0.02^a
SGR (%/d)	1.66 ± 0.05^c	1.74 ± 0.01^{bc}	1.73 ± 0.02^c	1.81 ± 0.02^{ab}	1.87 ± 0.01^a	1.83 ± 0.03^a
SR (%)	100	100	100	100	100	100
FI (g/fish)	9.90 ± 0.17^b	10.16 ± 0.04^b	10.12 ± 0.08^b	10.44 ± 0.06^a	10.66 ± 0.04^a	10.48 ± 0.02^a
FCR	1.66 ± 0.05^a	1.58 ± 0.01^{abc}	1.59 ± 0.02^{ab}	1.51 ± 0.01^{bcd}	1.47 ± 0.01^d	1.50 ± 0.03^{cd}

IBW initial body weight; FBW final body weight; WG weight gain; ADG average daily gain; SGR specific growth rate; SR survival rate; FI feed intake; FCR feed conversion ratio

Values represent means \pm SE ($n = 3$), and values within the same row with different letters were significantly different ($p \leq 0.05$, one-way ANOVA)

Table 4 Proximate profiles of the whole-body of thinlip mullet juveniles fed the experimental diets for 60 days under cold stress

Parameters	Dietary stevioside levels (mg/kg diet)					
	Cont 0 mg/kg	Stev 100 mg/kg	Stev 200 mg/kg	Stev 300 mg/kg	Stev 400 mg/kg	Stev 500 mg/kg
Moisture (%)	72.46 ± 0.42	72.29 ± 0.20	72.52 ± 0.16	72.63 ± 0.28	72.62 ± 0.32	72.12 ± 0.26
Protein (%)	19.33 ± 0.37	19.43 ± 0.47	19.44 ± 0.18	19.24 ± 0.04	19.23 ± 0.07	19.68 ± 0.14
Lipid (%)	6.02 ± 0.12	6.11 ± 0.02	6.11 ± 0.08	5.94 ± 0.16	6.00 ± 0.11	6.04 ± 0.13
Ash (%)	2.19 ± 0.09	2.16 ± 0.37	2.22 ± 0.07	2.22 ± 0.10	2.16 ± 0.17	2.17 ± 0.25

Values represent means \pm SE ($n = 3$), and values within the same row with different letters were significantly different ($p \leq 0.05$, one-way ANOVA)

Table 5 Impact of stevioside supplementation on serum biochemistry of *Liza ramada* exposed to chronic low temperature stress

Parameters	Dietary stevioside levels (mg/kg diet)					
	Cont 0 mg/kg	Stev 100 mg/kg	Stev 200 mg/kg	Stev 300 mg/kg	Stev 400 mg/kg	Stev 500 mg/kg
GLU (mmol/L)	9.22 ± 0.19 ^a	6.13 ± 0.10 ^b	6.10 ± 0.02 ^b	6.09 ± 0.06 ^b	6.08 ± 0.07 ^b	5.93 ± 0.09 ^b
TP (g/dl)	4.21 ± 0.08 ^c	4.80 ± 0.09 ^b	5.33 ± 0.16 ^a	5.42 ± 0.10 ^a	5.45 ± 0.11 ^a	5.44 ± 0.07 ^a
ALB (g/dl)	2.26 ± 0.38	2.38 ± 0.49	2.40 ± 0.49	2.34 ± 0.42	2.85 ± 0.38	3.08 ± 0.11
GLOB (g/dl)	1.95 ± 0.31 ^b	2.42 ± 0.40 ^{ab}	2.93 ± 0.33 ^{ab}	3.08 ± 0.41 ^a	2.60 ± 0.31 ^{ab}	2.37 ± 0.04 ^{ab}
T-CHOL (mg/dl)	19.33 ± 0.88 ^a	16.00 ± 0.58 ^{bc}	16.33 ± 0.88 ^b	16.00 ± 0.58 ^{bc}	14.00 ± 0.58 ^{cd}	13.67 ± 0.33 ^d
TG (mmol/L)	63.00 ± 1.73	63.33 ± 2.18	64.00 ± 1.53	64.67 ± 0.66	65.33 ± 2.40	65.00 ± 2.65
ALT (U/L)	22.07 ± 1.08	21.71 ± 1.67	20.29 ± 1.55	21.49 ± 0.82	20.28 ± 1.43	21.87 ± 1.30
AST (U/L)	34.62 ± 1.73	35.53 ± 1.66	33.25 ± 1.74	33.19 ± 1.13	33.78 ± 1.44	32.44 ± 2.08
Urea (mg/dl)	25.67 ± 0.88	25.33 ± 1.45	23.00 ± 0.99	24.33 ± 0.88	23.33 ± 0.88	23.00 ± 1.53
Creatinine (mg/dl)	0.51 ± 0.11	0.49 ± 0.07	0.48 ± 0.10	0.49 ± 0.04	0.48 ± 0.08	0.46 ± 0.06

GLU: glucose; TP: total protein; ALB: albumin; GLOB: globulin; T-CHOL: total cholesterol; TG: triglyceride; ALT: alanine aminotransferase; AST: aspartate aminotransferase. Values represent means ± SE (n = 3), and values within the same row with different letters were significantly different ($p \leq 0.05$, one-way ANOVA)

Activities of antioxidant enzymes

The antioxidant status in the liver of *L. ramada* after the 60-day feeding trial revealed that dietary stevioside supplementation significantly enhanced superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities compared to the control group (Fig. 1). Fish fed diets with higher stevioside levels (≥ 300 mg/kg) exhibited greater SOD and CAT activities than lower doses. However, GPx activity did not significantly differ across the various stevioside concentrations.

Immune system responses

The 60-day cold stress feeding trial showed that dietary stevioside supplementation significantly boosted the innate immune response in *L. ramada* (Table 6). Fish fed stevioside-supplemented diets exhibited higher lysozyme activity, bactericidal activity, nitro-blue tetrazolium (NBT) reduction ability, and alternative complement pathway (ACH50) activity compared to the control group. While bactericidal activity, NBT, and ACH50 did not differ substantially across stevioside doses, lysozyme activity peaked at doses over 400 mg/kg stevioside.

The expression of *IL-1 β* and *hepcidin* mRNA

Figure 2 shows the gene expression profiles of *interleukin-1 β* (*IL-1 β*) and *hepcidin* in *L. ramada* fish following a 60-day feeding trial under chronic cold stress. The gene expression analysis of *IL-1 β* and *hepcidin* revealed a dose-dependent modulation by dietary stevioside supplementation. Lower stevioside doses (100–300 mg/kg) resulted in downregulation of both *IL-1 β* and *hepcidin* genes compared to higher stevioside doses (≥ 400 mg/kg). The control group exhibited higher *IL-1 β* expression compared to treatment groups receiving stevioside at 100–400 mg/kg. Conversely, *hepcidin* expression

was gradually lowest at doses of 200 mg/kg, 100 mg/kg, 300 mg/kg, control, and 400 mg/kg, then peaked at stevioside doses up to 500 mg/kg.

Histological observation

The *L. ramada* intestine's histopathological structure showed that the intestinal wall and intestinal villi were intact in all the groups that were studied (Fig. 3A–F). The control group's intestine displayed short intestinal villi with slightly vacuolated enterocytes, somewhat separated lamina propria sub mucosa and thin intestinal wall. In the stevioside-supplemented groups, the histopathology investigation showed improved intestinal mucosa with well-arranged enterocytes. Moreover, the morphometric analysis displayed significant increase in villous height, villous width, and muscularis thickness with increasing stevioside dose (Fig. 3B–F and Table 7), especially at middle and high amounts of stevioside (300, 400, and 500 mg/kg, respectively) (Fig. 3D–F).

The histopathological examination of the liver in all experimental groups revealed a normal spongy appearance of hepatic parenchyma; polyhedral hepatocytes with vesicular nuclei arranged in hepatic cords around the hepatic central vein (Fig. 4A–F) with slight vacuolation in the control, low, and moderate levels of stevioside (Fig. 4A–D). The groups supplemented by high levels (400, 500 μ g/kg) of stevioside revealed an enhanced appearance of hepatic architecture with leukocytic aggregation (Fig. 4E) and increased glycogen deposition ($P < 0.05$) (Fig. 4F).

Discussion

The sustainable advancement of aquaculture relies on enhancing species' biological traits, management practices, aquatic environment, and feed regimes [49, 50].

