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Levamisole Enhances Growth, Haemato-biochemical Indicators, Resistance to *Aeromonas veronii* and Expression of Immune Genes in Asian Seabass, *Lates calcarifer*

Philominal Pathinathan[1], Uma Arumugam[1], Felix Nathan[1], Ahilan Baboonsundaram[1], Cheryl Antony[1]

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ABSTRACT

Background: The objective of this study was to assess the effects of dietary levamisole supplementation feed on the growth, hemato-biochemical parameters, disease resistance and the expression of genes related to growth and immunity in Asian seabass, *Lates calcarifer*.

Methods: Triplicate groups of fish (n=20) with an average weight of 3.29±0.4 g were fed with feed supplemented with levamisole (C, 0.0 mg/Kg; LVT1, 75 mg/Kg; LVT2, 150 mg/Kg; LVT3, 300 mg/Kg; LVT4, 600 mg/Kg) for 60 days. At the end of the experiment growth, haemato-biochemical parameters and disease resistance to *Aeromonas veronii* were assessed. Relative expressions of growth and immune genes were analyzed by quantitative real-time PCR (qPCR).

Result: Dietary incorporation with levamisole in the range of 75-600 mg/Kg diet improved the haemato-biochemical indices, survival rates and resistance to *Aeromonas veronii* challenge (CAK4/SRLAAH/2022) at a concentration of 2.5×10^4 cfu/ml. Levamisole incorporation at 300 mg/Kg feed (LVT3) resulted in significant ($p<0.05$) improvements in various parameters such as feed efficiency, growth rate, increased disease resistance and upregulated expression of both growth and immune-related genes in comparison to the control (C). Significant improvements in haemato-biochemical indices such as hemoglobin (Hb), white blood cells (WBC), packed cell volume (PCV), erythrocytes (Ery), glucose (GLU), cholesterol (Cho) and triglyceride (TG) levels were also recorded.

Key words: Asian seabass, Disease resistance, Gene expression, Growth performance, Haemato-biochemical, Levamisole.

INTRODUCTION

Diseases are the primary limiting factors for the development and expansion of aquaculture (Ringo *et al.* 2010). Traditionally, control and prevention of diseases were achieved using a wide range of antibiotics, pesticides, disinfectants and other chemicals. The use of chemotherapeutic drugs and antibiotics for treating illnesses has drawn a lot of criticism because of their unfavorable side effects, including residue buildup in tissue and the environment, the emergence of drug-resistant bacteria, immunosuppression and decreased customer demand for fish as a food source (Yasin *et al.*, 2023). Consequently, novel preventative approaches need to be developed to effectively address both emerging and established diseases (Ringo *et al.*, 2010). Immunostimulants serve as dietary supplements that enhance resistance to specific infections and activate the fish innate defense mechanisms. Levamisole is a synthetic anthelmintic used in mammals which also has the capability to elicit innate-specific immunity response in fishes (Reverter *et al.*, 2014). Levamisole has the capacity to stimulate the synthesis of interferon (IFN) and IL-6 through enhanced expression of MHC receptors (Holcombe *et al.*, 2006). Several studies demonstrated that levamisole boosted immunity by mimicking the action of the thymic hormone thymopoietin (TMPO) which is identified as a biologically active peptide comprising 49 amino acids and located within the thymus (Weber *et al.*, 1999). Hence levamisole has the potential to adopt a tertiary structure resembling thymopoietin and stimulate lymphocytes through its imidazole component also reported as a growth promotor in carp (Baba *et al.*, 1993; Gopalakannan and Arul, 2006), nile tilapia (Bedasso, 2017) and rainbow trout (Kajita *et al.*, 1990). Asian seabass (*Lates calcarifer*) is a commercially important species, known as barramundi, is widely cultured in Southeast Asia and Australia using fresh, brackish and marine water resources (Glencross *et al.*, 2016). Similar to many other aquaculture species, the adoption of intensive farming practices has led to a rise in the occurrence of bacterial,

[1]Dr. M.G.R. Fisheries College and Research Institute, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Ponneri-601 204, Tamil Nadu, India.

Corresponding Author: Philominal Pathinathan, Dr. M.G.R. Fisheries College and Research Institute, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Ponneri-601 204, Tamil Nadu, India. Email: drphilo555@gmail.com

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viral and parasitic infections in *L. calcarifer* farming (Anderson and Norton, 1991; Azad *et al*., 2004; Kumar *et al*., 2007). *Aeromonas veronii* stands out as a prevalent pathogen in aquaculture, with the ability to affect various aquatic species. Research has demonstrated that *A. veronii* exhibits a higher level of virulence compared to *Aeromonas hydrophila*. Consequently, this triggers the activation of the aer gene, leading to enhanced bacterial adhesion ability within host cells. Moreover, the quorum-sensing mechanism significantly contributes to the infection process of *A. veronii* in seabass. *Lateolabrax maculatus* infected with *A. veronii* exhibit acute mortality, marked by ulcerations on the body surface and congestion, as well as hemorrhaging in internal organs like the liver, kidney and spleen (Wang *et al*., 2021). Keeping this in view, this study was undertaken to evaluate the effect of dietary levamisole on the growth, haemato-biochemical parameters, disease resistance against *Aeromonas veronii* and immune-related gene expression in Asian seabass, *Lates calcarifer.*

