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Nutritional modulation with chitosan nanoparticles and α-tocopherol enhances growth, health and gut histology in butter catfish (*Ompok pabda*)

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Abstract

This study evaluated the effects of dietary chitosan nanoparticles (CNP), α-tocopherol (VE), and their nanocomposite (CNP-VE) on growth performance, haematology, biochemistry, liver function, immunoglobulin, and intestinal histology of butter catfish (*Ompok pabda*). Four diets were tested: a control (T1), 5 g kg^{-1} CNP (T2), 500 mg kg^{-1} VE (T3), and 5 g kg^{-1} CNP + 500 mg kg^{-1} VE (T4). A total of 240 adult fish (13.56 ± 0.33 cm and 14.98 ± 0.78 g) were randomly assigned to the treatments in triplicate groups ($n = 20$) for a 60-day feeding period. Total length, body weight, feed conversion ratio, and specific growth rate, significantly improved in supplemented groups, with the best performance observed in T4 ($p < 0.05$). Haematological analysis revealed significant increases in red blood cells, haemoglobin, haematocrit, and MCHC in T4. While total protein, albumin, and globulin remained unchanged, glucose and bilirubin levels were significantly lower in T4, indicating improved metabolic status. IgM level was enhanced across treatments, with the greatest increase in T4. Histological examination showed improved intestinal structure in T4, including uniform goblet cell distribution and well-formed villi. These findings suggest that combined supplementation of CNP and VE has a synergistic effect in enhancing growth, physiological health, immunity, and gut morphology in *O. pabda* under intensive culture.

Keywords *Ompok Pabda*, Chitosan-vitamin E nanocomposite, Haematology, Liver function, Immunity

1 Introduction

Aquaculture is one of the fastest-growing food sectors, expanding to 4.5% annually and valued at USD 312.8 billion in 2022 [1], driven by the need to meet global protein demands. In Bangladesh, catfish play a vital ecological and economic role, with *Ompok pabda* (butter catfish) recognized for its high market value, nutritional quality, rapid growth, and consumer preference. However, wild populations have declined significantly

due to overfishing, habitat degradation, and pollution, pushing the species toward endangered status [2–4]. This decline threatens inland biodiversity which may alter food web dynamics and reduces the availability of an important protein source. Intensive aquaculture offers a viable alternative for *O. pabda* production, though it often imposes physiological stress, disrupts metabolism, and increases production costs [5]. The scarcity of natural growth promotes further limits fish health and productivity [6], prompting interest in functional dietary additives.

Nanotechnology is employed to develop various nanomaterials for use in the aquatic industry, particularly as feed additives, nonmetals such as selenium, iron, copper, and silver, as well as nanosized chitosan used to coat certain medications [7]. Some of these nanomaterials can combat bacterial infections by interacting with bacterial cell walls and disrupting cellular contents. Their small size allows for greater bioavailability, enabling them to penetrate tissues more easily and remain active longer than their bulk counterparts. Additionally, nanomaterials have the potential to enhance immune responses in fish [8–16]. Compared to conventional approaches, nanotechnology presents a superior alternative across various scientific disciplines using biocompatible nanocomposites such as nanocapsules, nanoparticles, and conjugates for drug formulation [17]. Many biologists prefer chitosan nanoparticles (CNP) because of their small size and nanoscale nature, which increases the surface area available for interaction with biological support, boosts the bioavailability of essential compounds, and allows for deep penetration into the target sites and efficient uptake by body cells [18–20]. In addition, CNPs have shown antimicrobial, immune-enhancing, and growth-promoting effects in fish [21, 22], yet their impacts on haematology, metabolism, and gut health in *O. pabda* remain understudied.

α-tocopherol (Vitamin E), a potent lipid-soluble antioxidant required to prevent oxidation by free oxygen radicals (ROS) in the cell membranes' lipids [23, 24]. It functions as an antioxidant chain breaker and can scavenge ROS such as lipid peroxyl radicals [23–25]. It also plays a key role in fish immunity, growth, health, immunity, reproduction, and oxidative stress management [26, 27]. In farmed fish, optimal levels of Vitamin E (VE) are recorded to improve growth performance, antioxidant capacity, and stress tolerance [28–30]. However, its water instability can reduce bioavailability. Encapsulation using CNPs may enhance VE's stability, minimize leaching, and improve nutrient delivery [31].

The effects of CNP combined with VE may provide stronger immunostimulating properties. Applying CNP and/or VE as a feeding supplement is believed to improve fish productivity, immunity, and overall health [27]. Recently, it was reported that dietary supplementation of VE (VE) and chitosan VE nanocomposite (CVEN) can improve fish growth performance, immune status, antioxidant capacity, tissue architecture, and disease resistance to *Aeromonas sobria* [27]. Additionally, Frag et al. [32] discovered that Nile tilapia's growth performance and immunity were improved by dietary VE nanoparticles encapsulated in nano-chitosan. Considering this, *O. pabda* is a relatively delicate fish species, characterized by slow growth, susceptibility to environmental stress and poor health under culture conditions. The inclusion of CNP and VE may confer comparable or even greater benefits in improving overall health performance of *O. pabda* under aquaculture systems.