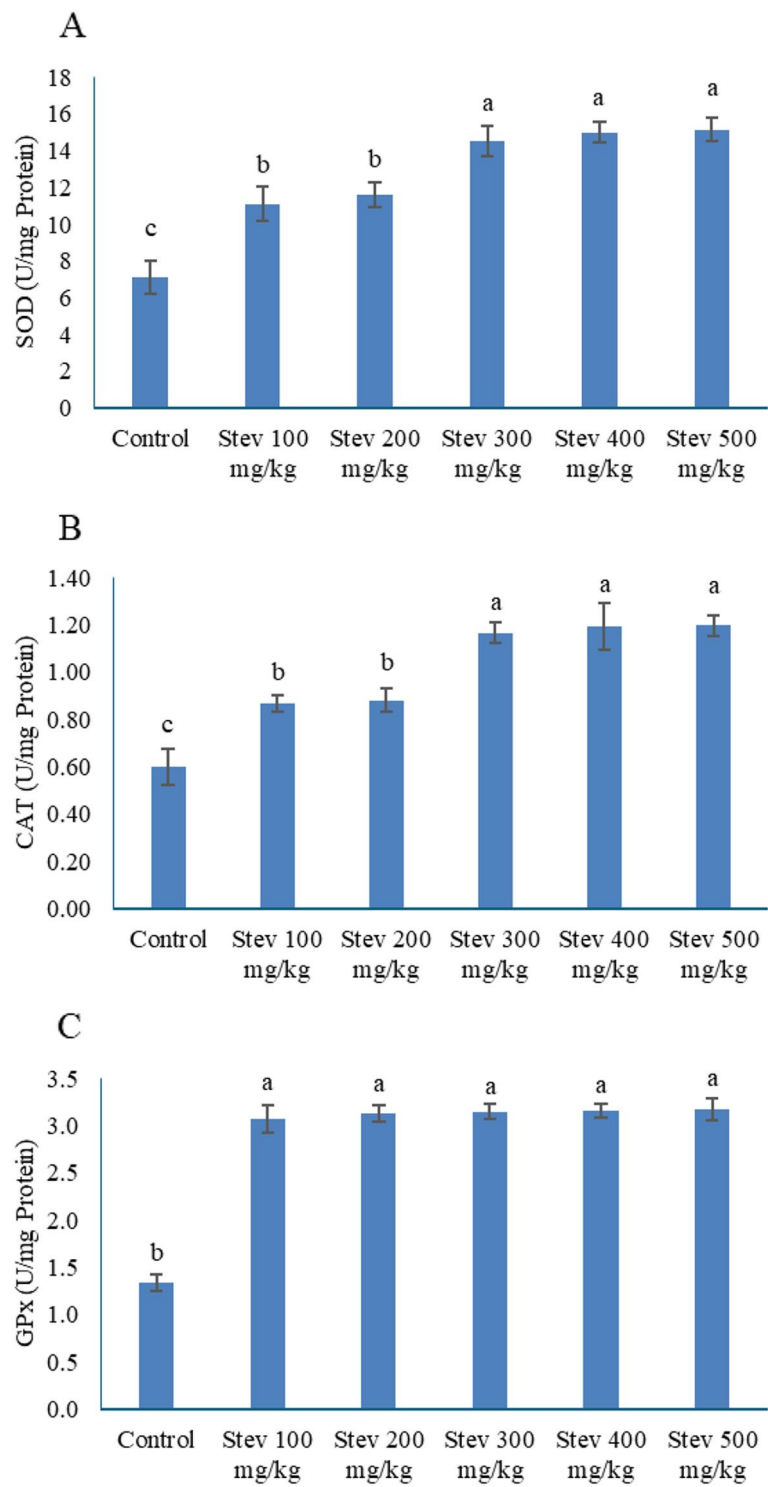


Fig. 1 Liver antioxidant parameters in *Liza ramada* after 60 days of feeding trial on stevioside levels. **A** SOD: Superoxide dismutase; **B** CAT: Catalase; **C** GPx: Glutathione peroxidase. Values represent means \pm SE (n = 3), and bars with different letters were significantly different ($p \leq 0.05$, one-way ANOVA)

Table 6 Immune system responses of *Liza ramada* fed diets supplemented with different levels of stevioside under cold stress for 60 days

Parameters	Dietary stevioside levels (mg/kg diet)					
	Cont 0 mg/kg	Stev 100 mg/kg	Stev 200 mg/kg	Stev 300 mg/kg	Stev 400 mg/kg	Stev 500 mg/kg
Lysozyme activity (U/ml)	184.28 ± 3.21 ^c	297.71 ± 6.86 ^b	300.30 ± 1.26 ^b	324.60 ± 3.02 ^a	328.50 ± 5.43 ^a	333.06 ± 5.35 ^a
Bactericidal activity %	6.60 ± 0.18 ^b	10.22 ± 0.65 ^a	10.41 ± 0.53 ^a	10.39 ± 0.32 ^a	10.43 ± 0.37 ^a	10.61 ± 0.64 ^a
NBT %	0.17 ± 0.01 ^b	0.26 ± 0.01 ^a	0.27 ± 0.01 ^a	0.28 ± 0.02 ^a	0.28 ± 0.01 ^a	0.29 ± 0.01 ^a
ACH50 (U/ml)	37.68 ± 1.45 ^b	53.32 ± 1.81 ^a	53.61 ± 1.79 ^a	55.88 ± 2.20 ^a	56.02 ± 2.38 ^a	56.87 ± 1.78 ^a

NBT Nitro-blue Tetrazolium; ACH50 Serum alternative complement pathway

Values represent means ± SE (n = 3), and values within the same row with different letters were significantly different ($p \leq 0.05$, one-way ANOVA)

To lay the groundwork, the use of feed additives plays a crucial role in improving fish health, promoting growth, enhancing antioxidant defence, and boosting immunity [51, 52]. Feed modifications, such as incorporating stevioside from *Stevia rebaudiana*, have shown potential in improving fish performance and immune responses [53]. This study investigates the effects of stevioside on the physiological and immunological responses of juvenile thinlip mullet (*L. ramada*) under chronic low-temperature stress.

Despite the growing popularity of *L. ramada* cultivation, critical knowledge required for its successful aquaculture remains limited. In aquaculture, growth performance parameters serve as essential metrics for evaluating production efficiency [7, 54]. These parameters are influenced by a complex interplay of environmental factors, genetic predisposition, and dietary quality and quantity. Consequently, they provide a valuable tool for assessing the effectiveness of various diets and supplements in optimizing fish farming practices [55, 56]. The present study demonstrates that dietary supplementation with Stev enhances growth and feed utilization of thinlip mullet. These findings align with the observations on *Cyprinus carpio*, where Stev supplementation similarly promoted growth performance [19].

Previous research has demonstrated that stevioside can enhance food palatability, leading to improved growth performance in piglets [57]. Research conducted on common carp showed that growth indices were greatly improved by a stevia extract concentration of 2000 mg/kg [58]. The attraction activity for aquatic animals tested is parallel with the concentrations of stevioside [59]. Nevertheless, Stev supplementation had no significant effect on tilapia growth, feed intake, or proximate composition [60]. Animal species variations in attraction activity may explain the observed discrepancies [19, 61–63]. Different pathways claim that the inclusion of Stev in *L. ramada* diets promotes growth. These factors result in increased food palatability, which leads to a higher

average daily amount of feed consumed [64], attractive properties influencing aquatic animals [59], different animals' digestive enzymes can't break down Stev into Steviol [65], and they change the gut microbiota in ways that help good bacteria grow and make digesting enzymes work better [66, 67], safeguarding the stomach with bioactive compounds for improved nutrient assimilation [68, 69], bactericidal traits in acidic conditions, and the dampening of the pro-inflammatory response [70–72]. Furthermore, researchers hypothesize that stevioside promotes an enhanced immune response and growth [73].

The whole-body nutritional formation of juvenile thinlip mullet (*L. ramada*) fed diets supplemented with varying amounts of stevioside for 60 days was assessed. Our findings show a negligible improvement in the whole-body analysis, which revealed consistent values for moisture, protein, fat, and ash content across all treatments. Additionally, previous research indicated that stevioside had no significant effect on the moisture, protein, fat, or ash content in mirror carp [19].

Serum biochemical indicators serve as reflections of the physiological and metabolic condition of fish [74]. It is also an indicator of liver health and toxicological status [75]. The serum biochemical profiles of *L. ramada* were determined after a feeding study lasting sixty days. When compared to the control diet, the treatment groups showed noticeably lower blood glucose and cholesterol values. Across the different stevioside concentrations, blood glucose levels showed no variations when stevioside supplementation was implemented. Furthermore, there was a steady decrease in blood cholesterol levels as stevioside concentrations rose. Furthermore, diet-fed groups that received stevioside at doses greater than 100 mg/kg showed the highest levels of total protein in their blood serum due to stevioside supplementation. It's interesting to note that across all experimental diets, blood concentrations of globulin, albumin, triglycerides, ALT, AST, urea, and creatinine did not change. Consistent