MATERIALS AND METHODS

Fish husbandry

Asian seabass (*Lates calcarifer*) seeds (2.4±0.3 cm; 1.2±0.2 g) were purchased from the hatchery located at Rajiv Gandhi Centre for Aquaculture in Sirkazhi, Tamil Nadu, India. The health status of the fish were examined immediately upon arrival to the experimental facility. The fishes were acclimatized for 30 days in Fiber-reinforced plastic (FRP) tanks (1000L capacity each) in a controlled laboratory environment, by feeding a diet with 45% crude protein.

Experimental diet preparation

A basal feed with 45% crude protein and 10% crude lipid was prepared. Levamisole (Sigma Aldrich) was incorporated into the basal diet at varying levels, *viz*., 0.0 mg/Kg (C), 75 mg/Kg (LVT1), 150 mg/Kg (LVT2), 300 mg/Kg (LVT3) and 600 mg/Kg (LVT4) based on the methods of Li *et al.* (2006); Kumari and Sahoo (2006); Lim *et al.* (2019) and Pahor-Filho *et al.* (2017). Briefly, the diets were prepared by mixing the ingredients (Table 1). Preparation of dough by adding water and grinding in a meat grinder followed by steam cooking for 20 min and pelletizing through 2-mm dia die in a sinking feed pelletizer (Jinan Sunpring, China). The feeds were stored in airtight containers at room temperature for later use (Prabu *et al*., 2021).

Experimental setup

For the experimental trial, fish with an average weight of 3.29±0.4 g were segregated into 15 groups with triplicates for the control and four treatment groups (C, LVT1, LVT2, LVT3 and LVT4) with 20 fish each. Subsequently, these fish were transferred to rectangular FRP tanks (250 L capacity) provided with ample aeration. During the 60-day experimental trial, the fish were fed daily with four feeding sessions (at 8:00 am, 12:00 pm, 4:00 pm and 8:00 pm). The values of water quality parameters *viz*., water temperature (28±1°C); dissolved oxygen (6.6±0.4 mg/L); pH (7.8±0.2), salinity (0 ppt), hardness (132±3 ppm) and alkalinity (97±2 ppm) (Boyd, 2012) were recorded daily and maintained at optimal ranges.

Fish growth and feed utilization

After the feeding trial, fish specimens were chosen randomly for comprehensive evaluation of growth performance, haemato-biochemical indices and analysis of growth and immune gene expressions related to both growth and immune functions. Biogrowth parameters such as the feed conversion ratio (FCR), weight gain (g), survival rate (%) and protein efficiency ratio (PER) were also recorded (Abdel-Tawwab *et al*., 2010).

Hemato-biochemical indicators

After the 60-day experimental feeding trial, the serum was collected from the experimental fishes by anesthetizing with 100 mg/L of MS-222 (Sigma-Aldrich, USA). The blood samples were collected using a 1 ml syringe by kept at the ambient temperature for 1h and at 4°C for 4 h followed by centrifugation (4°C, with 1500 × g for 5 min). The supernatant was collected and stored at -20°C until needed. Neubauer-hemocytometer was used to count the erythrocytes (Ery) and WBC followed by hemoglobin (Hb [g/dl]) level (Erdal *et al*., 1991). Blood indices, including mean corpuscular hemoglobin concentration (MCHC [g/dl]), mean corpuscular hemoglobin (MCH [pg]) and mean corpuscular volume (MCV [fl]) were also assessed (Wintrobe, 1934). The values of glucose (GLU) (Sigma Diagnostics, India), Serum cholesterol (CHO) (Parekh and Jung, 1970), Triglycerides (TG) (Rice, 1970) and serum total protein (Reinhold, 1953) were also analyzed.

Bacterial pathogen

A. veronii (CAK4/SRLAAH/2022) (Gen Bank Acc no. OP752155) isolated from an infected Asian seabass, identified and maintained at the microbial archive of State Referral Laboratory for Aquatic Animal Health, TNJFU-Madhavaram campus was used for the experimental challenge in this study. The isolate was revived by growing for 24 h at 30°C in nutrient broth (HiMedia). Growth was assessed in a spectrophotometer at OD 600 and centrifuged at 3000 × g for 15 min. Bacterial pellets were obtained, dissolved and vortexed in sterile 0.15 M physiological buffer saline (PBS) solution and preserved by storing them in tryptic soy broth (TSB) supplemented with 15% (v/v) glycerol and this was kept at a temperature of -70°C.