Although the individual effects of CNP or VE have been investigated in some fish species, their potential synergistic effects have not been evaluated in *O. pabda* [21, 32, 33]. To fill this gap, this study aimed to investigate the effect of dietary supplementation of chitosan nanoparticles, VE, and chitosan-vitamin E nanocomposite (CNPVE) on growth performance, hematological and biochemical parameters, liver function, immunoglobulin, and histological changes in the intestinal tissue of *Ompok pabda*. The outcome of this research supports the broader use of nanoparticle based elements, such as chitosan nanoparticles and VE, as growth promotors in *O. pabda* catfish.

2 Materials and methods

2.1 Chemicals used and preparation of fish feed

The chitosan nanoparticles (CNP) were obtained from NANOSHELTM, Intelligent Materials Pvt. Ltd. (Punjab, India) in the form of powder that is light brown in color. Based on elemental analysis, the degree of deacetylation and average molecular weight were found to be 161 g/mol, assay > 99%, and APS 80–100 nm. According to Du et al. [34], CNPs were formulated based on the ionotropic gelation of chitosan and sodium tripolyphosphate (Sigma, USA). Using reagent-grade water (Milli-Q SP ultrapure water system, Nihon Millipore Ltd., Tokyo), the CNP underwent rigorous purification before being frozen (Virtis Advantage EL, SP Industries Company, USA). α-tocopherol (VE) was obtained from Sigma-Aldrich, USA.

First, the commercial fish feed pellets (Spectra Hexa Feeds Ltd., Bangladesh) were soaked in water and blended to create a paste to assess their viscoelasticity and consistency. To enhance the consistency, CNP and VE were added to the food paste, followed by thoroughly mixing with 5% (w/w) gelatin (Nutri-B-Gel, Canal Aqua Cure, Port-Said, Egypt) [35]. Following a feed mill to pelletize the grain, room temperature air drying was done, and it was kept refrigerated at 4 °C until feeding time. The proximate composition of the feed is presented in Table 1. Fish feed was allowed with apparent satiation twice daily at 09:00 and 16:00.

2.2 Field sampling and experimental setup

A total of 240 (13.56 ± 0.33 cm and 14.98 ± 0.78 g) mature mix sexed healthy *O. pabda* fish were acquired from the local hatchery in Mymensingh and brought to the GFBL, GAU. Prior to the start of the experiment, the fish underwent a three-week adaptation period in rectangular cement tanks (size: 2.2 × 1.6 × 0.6 m) During which they were fed a conventional basal diet. Following their acclimation, the fish were split into twelve circular experimental tanks with 500 L capacity. Fish were distributed at random, and each tank holding 20 fish (Fig. 1). The tank and aquaria were filled with dechlorinated tap water and aerated using air stones connected to an electric compressor. To maintain optimal water quality for fish culture and prevent waste accumulation, one-third of the water was replaced daily with clean, fresh, dechlorinated water.

Table 1 Proximate composition of the commercial diet (Spectra hexa feeds ltd., Bangladesh) used in the study

Type of feed	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)	Fibre (%)	Digestible energy (Kcal/Kg)
Starter	9.9	38.6	6.78	6.77	6	2950

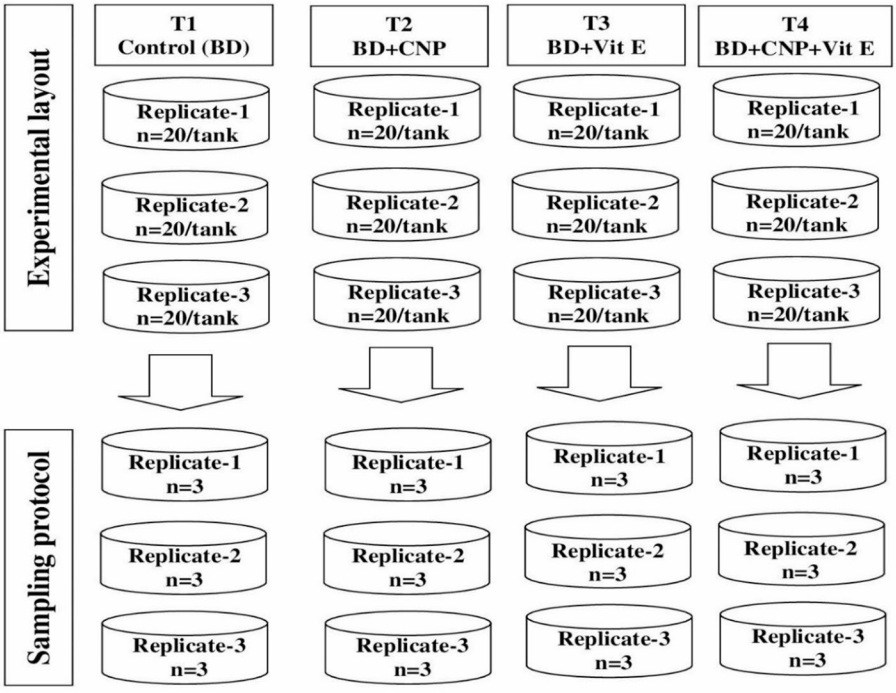

Fig. 1 Experimental protocol. The experiment consisted of four treatments with triplicate, in which *O. pabda* was maintained for 60 days

In the experimental design, a complete randomized design was used containing one control group and three treatments: T1: the basal diet with no supplement (control); T2: basal diet with 5 g kg^{-1} CNP; T3: basal diet with 500 mg kg^{-1} VE, and T4: CNP-VE nanocomposite group consisted of 5 g kg^{-1} CNP + 500 mg kg^{-1} VE. Each group consisted of three replications. The fish were kept in a flow-through system for 60 days [25]. Utilizing porous stones and an air pump, the water was continuously aerated. The population from each tank was measured and checked every alternate week. We used natural light during the experimental period.