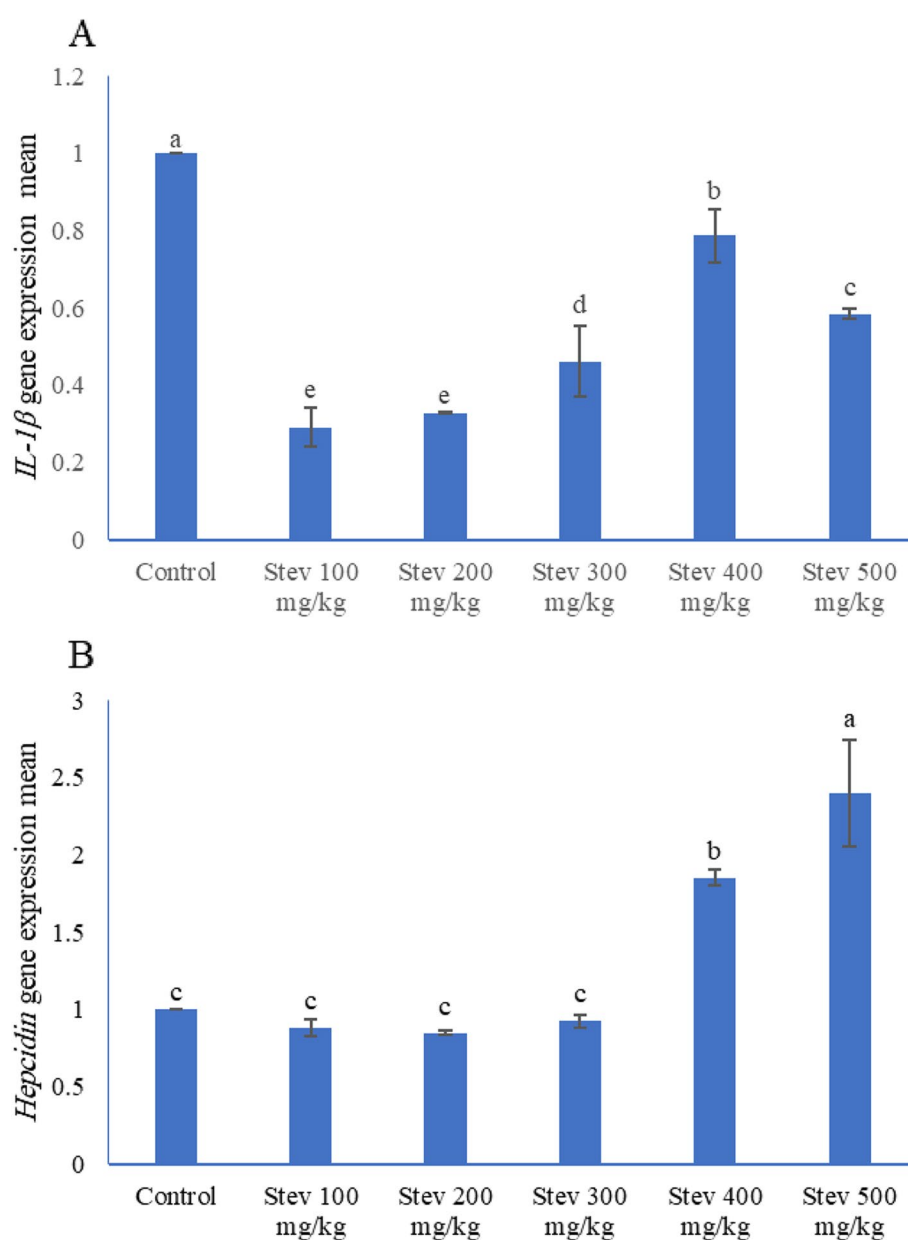


Fig. 2 Gene expression of (A) *IL-1 β* : interleukin-1 beta and (B) *Hepcidin* genes on stevioside levels in Thinlip Mullet (*Liza ramada*) juveniles after a 60-day feeding trial. *IL-1 β* , Interleukin-1 β . Values represent means \pm SE (n = 3), and bars with different letters were significantly different ($p \leq 0.05$, one-way ANOVA)

with our results, recent studies showed that the addition of Stev did not affect mirror carp blood glucose levels, which were similar to those of the control group [60]. The elevation of serum ALT and AST levels is often indicative of liver injury, as these transaminases are released into circulation when hepatocytes are damaged [76]. Wang et al. found that varying amounts of Stev had no impact on the activity of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides (TG),

acid phosphatase (ACP), and alkaline phosphatase (ALP) [19].

SOD, CAT, and GPx are key antioxidant enzymes that play critical roles in mitigating oxidative stress in biological systems [54, 77]. SOD is responsible for dismutating superoxide radicals into hydrogen peroxide and oxygen [39], while CAT efficiently decomposes hydrogen peroxide into water and oxygen [78]. GPx, on the other hand, protects cells from oxidative damage by reducing lipid

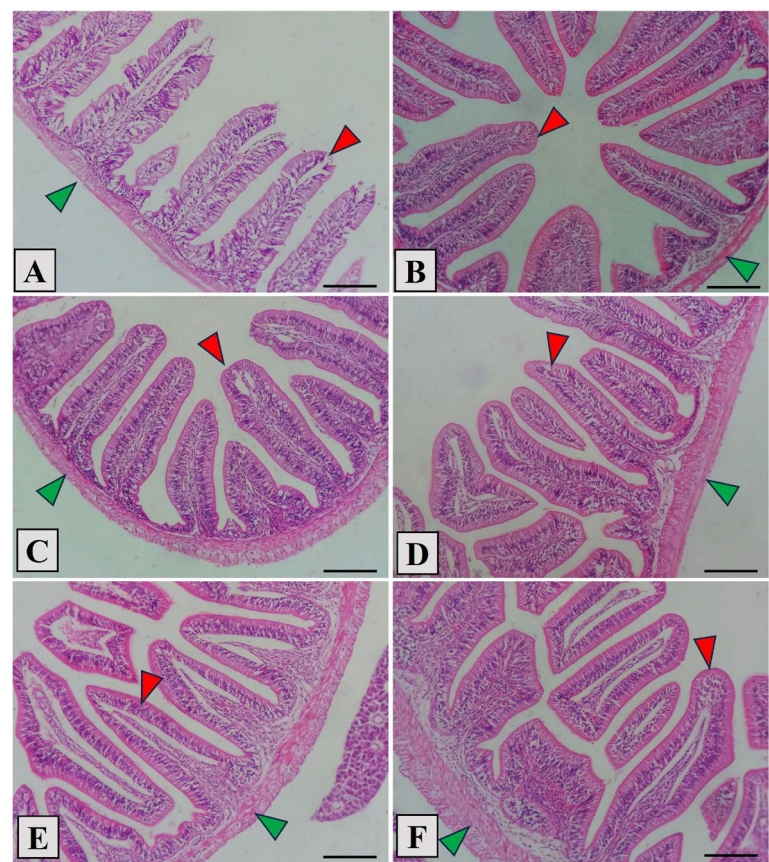


Fig. 3 Photomicrograph showing the histological structure of middle segment of *Liza ramada* juveniles' intestine in the control group (A), as well as stevioside-treated groups at ascending levels (B; 100, C; 200, D; 300, E; 400, F; 500 mg/kg). The control group displayed short intestinal villi (red arrowhead), vacuolated enterocytes. The intestinal wall (green arrowhead) and villi exposed evident normal structure with improved histomorphology by increased levels of stevioside. Stain H and E. Bar = 100 μm

Table 7 Intestinal morphometry indices of *Liza ramada* fed diets supplemented with different levels of stevioside under cold stress for 60 days

Parameters	Dietary stevioside levels (mg/kg diet)					
	Cont 0 mg/kg	Stev 100 mg/kg	Stev 200 mg/kg	Stev 300 mg/kg	Stev 400 mg/kg	Stev 500 mg/kg
Villus height (μm)	135.53 ± 8.52 ^e	258.73 ± 14.32 ^d	287.59 ± 8.69 ^d	370.41 ± 15.62 ^c	429.97 ± 9.94 ^b	473.04 ± 8.31 ^a
Villus width (μm)	72.01 ± 5.34 ^c	77.09 ± 1.62 ^{bc}	80.70 ± 2.58 ^{bc}	89.33 ± 4.50 ^b	104.41 ± 7.96 ^a	116.28 ± 3.82 ^a
Muscularis thickness (μm)	32.65 ± 1.93 ^d	29.64 ± 2.07 ^d	42.07 ± 1.37 ^c	53.92 ± 3.71 ^b	52.93 ± 1.16 ^b	68.93 ± 1.47 ^a

Values represent means ± SE (n = 3), and values within the same row with different letters were significantly different (p ≤ 0.05, one-way ANOVA)

hydroperoxides and hydrogen peroxide, using glutathione as a substrate [79]. These enzymes collectively form a robust defense mechanism against reactive oxygen species, helping to maintain cellular redox balance and protect organisms from oxidative damage. Supplementing with stevioside significantly increased the levels of SOD, CAT, and GPx activity compared to the control group, when assessing the activity of antioxidant enzymes. Fish groups fed a diet richer in stevioside (≥ 300 mg/kg) exhibited higher

levels of catalase and SOD activity compared to the other groups. Notably, there were no appreciable variations in GPx activity across the different stevioside concentrations. The findings of a recent experiment demonstrated that Stev might boost the activity of SOD in the fish liver, but it had no effect on CAT activity or GPx concentration. Thus, the influence of Stev on antioxidants might be one of the reasons for hepatoprotective effects [80]. The findings from a prior study revealed that the addition of Stev increased the