Experimental bacterial challenge

After the 60-day feeding trial, ten fish from each of the groups were experimentally challenged by intraperitoneal injection with *A.veronii* 0.1 mL at 2.5×10^4 cells/ml estimated by the LD50 method in the laboratory. The fish were observed for 15 days for the clinical signs and mortality. Reisolation of the pathogen was carried out on Aeromonas Isolation

Medium Base (HIMEDIA). To confirm the identity of the bacterial pathogen, 16S rRNA PCR and subsequent sequence analysis were performed (Weisburg *et al*., 1991)

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from aseptically collected fish muscle and kidney tissues using the RNA iso-plus kit (Takara Bio Inc. Japan), following the manufacturer's provided guidelines. Subsequently, 2 µg of total RNA was utilized to synthesize the first-strand complementary DNA (cDNA), following the manufacturer's instructions (Thermo Scientific, USA). The relative expressions of Hsp70 and Hsp90 were assessed following the procedures described by Glencross *et al.* (2016) and Fu *et al.* (2021) respectively. The GH and MSTN expressions were assessed by the methodology of Ezhimathi *et al.* (2022). The methodologies of Korni *et al.* (2021) and Hsieh *et al.* (2010) were employed to analyze TLR2 and TLR4 expression. The assessment of the ß-actin transcript was used as an internal control (Wahyudi *et al*., 2018). The details of primers, base pair and protocols are presented in Table 2.

PCR amplification was conducted using the C1000 Touch thermal cycler with the CFX96 Real-time PCR system (Bio-Rad, USA). For qPCR, the reaction mixture comprised approximately 20ng of cDNA template, 10 µM each of forward and reverse primers and 1x SYBR Green PCR Master Mix Kit (Takara Bio Inc, Japan), along with 20 µl of nuclease-free water. The qPCR cycle threshold (Ct) values were used to calculate the relative gene expression level displayed as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Statistical analysis

To assess the significant differences between the means, one-way analysis of variance (ANOVA) followed by Tukey's multiple range tests using IBM-SPSS Statistics version 20 were performed. The data are presented with mean values and their respective standard deviations (SD). A significance level of $p<0.05$ was applied to identify statistically significant differences among the treatment groups.

RESULTS AND DISCUSSION

Growth performances and feed utilization

Levamisole supplemented diet fed groups showed significant ($p<0.05$) improvement in feed efficiency and growth performance in *L.calcarifer* (Table 3). Fish fed with LVT3 diet exhibited significantly ($p<0.05$) higher final body weight, improved feed efficiency ratio and lesser feed conversion ratio compared to control and other dietary groups were shown in Fig 1A, B and C. Similarly, levamisole-fed *Cyprinus carpio* displayed a lower feed conversion ratio and a higher specific growth rate (Maqsood *et al*., 2009). Caspian brown trout (*Salmo caspius*), juvenile *Piaractus mesopotamicus* pacu fish and beluga fish that were fed with levamisole supplemented diet showed an improved protein efficiency ratio (Yuji Sado *et al*., 2010; Eslami and Bahrekazemi, 2019). Several studies have documented the immune-enhancing properties and growth-promoting effects of levamisole in various fish species *viz*., *Cyprinus carpio*, angelfish (*Pterophyllum scalare*), gilthead seabream (*Sparus aurata* L.) and nile tilapia (*Oreochromis niloticus*) (Kajita *et al*., 1990; Maqsood *et al*., 2009; Mulero *et al*., 1998; Gopalakannan and Arul, 2006; Kasiri *et al*., 2011; Bedasso, 2017).

Haemato-biochemical parameters

In comparison with the control group, the LVT3 fish group showed increased levels of hemoglobin (Hb), packed cell volume (PCV), erythrocyte count, mean corpuscular

Table 1: Formulation and chemical composition of the experimental diets (%).

Ingredients	Control	Levamisole 75 mg/kg	Levamisole 150 mg/kg	Levamisole 300 mg/kg	Levamisole 600 mg/kg
Fish meal	42	42	42	42	42
Squid meal	12.5	12.5	12.5	12.5	12.5
Soybean meal	16.5	16.5	16.5	16.5	16.5
Wheat flour	12	12	12	12	12
Cassava flour	8	8	8	8	8
Levamisole	-	7.5	15	30	60
Fish oil	4	4	4	4	4
Soy lecithin	2	2	2	2	2
Dicalcium phosphate	2	2	2	2	2
Vitamin premix	0.5	0.5	0.5	0.5	0.5
Mineral premix	0.5	0.5	0.5	0.5	0.5
Calculated chemical composition (% of diet)					
Moisture	9.2	9.1	9.4	9.2	9.3
Crude protein	45.24	45.31	45.17	45.22	45.27
Crude lipid	10.77	10.72	10.71	10.74	10.73
Ash	9.65	9.55	9.73	9.62	9.66