2.3 Hydrobiology

During the experimental period, temperature and pH were monitored on every alternate day, while ammoniacal nitrogen (NH_3-N), total dissolved solids (TDS), and nitrate (NO_3) were measured weekly. A YSI 59 multiparameter water quality sampler (Yellow Springs Instrument Company, OH, USA) was used to measure the parameters.

2.4 Growth and feed utilization

At the beginning and subsequent weeks, each fish in every tank was weighed. Prior to sampling, a mild anesthesia was applied to the fish using α-methyl quinoline (Transmore®, Nika Trading, Puchong, Malaysia) at 0.22 ml L^{-1} [36]. Final lengths and weights were measured following the feeding trial's 60-day duration, and calculated the growth parameters:

Weight gain (WG, %) = 100 × (final weight - initial weight)/initial weight [37]
Feed conversion ratios (FCR) = dry feed intake/(final wt - initial wt) [38]
Feed conversion efficiency (FCE) = (final weight - initial weight)/dry feed intake [39]

Specific growth rate (SGR) = 100 × (ln final weight - ln initial weight)/time	[39]
Daily growth rate (DGR) = [(final weight - initial weight)/t] x 100	[40]
Relative growth rate = 100 × (final wt - initial wt)/initial wt	[40]
Survival (%) = 100× final no. of fish/initial no. of fish	[41]

2.5 Haematological and biochemical indices

At the end of the trial, fish were fasted for 24 h, anesthetized, and blood samples were drawn non-lethally from the caudal vein of five fish per group using sterile 2.5 mL syringes. Blood was divided into two portions: one for hematological tests stored at 4 °C with EDTA (1.26 mg/0.6 mL), and another for serum separation (centrifuged at 12,000 × g, 15 min, 4 °C). Hematological assessments included RBC and WBC counts (Neubauer hemocytometer), hemoglobin (Hb; cyanmethemoglobin method), and hematocrit (Hct; microhematocrit method). Biochemical parameters included glucose, total protein (TP), albumin (ALB), globulin (GLB), albumin/globulin ratio (A/G ratio), bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), cholesterol, and triglycerides were measured from 800 µL serum samples using assay kits (Biosino Bio-Technology and Science Inc., Beijing, China) [42].

2.6 Immune assay

IgM (g L^{-1}) concentrations were quantified using a CUSABIO colorimetric ELISA kit (Wuhan, China) as per the manufacturer's protocol. Plasma samples (50 µL) were read at 450 nm after 10 min [43].

2.7 Histopathological investigation

A histopathological investigation was performed after partially modified by Karim et al., [44]. Fish samples were anesthetized with α-methyl quinoline (Transmore®, Nika Trading, Puchong, Malaysia), and the intestines were collected carefully from each sample. Then intestinal tissues were transferred into 10% neutral buffer formalin (10% NBF) and fixed for future preservation. Tissue samples were then subjected to varying degrees of alcohol treatment and infiltrated with Paraffin 135 Wax at 65 °C for an hour in order to dehydrate them. The inserted tissues were further thinned with a rotating microtome apparatus to a thickness of 5 µm. Hematoxylin and eosin was then applied to the tissue sample sections in accordance with standard protocol, and the samples were examined under an electron microscope (MCX100, Micros Austria). Using an AmScope 1000 video tracking camera connected to a photomicroscope was used to photograph histopathological changes caused by the different treatments in the tissues of different organs of the fish.

2.8 Statistical analysis

Since no significant differences were detected among replicate means ($P > 0.05$), the replicate data were pooled and averaged. Prior to statistical analysis, data sets were tested for homogeneity of variances using Bartlett's test [45] and for normality using the Kolmogorov-Smirnov test [46]. Comparisons among experimental treatments were then conducted using one-way ANOVA, followed by Student's t-test, with Origin™ 2016 and Minitab 17 software Results were considered statistically significant at $p < 0.05$, and data are presented as means ± standard error (SE).

3 Results

3.1 Hydrobiology

Table 2 presents the physico-chemical parameters of the water measured across the experimental tanks throughout the study period. All parameters except pH and NH_3-N remained stable and showed no significant differences among the treatment groups when maintained at nominal levels. The recorded values were within the acceptable ranges for the optimal culture of this species, as reported by Dulić et al. [47]. pH levels were consistent (7.35 ± 0.17 to 7.69 ± 0.13) across treatments, indicating optimal growth conditions. The measured concentrations of NH_3-N, ranging from 0.23 ± 0.02 to 0.28 ± 0.03 mg L^{-1}, remain well below the toxic thresholds for many aquatic species, thereby signifying a healthy nitrogen regime. Nitrate levels also varied, with T4 presenting at 25.5 ± 5.24 mg L^{-1} and T3 having 21.375 ± 4.18 mg L^{-1} nitrates, and no noticeable differences were revealed among the treatments, indicating a strong bioconversion process supported by these diets. The TDS remained stable across treatments, reinforcing the diets' adequacy for maintaining favorable ionic conditions for *O. pabda*.