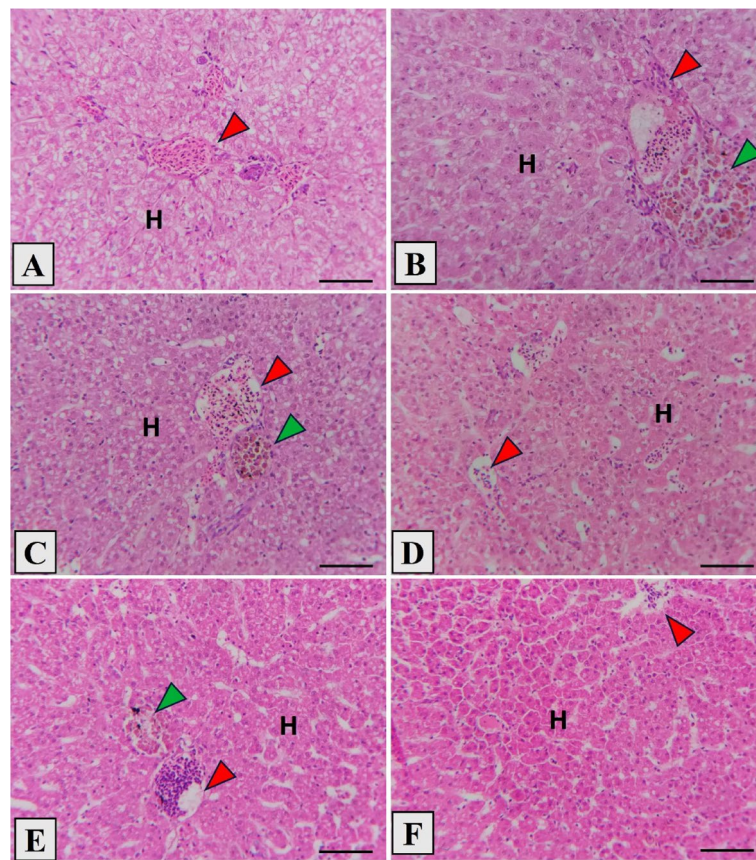


Fig. 4 Photomicrograph showing the histological structure of *Liza ramada* juveniles' liver in the control group (A), as well as stevioside-treated groups at ascending levels (B; 100, C; 200, D; 300, E; 400, F; 500 mg/kg). The liver presented normal appearance of hepatocytes (H) arranged in hepatic cords around hepatic central vein (red arrowhead), Melanomacrophage (green arrowhead) with slight vacuolation of hepatocytes in A-D. The high levels of stevioside (E, F) revealed normal hepatic architecture with leukocytic aggregation and increased glycogen deposition. Stain H and E. Bar = 50 μ m

activity of SOD in the liver of *Cyprinus carpio* (common carp), while it did not affect CAT activity or MDA content [19], indicating a nuanced and selective impact of Stev on specific components of the antioxidant defense system in this species.

The impact of stevioside supplementation on the immunological response of *L. ramada* during a 60-day feeding study. Serum lysozyme activity holds significance as a key element in the innate immune response against microbial pathogens [54]. Lysozymes play a vital role in restraining bacterial proliferation and colonization by actively targeting cell wall polysaccharides. This action results in the breakdown of the bacterial cell wall, ultimately leading to the demise of the microorganisms [71, 81].

The groups that received stevioside supplementation showed substantially elevated levels of lysozyme, bactericidal, NBT%, and ACH50 activity compared to the control group. Supplementation of mullet diets with stevioside yielded comparable impacts on the immune system [28].

The field of gene expression studies has evolved from the traditional reductionist approach of single-gene sequencing to advanced high-throughput techniques, including omics technologies such as nutrigenomics in aquaculture. This progressive shift in methodology has not only deepened our comprehension of biological markers associated with nutrition-related diseases but has also bolstered our capacity to recommend judicious feed additives for maintaining a stable immune status in aquatic animals [82]. Research has shown that down-regulated cytokines in fish are associated with pro-inflammatory responses, whereas up-regulated anti-inflammatory cytokines can reduce immune inflammatory reactions [83]. Specifically, *IL-1 β* , a pro-inflammatory cytokine, exerts influence over eicosanoid production, phagocyte function, lymphocyte proliferation, and stimulation through MHC-presented antigens [84]. Serving as a crucial mediator in inflammation, *IL-1 β* plays a pivotal role. Additionally, *hepcidin*, responsible for iron regulation [85], functions as an indispensable

antimicrobial peptide, playing a critical role in innate immunity against pathogens [86]. The present study highlights a considerable decrease in the mRNA levels of *IL-1 β* and *hepcidin* among fish subjected to a stevioside-enriched diet. This indicates a significant influence of stevioside on inflammatory cytokines. Furthermore, the integration of stevioside into the diet led to a notable reduction in the expressions of *IL-1 β* and *hepcidin* genes in *L. ramada*. Interestingly, a distinct down-regulation of all examined genes was evident in groups administered lower dosages (100–300 mg Stev/kg). Conversely, with increasing dosage, a dose-dependent up-regulation emerged, particularly pronounced in the 400–500 mg Stev/kg dosages.

The differential regulation of inflammatory cytokines and antimicrobial peptides in response to varying stevioside concentrations can be attributed to its dose-dependent immunomodulatory effects. At lower doses (100–300 mg Stev/kg), stevioside likely acts as an anti-inflammatory agent, potentially through the inhibition of NF- κ B signaling pathways, which are crucial for the transcription of pro-inflammatory genes such as *IL-1 β* [87]. This inhibition could lead to the observed down-regulation of *IL-1 β* and *hepcidin*, an antimicrobial peptide also regulated by inflammatory stimuli. The reduction in *hepcidin* expression may additionally be linked to stevioside's potential antioxidant properties, as oxidative stress is a known inducer of *hepcidin*. However, at higher doses (400–500 mg Stev/kg), the dose-dependent up-regulation suggests a hormetic effect, where stevioside may act as a mild stressor, activating adaptive stress response pathways such as Nrf2-mediated antioxidant responses or mild inflammatory responses that can enhance overall immune function [87]. This biphasic response underscores the complexity of stevioside's interactions with the fish immune system and highlights the importance of dose optimization in aquaculture applications to achieve desired immunomodulatory effects without triggering excessive inflammation.

Prebiotics have demonstrated immunostimulatory effects across diverse fish species, including Caspian trout (*Salmo trutta caspius*) with β -glucan and mannan oligosaccharides (MOS) [88], Zebrafish (*Danio rerio*) utilizing galacto-oligosaccharides (GOS) [89], Common carp (*Cyprinus carpio*) incorporating GOS, fructo-oligosaccharides (FOS), and inulin [90], Nile tilapia (*Oreochromis niloticus*) responding to β -glucan [91], and European sea bass (*Dicentrarchus labrax*) with MOS [92]. Atlantic cod (*Gadus morhua*) has also shown immunostimulating effects with the use of β -glucan and MOS [93]. Further studies are essential to clarify the comparison between the findings in the current study and those of others, as the observed outcomes may vary depending

on factors such as animal species, rearing conditions, age, size, trial duration, etc.

The intestine plays a crucial role in the overall well-being of fish, being intricately linked to immune response, metabolism, and the capacity to withstand environmental stress [74]. The health of the gut is paramount for animals in terms of digesting and absorbing nutrients effectively [94]. Specifically in fish, the condition of the intestines is closely tied to dietary nutrients, influencing growth, development, structural and functional aspects, as well as the processes of digestion and absorption [95]. The intestinal histological examination of *L. ramada* revealed that the intestinal wall and intestinal villi were intact in all groups examined. In the control group, the intestinal structure consisted of the tunica mucosa, composed of simple columnar enterocytes arranged in a normal arrangement, the propria submucosa, the tunica muscularis, and the outer serosa. Improved intestinal morphometry refers to the increase of the surface area of intestinal villi, which plays a key role in absorbing digested nutrients within the intestine [96]. This could be attributed to the enhanced feed efficiency which was linked to a larger surface area of intestinal villi [97]. The histological analysis revealed a dose-dependent progressive improvement in the structure of intestinal villi as the absorptive villous area increased. This improvement was most pronounced at moderate and high concentrations (300, 400, and 500 mg/kg, respectively) of stevioside. Boonkaewwan, et al. [98] observed that stevioside influenced the structure of intestinal villi in a dose-dependent manner. Stevioside and its metabolite, steviol, caused the greatest enhancement at moderate and high concentrations (300, 400, and 500 mg/kg). Stevioside and its metabolite, steviol, possess immunomodulatory and secretory properties, which contribute to the observed enhancement. Stevioside and steviol increase chloride secretion while decreasing TNF- α -stimulated IL-8 production, stevioside and steviol could help mitigate inflammation and promote a healthier intestinal environment. This, in turn, may support the observed changes in the structure of intestinal villi, indicating improved nutrient absorption and overall gut health.