hemoglobin concentration (MCHC), cholesterol, triglycerides, lymphocytes, total protein, albumin and globulin. Experimental challenge with *A. veronii* resulted in a significant variation ($p<0.05$) in the haemato-biochemical parameters such as increased glucose level and lowest level of packed cell volume (PCV), hemoglobin (Hb), erythrocyte count (Ery), mean corpuscular hemoglobin concentration (MCHC), triglycerides (TG), total protein, albumin and globulin in the control group. Whereas levamisole-supplemented diet-fed LVT3 groups did not show significant variation compared to other treatments after experimental infection with *A. veronii* depicted in Table 4,5. After the experimental challenge, a notable rise in WBC counts and levels of polymorphs, lymphocytes, eosinophils and monocytes, was noted in the LVT3 group. Similarly, enhancements in non-specific immune responses, including increased counts of "natural killer" cells and heightened phagocytic activity were observed in levamisole-injected *Oncorhynchus mykiss* after challenge with virulent *Vibrio anguillarum* (Kajita *et al*., 1990). According to Kowalska *et al.* (2015) and Wijendra *et al.* (2007), the addition of levamisole to the diet of pikeperch (*Sander lucioperca*) and *Labeo rohita* respectively, resulted in a significant increase in hematological indices such as hemoglobin, red blood cell (RBC) and white blood cell (WBC) counts.

Disease resistance

The LVT3 group exhibited the lowest cumulative mortality 13.34% when challenged with *A. veronii* whereas the LVT2 group showed a 20% mortality rate. The fish fed with 600 mg/Kg (LVT4) levamisole in their diet had a mortality rate of 33.3% indicating higher concentrations of levamisole caused agranulocytosis and leukopenia in fishes (Midthun *et al*., 2021) whereas the low concentration of levamisole 75 mg/Kg (LVT1) caused 26.67% mortality when challenged with *A. veronii*. Fishes fed with the diet (C) showed significantly ($p<0.05$) higher mortality (53.34%) (Fig 1D). Extensive research on feeding levamisole in some fishes showed that it improves the functioning of certain components of the innate immune system as observed in the present study (Kajita *et al*., 1990; Siwicki *et al*., 1989; Mulero *et al*., 1998).

Growth gene and immune-related gene expression

The relative expression of growth genes (GH, MSTN), stress genes (Hsp90, Hsp70) and immune genes (TLR2, TLR4) in juvenile *L. calcarifer* fed diets containing levamisole

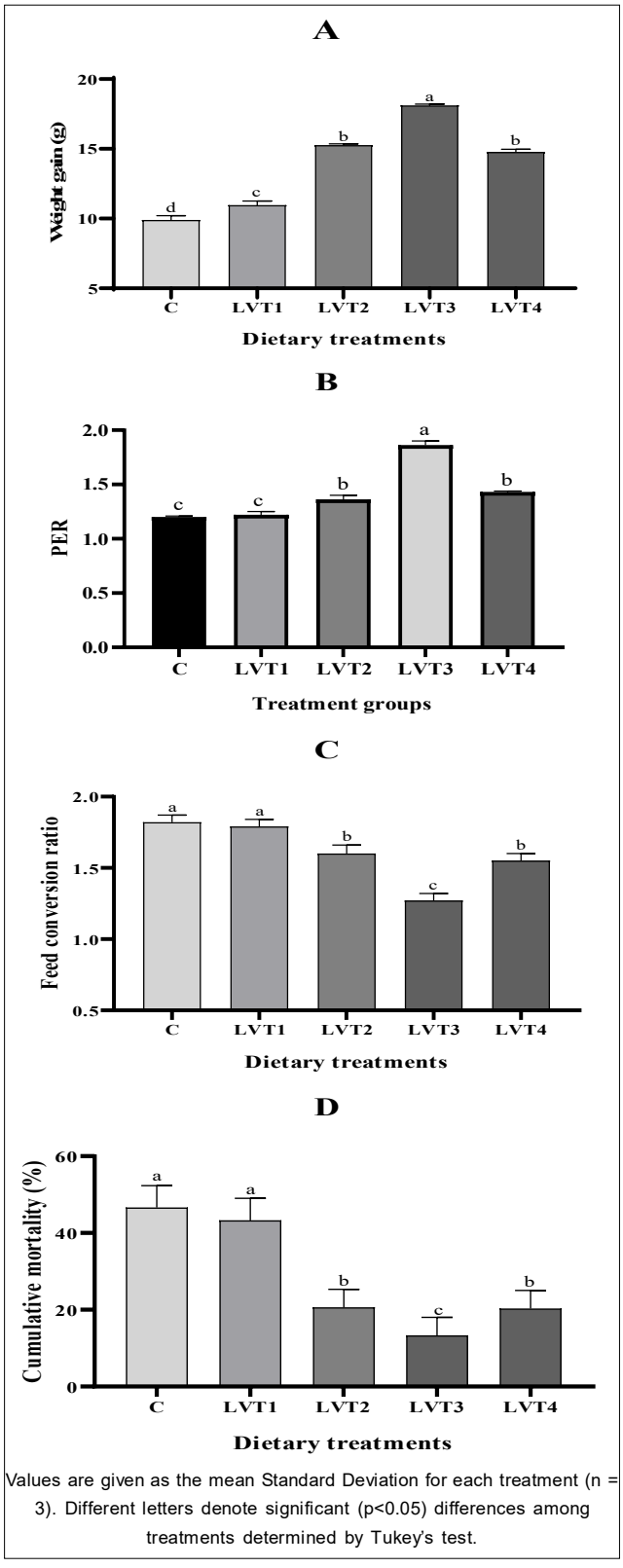
Table 2: Primers used for qPCR analysis of selected genes of Asian seabass fed with graded levels of levamisole.