3.2 Growth performances

Fortnightly growth measurements indicated that fish length did not differ significantly among dietary treatments during the first two weeks. However, from the fourth week onward, a significant reduction in length ($p < 0.05$) was observed in certain treatments, and this trend persisted until the end of the experiment (Fig. 2a). A similar pattern was noted in mean weight gain. No significant differences were found among treatments During the initial 30 days ($p > 0.05$). From day 30 onwards, however, average weight gain significantly increased compared to the control ($p < 0.05$), with the highest gain recorded in treatment T4 (Fig. 2b).

The enhanced growth in T3 and T4 suggests the betterment of the fish, leading to improved feed conversion, nutrient absorption and somatic growth. From an aquaculture point of view, identifying and maintaining better conditions like T3 and T4 is crucial for maximizing fish production.

Growth efficiency, feed utilization, and ANOVA analysis of *O. pabda* reared with control and the other three treatments are displayed in Table 3. No significant variations were found in the initial total length and weight. Fish supplemented with CNP & VE had higher ($p < 0.05$) total length gain (TLG) than other groups. Body weight gain (BWG) in T2 and T4 was higher than control but similar to T3. The lowest FCR was revealed in the T4. The FCR has also lowered the other diets supplemented with either CNP or VE ($p < 0.05$). However, feed conversion efficiency (FCE) and specific growth rate (SGR)

Table 2 Physico-chemical status of culture environment during the study period

Parameters*	Diet groups			
	T1	T2	T3	T4
Temperature (°C)	26.01 ± 0.12^{a}	26.10 ± 0.14^{a}	26.03 ± 0.16^{a}	26.08 ± 0.12^{a}
pH	7.35 ± 0.17^{b}	7.54 ± 0.17^{ab}	7.69 ± 0.13^{a}	7.58 ± 0.24^{ab}
DO (mg L^{-1})	5.70 ± 0.27^{a}	5.60 ± 0.40^{a}	5.29 ± 0.28^{a}	5.60 ± 0.37^{a}
NH_3-N (mg L^{-1})	0.27 ± 0.04^{ab}	0.25 ± 0.02^{ab}	0.23 ± 0.02^{b}	0.28 ± 0.03^{a}
TDS (mg L^{-1})	126.50 ± 3.54^{a}	127.63 ± 3.80^{a}	125.75 ± 3.53^{a}	127.38 ± 5.74^{a}
NO_3 (mg L^{-1})	22.5 ± 2.35^{a}	22.375 ± 5.24^{a}	21.375 ± 4.18^{a}	25.5 ± 5.24^{a}

*DO: dissolved oxygen, NH_3-N: ammoniacal nitrogen, TDS: total dissolved solids, and NO_3: Nitrate. Different superscript letters in the same row show significant differences ($p < 0.05$)

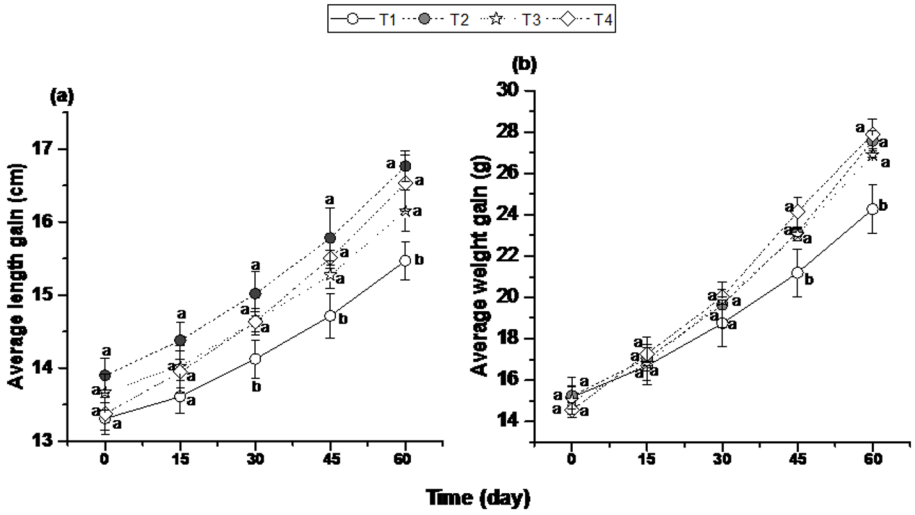

Fig. 2 Variations in the average length (**a**) and body (**b**) gain of *O. pabda* over 60 days of four distinct diets. The data is displayed as mean ± SE ($n = 20$). Groups denoted with the same letters are insignificant (Tukey's multiple comparison test, $p > 0.05$)

Table 3 Effects of dietary CNP, VE, and CNP-VE nanocomposite on growth and feed utilization of *O. pabda* reared for 60 days