The liver histopathological analysis across experimental groups revealed a typical spongy appearance, normal polyhedral hepatocytes, and vesicular nuclei arranged in hepatic cords around the central vein. Slight vacuolation was observed in control, low, and moderate stevioside levels. Notably, high stevioside supplementation (400, 500 μ g/kg) showed a notable enhancement in hepatic architecture, accompanied by leukocytic aggregation and increased glycogen deposition. These findings suggest a potentially positive impact of high-level stevioside supplementation on liver health, indicating improved structural characteristics and immune response. This is further supported

by the favorable AST and ALT indicators results, signifying overall health [19, 99]. While AST and ALT levels remained unchanged in this study, it suggests that the cold stress may not have significantly impacted liver health in juvenile thinlip mullet. The stable ALT and AST levels indicate that there was no liver damage or stress resulting from the experimental conditions. Furthermore, the stevioside supplementation appears to have helped the fish cope with cold stress, supporting their overall health and resilience. However, further immunohistochemical studies are needed to support the current study.

Conclusion

Stevioside supplementation (300–500 mg/kg) significantly improved growth, feed efficiency, antioxidant enzymes, and immune functions in Thinlip mullet under low-temperature stress. Future studies should explore stevioside's effectiveness under various conditions and its impact on stress responses in fish. Long-term studies and scalability evaluations are necessary for its practical application in large-scale aquaculture.

Abbreviations

Hep	Hepcidin
il1 β	Interleukin 1- β
IBW	initial body weight
FBW	final body weight
WG	weight gain
ADG	average daily gain
SGR	specific growth rate
SR	survival rate
FI	feed intake
FCR	feed conversion ratio
GLU	glucose
TP	total protein
ALB	albumin
GLOB	globulin
T-CHOL	total cholesterol
TG	triglyceride
ALT	alanine aminotransferase
AST	aspartate aminotransferase
NBT	Nitro-blue Tetrazolium
ACH50	Serum alternative complement pathway

Authors' contributions

Akram Ismael Shehata: Conceptualization; Methodology; Data curation; Data Analysis; Data Interpretation; Original draft preparation; Writing-Review and Editing; Supervision. Mohammed F. El Basuini: Methodology; Formal analysis; Data Analysis; Original draft preparation; Writing- Review and Editing. Ayaat M. Elmaghraby: Methodology; Resources; Data Analysis; Writing-Review and Editing. Mayada Alhoshy: Data curation; Writing-Review and Editing. Ali A. Soliman: Methodology; Resources; Writing-Review and Editing. Asem A. Amer: Resources; Methodology; Writing-Review and Editing. Nermin A Ibrahim: Methodology; Data Analysis; Writing-Review and Editing. Yusuf Jibril Habib: Writing-Review and Editing. Mahmoud S. Gewaily: Methodology; Data Analysis; Writing-Review and Editing. Islam I. Teiba: Data Analysis; Writing- Review and Editing. Shimaa A. Shahin: Methodology; Resources; Writing-Review and Editing.

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Data availability

The datasets underpinning this study are accessible from the corresponding author upon request.

Declarations

Ethics approval and consent to participate

This research has received ethical approval from the College of Agriculture Committee for Animal Care at Alexandria University, Egypt, with reference number AU: 19/23/07/24/3/33. All study techniques were conducted in strict adherence to the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines v2.0. In the "Ethics and consent to participate" section, we obtained informed consent from all owners to use the fish in this feeding study.

Consent for publication

All authors approved the final manuscript and the submission to this journal.

Competing interests

The authors declare no competing interests.

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References

- Clavelle T, Lester SE, Gentry R, Froehlich HE. Interactions and management for the future of marine aquaculture and capture fisheries. *Fish Fish*. 2019;20(2):368–88. <https://doi.org/10.1111/faf.12351>.
- Morro B, Davidson K, Adams TP, Falconer L, Holloway M, Dale A, Aleynik D, Thies PR, Khalid F, Hardwick J, et al. Offshore aquaculture of finfish: Big expectations at sea. *Rev Aquac*. 2022;14(2):791–815. <https://doi.org/10.1111/raq.12625>.
- El Basuini MF, Hussein AT, El-Hais AM, Elhetawy AIG, Soliman AA, Gabr SA, Abu-Elala NM, Luo Z, Zaineldin AI, Teiba II et al: Impacts of dietary Ajwain (*Trachyspermum ammi* L.) on growth, antioxidative capacity, immune responses, and intestinal histology of grey mullet (*Liza ramada*). *Aquaculture* 2025, 595:741706. <https://doi.org/10.1016/j.aquaculture.2024.741706>
- Basuini MFE, Shaban MMEA, El-Hais AM, Soliman AA, Abu-Elala NM, Teiba II, Alhoshy M, Sallam GR, Shadrack RS, Mzengereza K, et al. Exploring the Dual Benefits of Fermented and Non-Fermented Garlic Powder on Growth, Antioxidative Capacity, Immune Responses, and Histology in Gray Mullet (*Liza ramada*). *Fishes*. 2024;9(10):401.
- Shadrack RS, Kotra KK, Gereva S, Teiba II, El-Ratel IT, El Basuini MF. Utilizing dietary probiotics can boost amberjack (*Seriola dumerili*) lysozyme activity, antioxidant capacity, and gut microbiota. *Scientific African*. 2023;22:e01905. <https://doi.org/10.1016/j.sciaf.2023.e01905>.
- Hussein EE, Habiba MM, Ashry AM, Al-Zayat AM, Teiba II, Shehata AI, Shahin SA, El-Ratel IT, Mzengereza K, Tembo M, et al. Effects of dietary supplementation with organic acids mixture on growth, feed efficiency, hematobiochemical parameters, immunity, and intestinal microbiota of Gilthead seabream (*Sparus aurata*) juveniles. *Aquac Rep*. 2023;33: 101846. <https://doi.org/10.1016/j.aqrep.2023.101846>.