Gene name	Primer sequence (5′-3′)	Amplicon (base pair)	Reference
Growth Hormone	F: TCGACAAACACGAGACGCA	86	Ezhilmathi *et al*., 2022
	R: CCCAGGACTCAACCAGTCCA		
Myostatin-1	F: ATGTAGTTATGGAGGAGGATG	227	Ezhilmathi *et al*., 2022
	R: CTTGGACGATGGACTCAG		
Heat Shock Protein 90	F: ACGATGATGAGCAGTATGCC	204	Fu *et al*., 2021
	R: CAAACAGGGTGATGGGGTA		
Heat Shock Protein 70	F: CAAGGTGATTTCAGATGGAGG	201	Glencross *et al*., 2016
	R: CTTCATCTTCACCAGGACCA		
Toll like Receptor 2	F: CCCACAATGGATTCACCAG	112	Korni *et al*., 2021
	R: AAAGATCAAGACTCAAGGCACTG		
Toll like Receptor 4	F: TGTTCAAGATGCCACATCAG	138	Hsieh *et al*., 2010
	R: TCCACAAGAACAAGCCTTTG		
β-actin	F: TACCACCGGTATCGTCATGGA	176	Wahyudi *et al*., 2018
	R: CCACGCTCTGTCAGGATCTTC		

Table 3: Growth performance of asian seabass fed with graded levels of levamisole-supplemented diets.

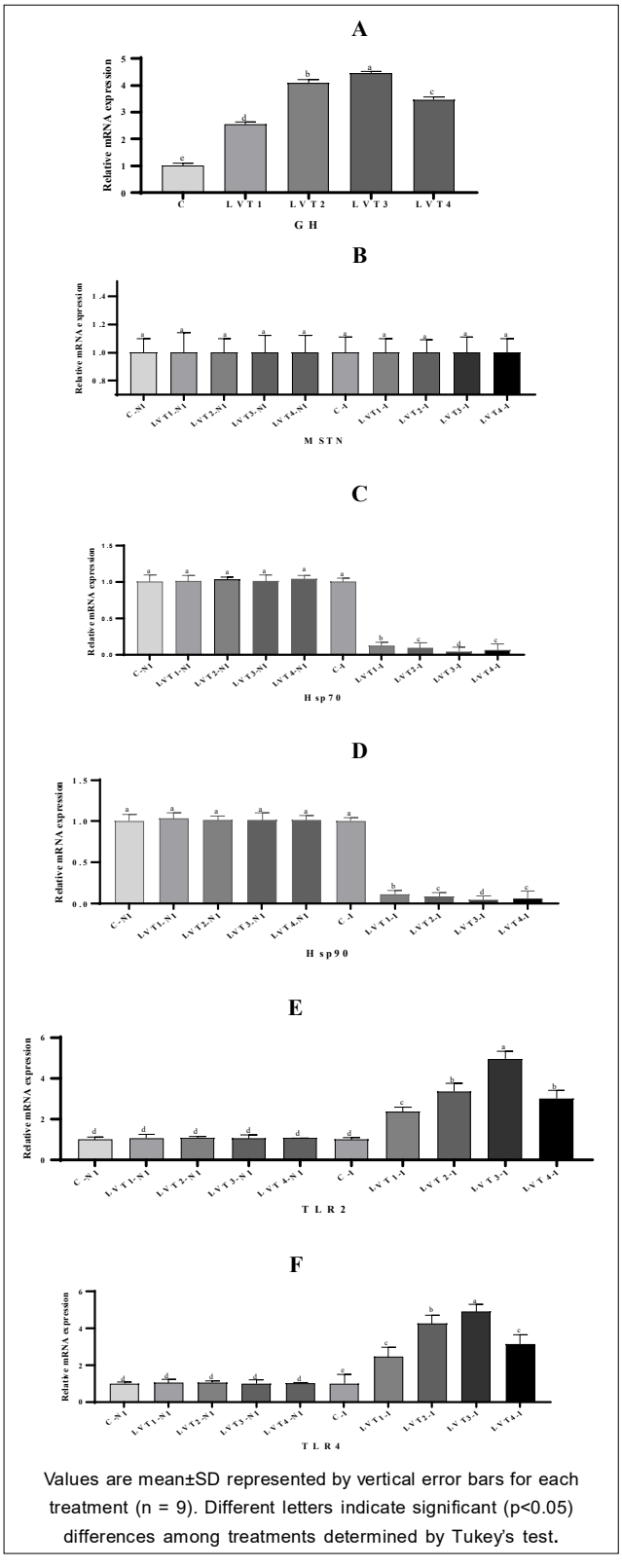
	Control	LVT1	LVT2	LVT3	LVT4
Initial weight (g)	3.29±0.4	3.53^{a}±0.21	3.25^{a}±0.14	3.31^{a}±0.185	3.33^{a}±0.25
Final weight (g)	13.2^{d}±0.20	14.5^{c}±0.10	18.51^{b}±0.06	21.4^{a}±0.26	18.1^{b}±0.09
Weight gain (g)	9.89^{d}±0.51	10.96^{c}±0.29	15.26^{b}±0.08	18.11^{a}±0.11	14.77^{b}±0.20
Survival (%)	98.3^{a}±2.8	96.66^{a}±2.8	96.66^{a}±5.7	98.3^{a}±2.8	96.6^{a}±5.77
Feed intake B/W	2.26^{a}±0.03	2.26^{a}±0.06	2.20^{a}±0.09	1.65^{b}±0.04	2.10^{a}±0.05
FCR	1.82^{a}±0.02	1.79^{a}±0.05	1.60^{b}±0.05	1.27^{c}±0.02	1.55^{b}±0.01
PER	1.20^{c}±0.01	1.22^{c}±0.03	1.46^{b}±0.04	1.86^{a}±0.04	1.33^{c}±0.01
FER	0.54^{c}±0.00	0.55^{c}±0.01	0.62^{b}±0.02	0.84^{a}±0.01	0.65^{b}±0.00