Parameters	Diet groups				Analysis of variance			
	T1	T2	T3	T4	Adj SS	Adj MS	F	*P*
L_1 (cm)	13.31 ± 0.23^{c}	13.90 ± 0.24^{a}	13.67 ± 0.25^{b}	13.38 ± 0.23^{c}	4.49	1.50	26.21	0.000
L_2 (cm)	15.47 ± 0.37^{c}	16.77 ± 0.53^{a}	16.16 ± 0.50^{b}	16.54 ± 0.66^{ab}	19.25	6.42	23.49	0.000
W_1 (g)	15.17 ± 0.57^{ab}	15.23 ± 0.91^{a}	15.00 ± 0.68^{ab}	14.55 ± 0.81^{b}	5.722	1.91	3.38	0.023
W_2 (g)	24.28 ± 2.53^{b}	27.57 ± 5.50^{a}	26.90 ± 4.05^{a}	27.90 ± 4.72^{a}	162.4	54.14	2.83	0.044
TLG (cm)	2.16 ± 0.18^{d}	2.87 ± 0.30^{b}	2.49 ± 0.28^{c}	3.16 ± 0.43^{d}	11.45	3.82	40.13	0.000
BWG (g)	9.11 ± 2.04^{b}	12.35 ± 4.74^{a}	11.905 ± 3.13^{ab}	13.36 ± 4.08^{a}	198.1	66.02	4.98	0.003
FCR	1.75 ± 0.004^{a}	1.48 ± 0.003^{c}	1.54 ± 0.08^{b}	1.23 ± 0.003^{d}	2.77	0.92	622.63	0.000
FCE	0.57 ± 0.001^{d}	0.68 ± 0.001^{b}	0.65 ± 0.03^{c}	0.81 ± 0.002^{a}	0.61	0.20	706.78	0.000
SGR (% day^{-1})	0.55 ± 0.10^{c}	0.96 ± 0.24^{a}	0.71 ± 0.12^{b}	1.07 ± 0.19^{a}	3.31	1.10	38.02	0.000
RGR (%)	59.68 ± 11.53^{b}	79.60 ± 25.84^{a}	64.91 ± 17.30^{a}	90.72 ± 22.34^{a}	9957	3319.0	8.30	0.000
DGR (% day^{-1})	91.10 ± 20.40^{b}	123.45 ± 47.40^{a}	98.55 ± 31.28^{ab}	133.55 ± 40.77^{a}	19,807	6602	4.98	0.003
Survival (%)	90.00 ± 0.00	95.00 ± 0.00	90.00 ± 0.00	100.00 ± 0.00	1375.00	458.333	-	-

Values are mean ± SE ($n = 20$). Different superscript letters in the same row show significant differences ($p < 0.05$)

were higher in all the treatments compared to the control and T4 possessed the highest FCE and SGR than other treatments. The value of RGRs also increased substantially among the treatments with supplementations than without supplementation (T1). Similarly, the highest DGR was also found in T4 ($p < 0.05$) but other treatments did not demonstrate any notable variations ($p > 0.05$). The survival rate of fish in all the treatments was > 90% but in T4, it was 100%. The study reveals that the CNP-VE supplemented diet (T4) significantly improved both growth (highest BWG, SGR, RGR) and feed efficiency (lowest FCR, highest FCE), while also ensuring 100% survival. Thus, T4 offers the best balance of growth performance and cost-effective feed utilization, making it most suitable for aquaculture productivity enhancement.

3.3 Haematological parameters

Haematological parameters varied significantly with CNP and/or VE supplementation, particularly in the T4 group. Red blood cell (RBC) counts increased when fish were supplemented, and the highest was noted in T4 ($p < 0.05$). Hemoglobin (Hb) levels were elevated in all treatment groups compared to T1 and reached the highest in T4. Similarly, hematocrit (HCT) values were significantly higher in T3 and T4, while no significant differences were observed between T1 and T2. The mean corpuscular volume (MCV) in T4 was significantly different from the control (T1) and the groups receiving individual additives (T2: CNP; T3: VE). In case of mean corpuscular hemoglobin (MCH), the highest value was noted in T2 (17.80 ± 0.85) while the lowest was in T4 (11.02 ± 0.16). However, no remarkable difference was revealed among the treatments T1, T3, and T4 ($p > 0.05$). For MCHC, no significant difference was observed between T2 and T4, or between T1 and T3. However, the higher values were observed in CNP and CNPVE composite groups. An increasing trend was observed in white blood cell (WBC) count in fish fed with supplements compared to those without supplementation (control T1). The highest and lowest WBC counts were recorded in T2 ($5.73 \pm 0.49 \times 10^9$ L) and T1 ($3.73 \pm 0.45 \times 10^9$ L), respectively. However, platelet counts remained unchanged across all treatments (Table 4). The lack of significant change in platelets can be interpreted as a positive outcome, indicating that the treatments did not compromise circulatory or immune health.