7. Shehata AI, Shahin SA, Elmaghraby AM, Alhoshy M, Soliman AA, Amer AA, Habib YJ, Gewaily MS, El Basuini MF. Synergistic benefits of dietary silymarin and selenium on growth, immune functions, antioxidants, and gut/liver health of Thinlip mullet (*Liza ramada*) juveniles. *Ann Anim Sci*. 2024. <https://doi.org/10.2478/aoas-2024-0044>.
8. Yu Z, Li M. Application of nutrition interventions strategy to enhance fish flesh quality. *J Food Compos Anal*. 2025;138: 107010. <https://doi.org/10.1016/j.jfca.2024.107010>.
9. Li M-Y, Liu Y-Z, Chen X-M, Niu X-T, Chen L, Zhao L, Wang G-Q. Astaxanthin ameliorates high-carbohydrate diet-induced ER stress, immunosuppression and hepatic glucose metabolism through AMPK/autophagy pathway in *Channa argus*. *Aquaculture*. 2025;598: 742010. <https://doi.org/10.1016/j.aquaculture.2024.742010>.
10. Kaushik P, Khandelwal R, Jain N, Keelka S, Jain P: Prospectives of Prebiotics, Probiotics, and Synbiotics for Sustainable Development of Aquaculture. In: *Prebiotics, Probiotics and Nutraceuticals*. edn. Edited by Behera KK, Bist R, Mohanty S, Bhattacharya M. Singapore: Springer Nature Singapore; 2022: 301–340. https://doi.org/10.1007/978-981-16-8990-1_15
11. Abdelmalek BE, Driss D, Kallel F, Guargouri M, Missaoui H, Chaabouni SE, Ayadi MA, Bougatef A. Effect of xylan oligosaccharides generated from corn cobs on food acceptability, growth performance, haematology and immunological parameters of *Dicentrarchus labrax* fingerlings. *Fish Physiol Biochem*. 2015;41(6):1587–96. <https://doi.org/10.1007/s10695-015-0110-5>.
12. Akter MN, Sutriana A, Talpur AD, Hashim R. Dietary supplementation with mannan oligosaccharide influences growth, digestive enzymes, gut morphology, and microbiota in juvenile striped catfish. *Pangasianodon hypophthalmus* Aquac Int. 2016;24(1):127–44. <https://doi.org/10.1007/s10499-015-9913-8>.
13. Ali SR, Ambasankar K, Praveena E, Nandakumar S, Syamadaya J. Effect of dietary mannan oligosaccharide on growth, body composition, haematology and biochemical parameters of Asian seabass (*Lates calcarifer*). *Aquac Res*. 2017;48(3):899–908. <https://doi.org/10.1111/are.12933>.
14. Sutriana A, Hashim R, Akter MN, Nor SAM. Galactooligosaccharide and a combination of yeast and β -glucan supplements enhance growth and improve intestinal condition in striped catfish *Pangasianodon hypophthalmus* fed soybean meal diets. *Fish Sci*. 2018;84(3):523–33. <https://doi.org/10.1007/s12562-018-1195-4>.
15. Akrami R, Nasri-Tajan M, Jahedi A, Jahedi M, Razeghi Mansour M, Jafarpour SA. Effects of dietary synbiotic on growth, survival, lactobacillus bacterial count, blood indices and immunity of beluga (*Huso huso* Linnaeus, 1754) juvenile. *Aquac Nutr*. 2015;21(6):952–9. <https://doi.org/10.1111/anu.12219>.
16. Xu B, Wang Y, Li J, Lin Q. Effect of prebiotic xylooligosaccharides on growth performances and digestive enzyme activities of allogynogenetic crucian carp (*Carassius auratus gibelio*). *Fish Physiol Biochem*. 2009;35(3):351–7. <https://doi.org/10.1007/s10695-008-9248-8>.
17. Hoseinifar SH, Khalili M, Sun Y-Z. Intestinal histomorphology, autochthonous microbiota and growth performance of the oscar (*Astronotus ocellatus* Agassiz, 1831) following dietary administration of xylooligosaccharide. *J Appl Ichthyol*. 2016;32(6):1137–41. <https://doi.org/10.1111/jai.13118>.
18. Gelibolu S, Yanar Y, Genc MA, Genc E. The effect of mannan-oligosaccharide (MOS) as a feed supplement on growth and some blood parameters of Gilthead Sea Bream (*Sparus aurata*). *Turk J Fish Aquat Sci*. 2018;18(6):817–23. https://doi.org/10.4194/1303-2712-v18_6_08.
19. Wang J, Li K, Wang L, Xu Q. Effects of different levels of stevioside on growth performance, digestive enzyme activity, antioxidant capacity and gene expression of juvenile mirror carp (*Cyprinus carpio*). *Aquaculture*. 2021;543: 737019. <https://doi.org/10.1016/j.aquaculture.2021.737019>.
20. Zhao L, Zhao J-L, Bai Z, Du J, Shi Y, Wang Y, Wang Y, Liu Y, Yu Z, Li M-Y. Polysaccharide from dandelion enriched nutritional composition, antioxidant capacity, and inhibited bioaccumulation and inflammation in *Channa asiatica* under hexavalent chromium exposure. *Int J Biol Macromol*. 2022;201:557–68. <https://doi.org/10.1016/j.jbiomac.2021.12.117>.
21. Hashmi SJ, Hashmi SS, Ghani A, Aqeel A, Ibrahim M, Taskeen SA, Salman S: Antioxidant and antimicrobial activities of so-steviol: a derivative of Stevioside. *Pure and Applied Biology*. Vol. 12, Issue 2, pp1076–1083. In.; 2023. <https://doi.org/10.19045/bspab.2023.120110>
22. Gunasena MDKM, Senarath RMUS, Senarath WTPSK: A Review on *Stevia rebaudiana* (Bert.) Bertoni, a natural sweetener. *Curr Res Pharm Sci*. 2023:103–131.
23. Xu Q, Liu M, Chao X, Zhang C, Yang H, Chen J, Zhou B. Stevioside Improves Antioxidant Capacity and Intestinal Barrier Function while Attenuating Inflammation and Apoptosis by Regulating the NF- κ B/ MAPK Pathways in Diquat-Induced Oxidative Stress of IPEC-J2 Cells. *Antioxidants*. 2023;12(5):1070.
24. Geuns JMC, Bruggeman V, Buyse JG. Effect of stevioside and steviol on the developing Broiler embryos. *J Agric Food Chem*. 2003;51(17):5162–7. <https://doi.org/10.1021/jf020931p>.
25. Abbas Momtazi-Borojeni A, Esmaeili S-A, Abdollahi E, Sahebkar A. A review on the pharmacology and toxicology of steviol glycosides extracted from *Stevia rebaudiana*. *Curr Pharm Des*. 2017;23(11):1616–22.
26. Kurek JM, Zielińska-Wasielica J, Kowalska K, Krecjpcio Z, Olejnik A. Modulating effects of steviol and steviol glycosides on adipogenesis, lipogenesis, glucose uptake and insulin resistance in 3T3-L1 adipocyte model. *Journal of Functional Foods*. 2022;94: 105141. <https://doi.org/10.1016/j.jff.2022.105141>.
27. Lailerd N, Saengsirisuwan V, Sloniger JA, Toskulkao C, Henriksen EJ. Effects of stevioside on glucose transport activity in insulin-sensitive and insulin-resistant rat skeletal muscle. *Metabolism*. 2004;53(1):101–7. <https://doi.org/10.1016/j.metabol.2003.07.014>.
28. Shehata AI, Shahin SA, Elmaghraby AM, Alhoshy M, Toutou MM, Soliman AA, Amer AA, Habib YJ, Gewaily MS, Teiba II, et al. Stevioside mitigates lead toxicity in thinlip mullet juveniles: Impacts on growth, metabolism, and immune function. *Aquat Toxicol*. 2024;271: 106910. <https://doi.org/10.1016/j.aquatox.2024.106910>.
29. El-Sayed A-FM, El-Ghobashy AE: Effects of tank colour and feed colour on growth and feed utilization of thinlip mullet (*Liza ramada*) larvae. *Aquac Res* 2011, 42(8):1163–1169. <https://doi.org/10.1111/j.1365-2109.2010.02704.x>
30. Toutou MM, Abdelhamid AM, Helmy AE, Abouzied AS, Refaey MM. Evaluation of alternative non-traditional lipid sources in diet for Thinlip grey mullet, *Liza ramada* fingerlings. *Egypt J Aquat Res*. 2023. <https://doi.org/10.1016/j.ejar.2023.02.003>.
31. El Basuini MF, Zaki MAA, El-Hais AM, Elhanafy MG, El-Bilawy EH, Zaineldin AI, Abdel-Aziz MFA, Abouelsaad IA, El-Ratel IT, Mzengereza K, et al. Microbial, immune and antioxidant responses of Nile tilapia with dietary nano-curcumin supplements under chronic low temperatures. *Aquac Fish*. 2024;9(1):57–65. <https://doi.org/10.1016/j.aaf.2022.03.011>.
32. Mousa MA, Khalil NA. Environmental and hormonal control of induced spawning in thin-lipped grey mullet, *Liza ramada* (Risso). *Egypt J Histol*. 2013;36(2):449–58. <https://doi.org/10.1097/01.EHX.0000428962.36676.05>.
33. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U et al: The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research*. *Journal of Cerebral Blood Flow & Metabolism* 2020, 40(9):1769–1777. <https://doi.org/10.1177/0271678X20943823>
34. Dye B, Kuijten S, Tulp I, Blom E, Poos J, Schram E: Influence of Salinity on the Thermal Preference of Juvenile Thinlip Mullet, *Chelon Ramada*; 2024. <https://doi.org/10.2139/ssrn.4727789>
35. Du J-H, Xu M-Y, Wang Y, Lei Z, Yu Z, Li M-Y. Evaluation of *Taraxacum mongolicum* flavonoids in diets for *Channa argus* based on growth performance, immune responses, apoptosis and antioxidant defense system under lipopolysaccharide stress. *Fish Shellfish Immunol*. 2022;131:1224–33. <https://doi.org/10.1016/j.fsi.2022.11.034>.
36. AOAC: Official methods of analysis of the Association of Official Analytical Chemists: Association of official analytical chemists; 2000.
37. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci*. 1991;74(10):3583–97. [https://doi.org/10.3168/jds.50022-0302\(91\)78551-2](https://doi.org/10.3168/jds.50022-0302(91)78551-2).
38. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72(1):248–54. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3).
39. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem*. 1972;247(10):3170–5. [https://doi.org/10.1016/S0021-9258\(19\)45228-9](https://doi.org/10.1016/S0021-9258(19)45228-9).