Note: Values were expressed as means±SD of three replicates per treatment (n = 3) and values with different superscripts indicate significant differences as determined by Tukey's test ($p<0.05$).



Values are given as the mean Standard Deviation for each treatment (n = 3). Different letters denote significant (p<0.05) differences among treatments determined by Tukey's test.

Fig 1: A- Weight gain, B- PER, C- Feed conversion ratio in Asian seabass fed with control and treatment diets, D- Cumulative mortality of Asian seabass challenged with *Streptococcus agalactiae*.



Values are mean±SD represented by vertical error bars for each treatment (n = 9). Different letters indicate significant (p<0.05) differences among treatments determined by Tukey's test.

Fig 2: A- Growth Hormone, B- Myostatin, C- Heat shock protein 70, D- Heat shock protein90, E- Toll-like receptor2, F- Toll-like receptor4 in Asian seabass fed with graded levels of lentinan for 60 days.

Table 4: Haematological parameters of Asian seabass fed graded levels of levamisole before challenge and after challenge against Aeromonas infection.

Haematology indicators	Control		LVT1		LVT2		LVT3		LVT4	
Hb (g dl^{-1})	$11.3^{e}\pm0.15$	$9.4^{d}\pm0.05$	$13.2^{d}\pm0.2$	$11.7^{c}\pm0.17$	$16.4^{c}\pm0.2$	$16.1^{b}\pm0.3$	$23.4^{a}\pm0.15$	$19.2^{a}\pm0.15$	$18.2^{b}\pm0.1$	$15.3^{b}\pm0.5$
WBC (1000/cumm)	$24.0^{a}\pm1.0$	$31.3^{d}\pm0.57$	$25.0^{a}\pm1.0$	$35.3^{c}\pm0.57$	$24.6^{a}\pm0.57$	$39.6^{b}\pm0.57$	$24.6^{a}\pm0.57$	$45.3^{a}\pm0.57$	$21.4^{b}\pm0.57$	$36.6^{c}\pm0.57$
Ery (million/ cumm)	$3.63^{e}\pm0.05$	$1.7^{d}\pm0.11$	$4.50^{d}\pm0.1$	$3.1^{c}\pm0.1$	$5.66^{c}\pm0.15$	$3.56^{b}\pm0.05$	$7.56^{a}\pm0.05$	$5.0^{a}\pm0.1$	$6.13^{b}\pm0.15$	$3.76^{b}\pm0.15$
PCV %	$32.3^{d}\pm0.57$	$28.0^{d}\pm1.0$	$35.1^{c}\pm0.05$	$31.4^{c}\pm0.1$	$36.7^{b}\pm0.15$	$34.9^{b}\pm0.1$	$42.4^{a}\pm0.10$	$40.2^{a}\pm0.1$	$34.9^{c}\pm0.45$	$30.4^{c}\pm0.2$
MCV (fl)	$88.9^{a}\pm0.17$	$162.0^{a}\pm12.5$	$78.09^{b}\pm1.62$	$101.3^{b}\pm2.9$	$64.9^{c}\pm1.49$	$97.7^{b}\pm1.6$	$56.03^{d}\pm0.4$	$80.4^{c}\pm1.5$	$57.02^{d}\pm0.72$	$80.8^{c}\pm3.71$
MCH (pg)	$31.2^{a}\pm0.16$	$54.6^{a}\pm4.12$	$29.3^{c}\pm0.56$	$37.8^{c}\pm1.73$	$28.9^{c}\pm0.4$	$45.14^{b}\pm0.4$	$30.8^{ab}\pm0.41$	$38.4^{c}\pm1.0$	$29.6^{bc}\pm0.64$	$40.8^{c}\pm1.24$
MCHC (g dl^{-1})	$35.05^{e}\pm0.23$	$33.7^{d}\pm1.21$	$37.57^{d}\pm0.57$	$37.2^{c}\pm0.65$	$44.6^{c}\pm0.36$	$46.1^{b}\pm0.93$	$55.2^{a}\pm0.45$	$47.8^{ab}\pm0.35$	$52.05^{b}\pm0.6$	$50.54^{a}\pm1.81$
Polymorphs %	$3.66^{a}\pm0.57$	$12.3^{d}\pm0.57$	$2.83^{bc}\pm0.28$	$17.6^{c}\pm0.57$	$2.53^{bc}\pm0.57$	$28.6^{b}\pm0.57$	$3.26^{ab}\pm0.05$	$35.3^{a}\pm0.57$	$2.36^{c}\pm0.23$	$26.6^{b}\pm0.57$
Lymphocytes %	$69.6^{d}\pm0.57$	$73.6^{e}\pm0.57$	$74.3^{c}\pm0.57$	$77.6^{d}\pm0.57$	$80.3^{b}\pm0.57$	$88.0^{b}\pm1.0$	$90.6^{a}\pm0.57$	$95.0^{a}\pm1.0$	$75.6^{c}\pm0.57$	$83.0^{c}\pm1.0$
Eosinophilis %	$0.76^{d}\pm0.20$	$1.33^{b}\pm0.57$	$1.23^{c}\pm0.11$	$2.43^{a}\pm0.57$	$1.46^{b}\pm0.11$	$2.70^{a}\pm0.10$	$2.30^{a}\pm0.10$	$3.06^{a}\pm0.05$	$1.34^{c}\pm0.05$	$2.66^{a}\pm0.05$
Monocytes %	$1.13^{c}\pm0.05$	$2.06^{d}\pm0.15$	$1.56^{b}\pm0.05$	$2.46^{c}\pm0.11$	$1.80^{a}\pm0.10$	$2.86^{b}\pm0.05$	$1.83^{a}\pm0.05$	$3.86^{a}\pm0.05$	$1.46^{b}\pm0.05$	$2.70^{bc}\pm0.10$