3.4 Levels of different biochemical parameters

Serum biochemical composition of fish reflects the internal physiological and metabolic state of the organism. Growth performance in fish is an outcome of efficient nutrient utilization, energy allocation, and overall health, all of which are closely linked to the fish's biochemical status. In the current study, serum glucose levels gradually decreased in fish fed diets supplemented with CNP and/or VE. The highest glucose level was observed in the control group (T1), followed by T2 (CNP), T3 (VE), and the lowest in T4 (CNP + VE) (Table 5). No significant differences ($p > 0.05$) were observed in total protein and albumin/globulin (A/G) ratio among the different treatment groups of *O. pabda*. Although higher albumin levels were recorded in fish fed with CNP (T2) or the combination diet (T4), these values did not differ significantly from the control (T1).

Table 4 Dietary CNP, VE, and their combination's effects on hematological indices of *O. pabda* reared for 60 days

Parameters	Treatments			
	T1	T2	T3	T4
RBC (x 10^{12} L)	5.93 ± 0.25^{c}	6.53 ± 0.74^{bc}	7.87 ± 0.33^{b}	11.33 ± 0.61^{a}
Hb (g dL^{-1})	7.23 ± 0.97^{b}	11.57 ± 0.78^{a}	10.33 ± 0.87^{a}	12.50 ± 0.83^{a}
HCT (%)	31.43 ± 3.48^{b}	39.07 ± 1.60^{ab}	41.47 ± 3.04^{a}	42.93 ± 1.54^{a}
MCV (fL)	52.82 ± 3.71^{a}	60.31 ± 4.61^{a}	52.66 ± 2.33^{a}	37.92 ± 0.76^{b}
MCH (pg)	12.14 ± 1.16^{b}	17.80 ± 0.85^{a}	13.11 ± 0.55^{b}	11.02 ± 0.16^{b}
MCHC (g dL^{-1})	22.95 ± 0.67^{b}	29.58 ± 1.02^{a}	24.93 ± 1.11^{b}	29.08 ± 0.88^{a}
WBC (x 10^{9} L)	3.73 ± 0.45^{b}	5.73 ± 0.49^{a}	4.86 ± 0.33^{ab}	4.57 ± 0.73^{ab}
Platelet (x 10^{9} L)	204.67 ± 34.93^{a}	188.33 ± 18.41^{a}	238.33 ± 67.37^{a}	250.33 ± 42.74^{a}

Values are mean ± SE ($n = 20$). Different superscript letters in the same row show significant differences ($p < 0.05$)

Table 5 Effects of dietary supplementations on different biochemical parameters of *O. pabda*

Parameter	Diet groups			
	T1	T2	T3	T4
Glucose (mmol dL^{-1})	7.20 ± 0.71^{a}	5.70 ± 0.51^{ab}	5.17 ± 0.33^{b}	3.43 ± 0.31^{c}
Total Protein (g L^{-1})	61.67 ± 2.49^{a}	62.00 ± 6.53^{a}	68.00 ± 4.90^{a}	66.67 ± 4.99^{a}
Albumin (g dL^{-1})	3.13 ± 0.82^{ab}	3.93 ± 0.57^{a}	1.34 ± 1.02^{b}	3.97 ± 0.59^{a}
Globulin (g dL^{-1})	2.23 ± 0.59^{ab}	2.96 ± 0.50^{ab}	1.54 ± 0.33^{b}	3.66 ± 0.45^{a}
A/G ratio	1.41 ± 0.03^{a}	1.34 ± 0.16^{a}	1.25 ± 0.08^{a}	1.09 ± 0.09^{a}

Table 6 Effects of dietary CNP, VE, and CNP-VE nanocomposite on liver function responses of *O. pabda* reared for 60 days

Parameter	Treatments			
	T1	T2	T3	T4
Bilirubin (mg dL^{-1})	0.31 ± 0.06^{bc}	0.45 ± 0.04^{a}	0.44 ± 0.04^{ab}	0.20 ± 0.01^{c}
ALT (SGPT) (U L^{-1})	10.43 ± 2.01^{a}	7.97 ± 0.34^{a}	7.87 ± 0.33^{a}	7.60 ± 0.41^{a}
AST (SGOT) (U L^{-1})	15.27 ± 0.71^{a}	14.13 ± 0.46^{a}	15.30 ± 0.67^{a}	14.53 ± 0.49^{a}
AP (IU L^{-1})	86.00 ± 4.08^{a}	79.00 ± 6.38^{a}	82.01 ± 3.28^{a}	90.00 ± 2.94^{a}
Cholesterol (mg dL^{-1})	157.00 ± 5.89^{a}	134.33 ± 7.04^{b}	130.33 ± 4.11^{b}	125.00 ± 3.27^{b}
Triglyceride (mg dL^{-1})	130.33 ± 4.11^{a}	130.67 ± 10.34^{a}	143.33 ± 8.50^{a}	136.67 ± 5.73^{a}

Values are mean ± SE ($n = 20$). Different superscript letters in the same row show significant differences ($p < 0.05$)

3.5 Effects on liver function test

The outcome of liver function parameters of *O. pabda* supplemented with CNP and VE are presented in Table 6. The bilirubin level was found to be higher in T2 and T3 (0.45 ± 0.04 and 0.44 ± 0.04 mg dL^{-1}, respectively) while the lowest was found in T4 (0.20 ± 0.01 mg dL^{-1}, $p < 0.05$). Dietary CNP (T2), VE (T3), and their combination (T4) had no significant effects ($p > 0.05$) on ALT, AST, alkaline phosphatase, and triglyceride. The ALT level was in a decreasing trend when the fish were fed supplemented diets with either CNP and/or VE, and the lowest was observed in T4 (7.60 ± 0.41 mg dL^{-1}). The highest and lowest AST levels were revealed in T1 and T1 (15.27 ± 0.71 and 14.13 ± 0.46 U L^{-1}) respectively. Cholesterol levels decreased substantially ($p < 0.05$) when the control diet was mixed with additives either individually or in combination. Nevertheless, cholesterol levels did not fluctuate remarkably among the supplements ($p > 0.05$).