40. Góth L. A simple method for determination of serum catalase activity and revision of reference range. *Clin Chim Acta*. 1991;196(2):143–51. [https://doi.org/10.1016/0009-8981\(91\)90067-M](https://doi.org/10.1016/0009-8981(91)90067-M).
41. Arun S, Krishnamoorthy P, Subramanian P. Properties of glutathione peroxidase from the hepatopancreas of freshwater prawn *Macrobrachium malcolmsonii*. *Int J Biochem Cell Biol*. 1999;31(6):725–32. [https://doi.org/10.1016/S1357-2725\(99\)00016-3](https://doi.org/10.1016/S1357-2725(99)00016-3).
42. Lygren B, Sveier H, Hjeltnes B, Waagbø R. Examination of the immunomodulatory properties and the effect on disease resistance of dietary bovine lactoferrin and vitamin C fed to Atlantic salmon (*Salmo salar*) for a short-term period. *Fish Shellfish Immunol*. 1999;9(2):95–107. <https://doi.org/10.1006/fsim.1998.0179>.
43. Wang J, He R-Z, Lu G-L, Luo H-L, Lu D-Q, Li A-X. Vaccine-induced antibody level as the parameter of the influence of environmental salinity on vaccine efficacy in Nile tilapia. *Fish Shellfish Immunol*. 2018;82:522–30. <https://doi.org/10.1016/j.fsi.2018.08.025>.
44. Gallage S, Katagiri T, Endo M, Futami K, Endo M, Maita M. Influence of moderate hypoxia on vaccine efficacy against *Vibrio anguillarum* in *Oreochromis niloticus* (Nile tilapia). *Fish Shellfish Immunol*. 2016;51:271–81. <https://doi.org/10.1016/j.fsi.2016.02.024>.
45. Secombes CJ. Isolation of salmonid macrophages and analysis of their killing activity. *Tech Fish Immunol*. 1990;1:137–63.
46. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻ΔΔCT method. *methods* 2001, 25(4):402–408.
47. Bancroft JD, Gamble M: Theory and practice of histological techniques: Elsevier health sciences; 2008.
48. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012;9(7):671–5.
49. El Basuini MF, Abdel Fattah AM, El-Hais AM, Soliman AA, Amer AA, Gewaily M, Zaki MAA, Zaineldin AI, Dossou S, Teiba II, et al. Dietary co-enzyme Q10 boosted the growth performance, antioxidative capacity, immune responses, and intestinal and hepatic histomorphology of grey mullet (*Liza ramada*). *Aquac Rep*. 2024;36: 102147. <https://doi.org/10.1016/j.aqrep.2024.102147>.
50. Zhao L, Duan H, Liu Y, Wang Y, Li M, Li M. Long-term exposure of zebrafish (*Danio rerio*) to Cr(VI): Reproductive toxicity and neurotoxicity. *Regional Studies in Marine Science*. 2024;74: 103559. <https://doi.org/10.1016/j.rsma.2024.103559>.
51. Li M-Y, Shi Y-C, Xu W-X, Zhao L, Zhang A-Z. Exploring Cr(VI)-induced blood-brain barrier injury and neurotoxicity in zebrafish and snakehead fish, and inhibiting toxic effects of astaxanthin. *Environ Pollut*. 2024;355: 124280. <https://doi.org/10.1016/j.envpol.2024.124280>.
52. Niu X-T, Sun C, Zhao L, Chen X-M, Wang G-Q, Li M-Y. The major role of glucocorticoid receptor (GR) in astaxanthin alleviates immune stress in *Channa argus* lymphocyte. *Aquaculture*. 2024;584: 740637. <https://doi.org/10.1016/j.aquaculture.2024.740637>.
53. Oliva-Teles A. Nutrition and health of aquaculture fish. *J Fish Dis*. 2012;35(2):83–108. <https://doi.org/10.1111/j.1365-2761.2011.01333.x>.
54. Yu Z, Zhao L, Zhao J-L, Xu W, Guo Z, Zhang A-Z, Li M-Y. Dietary *Taraxacum mongolicum* polysaccharide ameliorates the growth, immune response, and antioxidant status in association with NF-κB, Nrf2 and TOR in Jian carp (*Cyprinus carpio* var. Jian). *Aquaculture* 2022, 547:737522. <https://doi.org/10.1016/j.aquaculture.2021.737522>.
55. Oduleye SO. Growth and growth regulation in the cichlids. *Aquaculture*. 1982;27(3):301–6. [https://doi.org/10.1016/0044-8486\(82\)90065-5](https://doi.org/10.1016/0044-8486(82)90065-5).
56. Shehata AI, Wang T, Wang J, Zhang Z. Effect of feed additives in the diet on the growth and testicular development of male red claw crayfish (*Cherax quadricarinatus*) using orthogonal experiments. *Anim Feed Sci Technol*. 2022;283: 115180. <https://doi.org/10.1016/j.anifeeds.2021.115180>.
57. Wang LS, Shi Z, Shi BM, Shan AS. Effects of dietary stevioside/rebaudioside A on the growth performance and diarrhea incidence of weaned piglets. *Anim Feed Sci Technol*. 2014;187:104–9. <https://doi.org/10.1016/j.anifeeds.2013.10.014>.
58. Heidari N, Chelehmal M, Javaheri Baboli M: The effect of adding Stevia (*Stevia rebaudiana*) extract in the diet on the growth, survival and fillet chemical properties on common carp (*Cyprinus carpio*). *Appl Biol* 2018, 31(3):55–67. <https://doi.org/10.22051/JAB.2018.9284.1046>.
59. Harada K, Miyasaki T, Tamura Y: Feeding attraction activity of stevioside for aquatic animals. *Nippon Suisan Gakkaishi* 1993, 59(11).
60. Choy J, Romano N, Ebrahimi M, Kamarudin MS: Effects of dietary steviol glycosides on the growth, feed intake and intestinal short-chain fatty acids in red hybrid tilapia. *J Environ Biol* 2018, 39(5):761–766. [https://doi.org/10.22438/jeb/39/5\(SI\)/8](https://doi.org/10.22438/jeb/39/5(SI)/8).
61. Yodyingyud V, Bunyawong S. Effect of stevioside on growth and reproduction*. *Hum Reprod*. 1991;6(1):158–65. <https://doi.org/10.1093/oxfordjournals.humrep.a137251>.
62. Wood DJ, Lirette A, Crober DC, Ju HY. The effect of stevia as a feed sweetener on weight gain and feed consumption of broiler chickens. *Can J Anim Sci*. 1996;76(2):267–9. <https://doi.org/10.4141/cjas96-040>.
63. Munro PJ, Lirette A, Anderson DM, Ju HY. Effects of a new sweetener, Stevia, on performance of newly weaned pigs. *Can J Anim Sci*. 2000;80(3):529–31. <https://doi.org/10.4141/A00-00>.
64. Han X, Chen C, Zhang X, Wei Y, Tang S, Wang J, Tan Z, Xu L. Effects of dietary stevioside supplementation on feed intake, digestion, ruminal fermentation, and blood metabolites of Goats. *Animals*. 2019;9(2):32. <https://doi.org/10.3390/ani9020032>.
65. Koyama E, Kitazawa K, Ohori Y, Izawa O, Kakegawa K, Fujino A, Ui M. In vitro metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in human intestinal microflora. *Food Chem Toxicol*. 2003;41(3):359–74. [https://doi.org/10.1016/S0278-6915\(02\)00235-1](https://doi.org/10.1016/S0278-6915(02)00235-1).
66. Yanbo W, Zirong X. Effect of probiotics for common carp (*Cyprinus carpio*) based on growth performance and digestive enzyme activities. *Anim Feed Sci Technol*. 2006;127(3):283–92. <https://doi.org/10.1016/j.anifeeds.2005.09.003>.
67. Kiron V. Fish immune system and its nutritional modulation for preventive health care. *Anim Feed Sci Technol*. 2012;173(1):111–33. <https://doi.org/10.1016/j.anifeeds.2011.12.015>.
68. Ortiz LT, Rebolé A, Velasco S, Rodríguez ML, Treviño J, Tejedor JL, Alzueta C. Effects of inulin and fructooligosaccharides on growth performance, body chemical composition and intestinal microbiota of farmed rainbow trout (*Oncorhynchus mykiss*). *Aquac Nutr*. 2013;19(4):475–82. <https://doi.org/10.1111/j.1365-2095.2012.00981.x>.
69. Abasubong KP, Li X-F, Adjoumani J-JY, Jiang G-Z, Desouky HE, Liu W-b: Effects of dietary xylooligosaccharide prebiotic supplementation on growth, antioxidant and intestinal immune-related genes expression in common carp *Cyprinus carpio* fed a high-fat diet. *J Anim Physiol Anim Nutr*. 2022;106(2):403–18. <https://doi.org/10.1111/jpn.13669>.
70. Daneshyar M, Geuns JMC, Willemsen H, Ansari Z, Darras VM, Buyse JG, Everaert N. Evaluation of dietary stevioside supplementation on anti-human serum albumin immunoglobulin G, Alpha-1-glycoprotein, body weight and thyroid hormones in broiler chickens. *J Anim Physiol Anim Nutr*. 2012;96(4):627–33. <https://doi.org/10.1111/j.1439-0396.2011.01188.x>.
71. Hahor W, Thongprajukaew K, Suanyuk N. Effects of dietary supplementation of oligosaccharides on growth performance, gut health and immune response of hybrid catfish (*Pangasianodon gigas* × *Pangasianodon hypophthalmus*). *Aquaculture*. 2019;507:97–107. <https://doi.org/10.1016/j.aquaculture.2019.04.010>.
72. Tomita T, Sato N, Arai T, Shiraishi H, Sato M, Takeuchi M, Kamio Y. Bactericidal activity of a fermented hot-water extract from *Stevia rebaudiana* Berton towards enterohemorrhagic *Escherichia coli* O157: H7 and other food-borne pathogenic bacteria. *Microbiol Immunol*. 1997;41(12):1005–9. <https://doi.org/10.1111/j.1348-0421.1997.tb01961.x>.
73. Hosseini SM, Hoseinifar SH, Mazandarani M, Paknejad H, Van Doan H, El-Haroun ER. The potential benefits of orange peels derived pectin on serum and skin mucus immune parameters, antioxidant defence and growth performance in common carp (*Cyprinus carpio*). *Fish Shellfish Immunol*. 2020;103:17–22. <https://doi.org/10.1016/j.fsi.2020.04.019>.
74. Sun Z, Wei Z, Liu Q, Mai H, Liu Y, Liu B, Tan X, Ye C. Effects of dietary *Astragalus membranaceus* (Fisch.) Bge. root extract on growth performance, plasma biochemical parameters, fish composition, liver and intestinal morphology, and genes expression in head kidney of hybrid grouper (*Epinephelus lanceolatus* ♂ × *Epinephelus fuscoguttatus* ♀). *Aquaculture Reports* 2022, 22:100934. <https://doi.org/10.1016/j.aqrep.2021.100934>.
75. Shi Y-C, Zhao Y-R, Zhang A-Z, Zhao L, Yu Z, Li M-Y. Hexavalent chromium-induced toxic effects on the hematology, redox state, and apoptosis in *Cyprinus carpio*. *Regional Studies in Marine Science*. 2022;56: 102676. <https://doi.org/10.1016/j.rsma.2022.102676>.