Table 5: Haemato-biochemical parameters of Asian seabass fed graded levels of levamisole before challenge and after challenge against aeromonas infection.

Haemato -biochemical indicators	Control		LVT1		LVT2		LVT3		LVT4	
GLU (mg dl^{-1})	$104.5^{a}\pm0.86$	$141.1^{a}\pm0.95$	$104.6^{a}\pm0.7$	$127.2^{b}\pm0.2$	$106.4^{a}\pm0.1$	$125.8^{c}\pm0.2$	$104.8^{a}\pm1.1$	$105.0^{e}\pm0.15$	$104.6^{a}\pm0.72$	$112.6^{d}\pm0.5$
CHO (mg dl^{-1})	$240.3^{d}\pm0.57$	$231.3^{d}\pm0.57$	$245.6^{c}\pm0.57$	$240.3^{c}\pm0.57$	$252.6^{b}\pm0.57$	$246.3^{b}\pm0.57$	$262.0^{a}\pm1.0$	$257.3^{a}\pm0.57$	$253.3^{b}\pm1.52$	$246^{b}\pm1.0$
TG (mg dl^{-1})	$203.3^{e}\pm0.26$	$198.1^{e}\pm0.15$	$206.2^{d}\pm0.25$	$202.5^{d}\pm0.1$	$215.6^{c}\pm0.15$	$212.2^{b}\pm0.37$	$225.4^{a}\pm0.37$	$221.1^{a}\pm0.1$	$219.1^{b}\pm0.15$	$211.3^{c}\pm0.1$
Polymorphs %	$3.66^{a}\pm0.57$	$12.3^{d}\pm0.57$	$2.83^{bc}\pm0.28$	$17.6^{c}\pm0.57$	$2.53^{bc}\pm0.57$	$28.6^{b}\pm0.57$	$3.26^{ab}\pm0.05$	$35.3^{a}\pm0.57$	$2.36^{c}\pm0.23$	$26.6^{b}\pm0.57$
Lymphocytes %	$69.6^{d}\pm0.57$	$73.6^{e}\pm0.57$	$74.3^{c}\pm0.57$	$77.6^{d}\pm0.57$	$80.3^{b}\pm0.57$	$88.0^{b}\pm1.0$	$90.6^{a}\pm0.57$	$95.0^{a}\pm1.0$	$75.6^{c}\pm0.57$	$83.0^{c}\pm1.0$
Eosinophilis %	$0.76^{d}\pm0.20$	$1.33^{b}\pm0.57$	$1.23^{c}\pm0.11$	$2.43^{a}\pm0.57$	$1.46^{b}\pm0.11$	$2.70^{a}\pm0.10$	$2.30^{a}\pm0.10$	$3.06^{a}\pm0.05$	$1.34^{c}\pm0.05$	$2.66^{a}\pm0.05$
Monocytes %	$1.13^{c}\pm0.05$	$2.06^{d}\pm0.15$	$1.56^{b}\pm0.05$	$2.46^{c}\pm0.11$	$1.80^{a}\pm0.10$	$2.86^{b}\pm0.05$	$1.83^{a}\pm0.05$	$3.86^{a}\pm0.05$	$1.46^{b}\pm0.05$	$2.70^{bc}\pm0.10$
Total protein (g/dL)	$3.43^{d}\pm0.05$	$2.3^{c}\pm0.15$	$4.13^{c}\pm0.11$	$3.13^{b}\pm0.05$	$4.70^{b}\pm0.10$	$2.86^{c}\pm0.05$	$5.86^{a}\pm0.05$	$4.36^{a}\pm0.15$	$4.56^{b}\pm0.11$	$3.5^{b}\pm0.1$
Albumin (g/dL)	$0.80^{e}\pm0.10$	$0.33^{d}\pm0.1$	$1.33^{d}\pm0.05$	$0.73^{c}\pm0.15$	$2.16^{b}\pm0.05$	$1.30^{b}\pm0.10$	$2.80^{a}\pm0.17$	$1.76^{a}\pm0.15$	$1.86^{c}\pm0.05$	$0.70^{c}\pm0.10$
Globulin (g/dL)	$1.87^{e}\pm0.06$	$1.30^{c}\pm0.10$	$2.15^{d}\pm0.05$	$1.66^{c}\pm0.15$	$3.16^{b}\pm0.05$	$2.20^{b}\pm0.10$	$3.53^{a}\pm0.11$	$2.70^{a}\pm0.26$	$2.43^{c}\pm0.05$	$1.56^{c}\pm0.11$