3.6 Effects on CNP and VE on Immunoglobulin M

IgM plays a key role in the early immune response, so changes in its level can reflect how well the fish's immune system is functioning. In the present study, the IgM levels increased linearly, meaning there was a steady increase in IgM as the supplementation improved, from no supplement (T1) to the combined group (T4) and this trend was statistically significant ($p < 0.05$, Fig. 3). The highest IgM level was found in T4, the group that received both CNP and VE. This suggests a possible synergistic effect, where combining CNP and VE enhanced the immune response more than either supplement alone. The lowest IgM level was in the control group (T1), which is expected since this group received no immune-boosting additives. The fish in T2 (CNP) and T3 (VE) improved IgM levels compared to the control group (T1), showing that each supplement individually had a positive effect on immune function. However, there was no statistically significant difference between T2 and T3 ($p > 0.05$), meaning the individual effects of CNP and

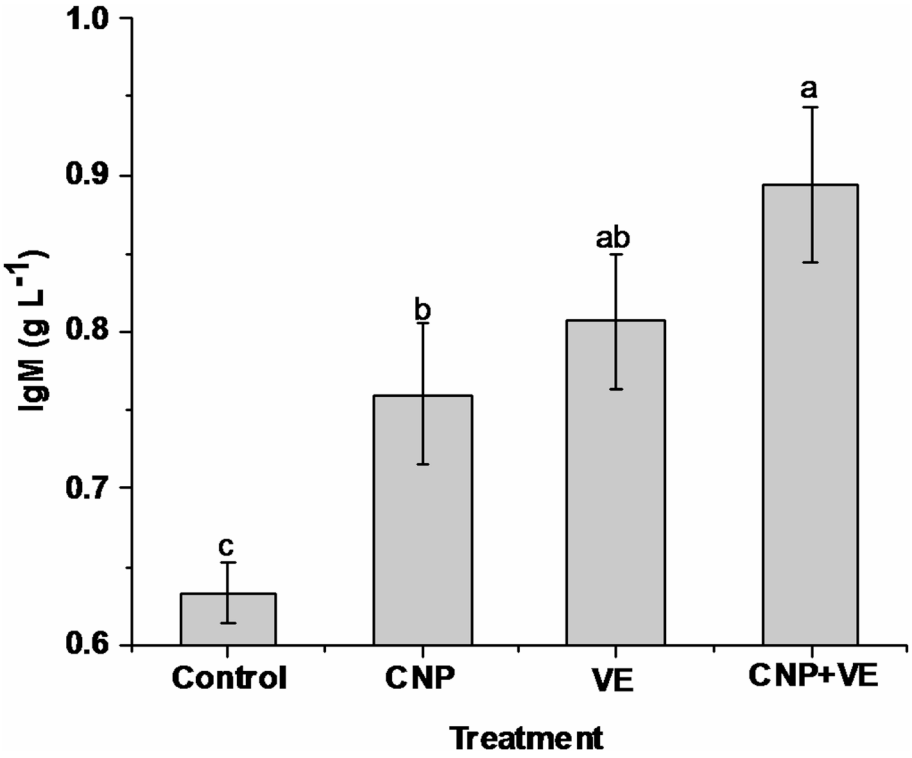

Fig. 3 Effects of CNP, VE and their combination on the IgM concentration of *O. pabda*. The data shown is mean ± SE ($n = 20$). Significant differences exist between groups with different letters (Tukey's multiple comparison test, $p < 0.05$)

VE were similar in magnitude and not enough to be considered distinct from each other in this context (Fig. 3).

3.7 Gut histology

The pathological changes in the intestinal tissues of fish subjected to CNP and/or VE supplementation (T1, T2, T3, and T4) were examined through histological analysis (Fig. 4). In T1, the intestinal villi are relatively short with a narrow lumen indicated by the presence of black star, which indicates the absorptive surface area appears limited and nutrient assimilation efficiency is moderate. When the fish was supplemented with CNP (T2), noticeable improvements in villus height (V.E) and intestinal wall width (I.W.W.). CNP likely enhanced mucosal development and surface area, supporting better absorption. In addition, VE supplemented group (T3), showed longer lumen diameter (L.D.) compared to T1, suggesting improved nutrient absorption potential due to VE's antioxidant and membrane protective effects. In T4, the gut tissues exhibited the most pronounced changes, revealed elongated, well organized villi with significantly larger intestinal diameter (I.D.). This reflects optimal absorptive capacity and healthier mucosal structure, supporting efficient digestion and nutrient uptake (Fig. 4).