76. Wang Y-J, Chien Y-H, Pan C-H. Effects of dietary supplementation of carotenoids on survival, growth, pigmentation, and antioxidant capacity of characins. *Hyphessobrycon callistus* Aquaculture. 2006;261(2):641–8. <https://doi.org/10.1016/j.aquaculture.2006.08.040>.
77. Gao X-Q, Fei F, Huang B, Meng XS, Zhang T, Zhao K-F, Chen H-B, Xing R, Liu B-L. Alterations in hematological and biochemical parameters, oxidative stress, and immune response in *Takifugu rubripes* under acute ammonia exposure. *Comp Biochem Physiol C: Toxicol Pharmacol*. 2021;243: 108978. <https://doi.org/10.1016/j.cbpc.2021.108978>.
78. Aebi H: Catalase. In: *Methods of Enzymatic Analysis (Second Edition)*. edn. Edited by Bergmeyer HU: Academic Press; 1974: 673–684. <https://doi.org/10.1016/B978-0-12-091302-2.50032-3>
79. Rotruck JT, Pope AL, Ganther HE, Swanson A, Hafeman DG, Hoekstra W: Selenium: biochemical role as a component of glutathione peroxidase. *Science* 1973, 179(4073):588–590. <https://doi.org/10.1126/science.179.4073>.
80. Özlüer Hunt A, Çetinkaya M, Özkan Yılmaz F, Yıldırım M, Berköz M, Yalın S: Effect of dietary supplementation of inulin on growth performance, digestion enzyme activities and antioxidant status of Rainbow Trout (*Oncorhynchus mykiss*). *Turk J Agric Food Sci Technol* 2019, 7(9):1344–1353. <https://doi.org/10.24925/turjaf.v7i9.1344-1353.2581>
81. Sotirov L, Dimitrov I, Djorboneva M. Serum lysozyme concentrations in different sheep breeds. *Bulg J Vet Med*. 2005;8:83–9.
82. Alhoshy M, Shehata AI, Habib YJ, Abdel-Latif HMR, Wang Y, Zhang Z. Nutrigonomics in crustaceans: Current status and future prospects. *Fish Shellfish Immunol*. 2022;129:1–12. <https://doi.org/10.1016/j.fsi.2022.08.056>.
83. Wang T, Secombes CJ. The cytokine networks of adaptive immunity in fish. *Fish Shellfish Immunol*. 2013;35(6):1703–18. <https://doi.org/10.1016/j.fsi.2013.08.030>.
84. Hong S, Zou J, Crampe M, Peddie S, Scapigliati G, Bols N, Cunningham C, Secombes CJ. The production and bioactivity of rainbow trout (*Oncorhynchus mykiss*) recombinant IL-1 β . *Vet Immunol Immunopathol*. 2001;81(1):1–14. [https://doi.org/10.1016/S0165-2427\(01\)00328-2](https://doi.org/10.1016/S0165-2427(01)00328-2).
85. Shike H, Shimizu C, Lauth X, Burns JC. Organization and expression analysis of the zebrafish hepcidin gene, an antimicrobial peptide gene conserved among vertebrates. *Dev Comp Immunol*. 2004;28(7):747–54. <https://doi.org/10.1016/j.dci.2003.11.009>.
86. Campoverde C, Milne DJ, Estévez A, Duncan N, Secombes CJ, Andree KB. Ontogeny and modulation after PAMPs stimulation of β -defensin, hepcidin, and piscidin antimicrobial peptides in meagre (*Argyrosomus regius*). *Fish Shellfish Immunol*. 2017;69:200–10. <https://doi.org/10.1016/j.fsi.2017.08.026>.
87. Cai T, Ye H, Jiang H, Lin C, Lou C, Wang W, Yan Z, Xue X, Pan X, Lin J. Steviolside targets the NF- κ B and MAPK pathways for inhibiting inflammation and apoptosis of chondrocytes and ameliorates osteoarthritis in vivo. *Int Immunopharmacol*. 2023;115: 109683. <https://doi.org/10.1016/j.intimp.2023.109683>.
88. Jami MJ, Abedian Kenari A, Paknejad H, Mohseni M. Effects of dietary β -glucan, mannan oligosaccharide, *Lactobacillus plantarum* and their combinations on growth performance, immunity and immune related gene expression of Caspian trout, *Salmo trutta caspius* (Kessler, 1877). *Fish Shellfish Immunol*. 2019;91:202–8. <https://doi.org/10.1016/j.fsi.2019.05.024>.
89. Yousefi S, Hoseinifar SH, Paknejad H, Hajimoradloo A. The effects of dietary supplement of galactooligosaccharide on innate immunity, immune related genes expression and growth performance in zebrafish (*Danio rerio*). *Fish Shellfish Immunol*. 2018;73:192–6. <https://doi.org/10.1016/j.fsi.2017.12.022>.
90. Hoseinifar SH, Ahmadi A, Khalili M, Raeisi M, Van Doan H, Caipang CM. The study of antioxidant enzymes and immune-related genes expression in common carp (*Cyprinus carpio*) fingerlings fed different prebiotics. *Aquac Res*. 2017;48(11):5447–54. <https://doi.org/10.1111/are.13359>.
91. Salah AS, El Nahas AF, Mahmoud S. Modulatory effect of different doses of β -1,3/1,6-glucan on the expression of antioxidant, inflammatory, stress and immune-related genes of *Oreochromis niloticus* challenged with *Streptococcus iniae*. *Fish Shellfish Immunol*. 2017;70:204–13. <https://doi.org/10.1016/j.fsi.2017.09.008>.
92. Torrecillas S, Montero D, Caballero MJ, Robaina L, Zamorano MJ, Sweetman J, Izquierdo M. Effects of dietary concentrated mannan oligosaccharides supplementation on growth, gut mucosal immune system and liver lipid metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. *Fish Shellfish Immunol*. 2015;42(2):508–16. <https://doi.org/10.1016/j.fsi.2014.11.033>.
93. Lokesh J, Fernandes JMO, Korsnes K, Bergh Ø, Brinchmann MF, Kiron V. Transcriptional regulation of cytokines in the intestine of Atlantic cod fed yeast derived mannan oligosaccharide or β -Glucan and challenged with *Vibrio anguillarum*. *Fish Shellfish Immunol*. 2012;33(3):626–31. <https://doi.org/10.1016/j.fsi.2012.06.017>.
94. Ramena Y, Rawles SD, Lochmann R, Gaylord TG, McEntire ME, Farmer BD, Baumgartner W, Webster CD, Beck BH, Green BW et al: Growth, nutrient retention, innate immune response, and intestinal morphology of juvenile, soy-naïve hybrid striped bass, *Morone saxatilis* x *M. chrysops* fed commercial-type, soy-based, ideal protein, fish meal replacement diets. *Aquaculture* 2020, 522:735150. <https://doi.org/10.1016/j.aquaculture.2020.735150>
95. Tian J, Gao W, Wen H. Research advances: intestinal health and feed additives in aquatic animals. *Chin J Anim Nutr*. 2018;30(1):7–13.
96. Zaki M, Salem ME-S, Gaber M, Nour A: Effect of chitosan supplemented diet on survival, growth, feed utilization, body composition & histology of sea bass (*Dicentrarchus labrax*). *World Journal of Engineering and technology*. 2015;3(4):38–47. <https://doi.org/10.4236/wjet.2015.34C005>.
97. Shukry M, Albogami S, Gewaily M, Amer AA, Soliman AA, Alsaied SM, El-Shehawi AM, Dawood MAO. Growth Performance, Antioxidative Capacity, and Intestinal Histomorphology of Grey Mullet (*Liza ramada*)—Fed Dietary Zinc Nanoparticles. *Biol Trace Elem Res*. 2022;200(5):2406–15. <https://doi.org/10.1007/s12011-021-02844-6>.
98. Boonkaewwan C, Ao M, Toskulkao C, Rao MC. Specific immunomodulatory and secretory activities of steviolside and steviol in intestinal cells. *J Agric Food Chem*. 2008;56(10):3777–84. <https://doi.org/10.1021/jf072681o>.
99. El Basuini MF, Teiba II, Zaki MAA, Alabssawy AN, El-Hais AM, Gabr AA, Dawood MAO, Zaineldin AI, Mzengereza K, Shadrack RS, et al. Assessing the effectiveness of CoQ10 dietary supplementation on growth performance, digestive enzymes, blood health, immune response, and oxidative-related genes expression of Nile tilapia (*Oreochromis niloticus*). *Fish Shellfish Immunol*. 2020;98:420–8. <https://doi.org/10.1016/j.fsi.2020.01.052>.

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