Hb: (Hemoglobin); WBC: (White blood cell); Ery: (Erythrocyte); PCV: (Packed cell volume); MCV: (Mean corpuscular volume); MCH: (Mean corpuscular hemoglobin); MCHC: (Mean corpuscular hemoglobin concentration); GLU: (Glucose); CHO: (Cholesterol) and TG: (Triglycerides).

Note: Values were expressed as mean±SD of three replicate per treatment (n=3) and values with different superscripts indicate significant differences as determined by Tukey's test ($p<0.05$).

supplementation showed in Fig 2. Fish raised in different treatments (LVT1, LVT2, LVT3, LVT4 and control) showed a distinct pattern of significant ($p<0.05$) expression. The relative expression of the GH (Growth Hormone) gene was up-regulated in the LVT3 group 4.46 fold ($p<0.05$) compared to the control (C) whereas, 2.54-fold in LVT1, 3.47-fold in LVT4 and 4.10-fold in LVT2 were recorded. Nevertheless, when different levels of dietary levamisole were administered, there were no notable changes observed in myostatin (MSTN) mRNA expression within the muscle tissues of *L. calcarifer.* Earlier studies have shown that dietary supplementation of olive leaf extract enhances GH gene expression in common carp *Cyprinus carpio* (Zemheri-Navruz *et al*., 2020). The expression levels of Hsp70 and Hsp90 exhibited a significant downregulation, with a fold change of 0.04 in the LVT3 group after being infected with *A. veronii* when compared to the control group. According to Hassaan *et al.* (2021), Hsp70 gene expression was significantly reduced ($p<0.05$) when fish were fed with β-carotene and phycocyanin in their diet. In the current research, it was observed that the addition of levamisole LVT3 (300 mg/Kg) to the diet of *L. calcarifer* decreased the mRNA expression levels of Hsp90. Tan *et al.* (2017) demonstrated that the mRNA expression levels of the Hsp90 gene were reduced in golden pompano when fed with hawthorn extract. This downregulation of Hsp90 expression was likely a result of the increased tolerance of the fish towards common stresses. The relative expression of TLR2 and TLR4 genes was upregulated in the LVT3 group with 4.94 and 4.90 fold ($p<0.05$), respectively. Upregulation of the TLR2 has been observed in the antimicrobial peptide Epinecidin-1-expressing Artemia cyst-fed Nile tilapia (Ting *et al*., 2018). TLR4 plays a major role in maintaining the immune system of animals, particularly in the gut (Cario and Podolsky, 2000). Previously, TLR4 upregulation had been documented in transgenic zebrafish following infection with *Vibrio vulnificus* (Hsieh *et al*., 2010). In the present study, LVT3 showed decreased stress gene expressions and enhanced immune gene expressions. Relative higher expression of the immune gene indicates, enhanced pathogen defense, improved clearance of infected cells and reduced disease severity (Mohanty and Sahoo, 2010) in levamisole diet-fed fishes after experimental infection with *A. veronii*, as a result, the mortality was lesser in the LVT3 group.

CONCLUSION

Levamisole shows great promise as an immunostimulant in *L. calcarifer*. In this study, dietary supplementation with levamisole exhibited improved growth performance, feed utilization and disease resistance against *A. veronii* in *L. calcarifer*. When compared to other dietary treatments and the control group, fish that were fed diets supplemented with 300 mg/Kg levamisole demonstrated superior performance in various aspects *viz*., enhanced growth, improved disease resistance, upregulated expression of growth and immune-related genes and higher survival rates when exposed to *A. veronii* infection. In conclusion, the findings of this study suggest that levamisole is a promising immunostimulant to be used for enhancing the growth and resistance to *A.veronii* infection of *L. calcarifer*.

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Conflict of interest

The authors have no conflicts of interest to declare.

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