4 Discussion

The physico-chemical characteristics of water in aquaculture systems are crucial for the effective fish cultivation [48]. Changes in water quality have a major impact on fish behavior, which is a key indicator of fish welfare and growth [49, 50]. In the present study, the hydrobiological metrics in different diet groups showed that the experimental

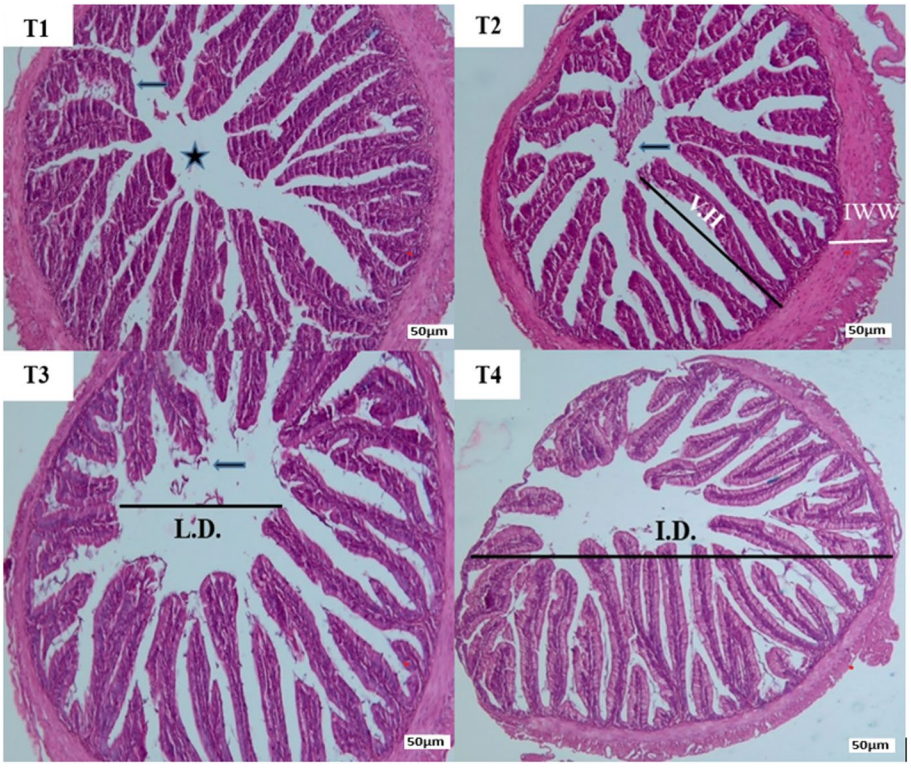

Fig. 4 Comparative microphotographs showcasing the variations in gut histological structures of *Ompok pabda* subjected to diet supplements (T1 = Control; T2 = Chitosan nanoparticles; T3 = VE; & T4 = Chitosan nanoparticles + VE). I.D. = intestine diameter, L.D. =lumen diameter, I.W.W. = intestinal wall width, V.H. = villi height. Scale bar: 50 µm; Stain: H & E

tanks maintained within the permissible bounds over the study period [48, 51]. No notable difference across the different treatments indicates that the food interventions did not affect the water quality measures in the tanks.

The growth and feed utilization indices were improved with dietary supplementation of CNP/VE either individually or combined, in contrast to the group that did not receive supplements. The best dietary performance was when fish were administered the CNP and VE combination. Since feed represents the largest operational cost in aquaculture, improving FCR while maintaining high BWG directly enhances economic returns and sustainability. In aquaculture, better BWG and lower FCR lead to faster fish growth, reduced feed costs, and increased productivity. The lowest bilirubin levels observed in the combined supplemented group (T4) suggesting the supports the hypothesis of improved oxidative balance and hepatic protection [52]. These effects include the stimulation of digestive enzyme secretion, inhibition of pathogenic bacterial growth, and increased absorptive surface of the intestine, which increases nutrient digestibility and cell access to VE [53–57]. The improvement in fish growth might be the result from a higher growth rate and weight gain. Dietary VE supports protein synthesis while chitosan enhances the bioavailability of VE, further contributing to growth. In its natural form, VE shows low absorption and high dispersion at the molecular level due to its nanoscale size [20]. According to Aranaz et al. [58], chitosan enhances VE's absorption while shielding it from the unfavorable circumstances in the gastrointestinal tract. Previous research [59] found that vitamin and chitosan supplementation improved the growth and survival of *Cyprinus carpio* haematopterus fish without having a negative

impact on their health. Similar outcomes were also seen by Abdel-Tawwab et al. [55], who noted that adding CNPs to *O. niloticus* bolstered both growth and health. By enhancing the functions of digestive enzymes like lipase and amylase, preventing the expansion of intestinal pathogenic bacteria populations, and enhancing a few innate immune markers, it also promoted growth and feed utilization [54]. According to Ibrahim et al. [60], feeding *O. niloticus* a chitosan-vitamin C nanocomposite enhanced fish development, antioxidant status, immunological response, illness resistance, and intestinal histomorphology. The synergistic effect of CNP and VE because CNP enhances the solubility, stability, and absorption of VE, while also contributing independent antioxidant and immunostimulant properties. This leads to improved bioavailability, oxidative stress reduction, immune modulation, and growth performance [27]. These effects are far stronger than when either supplement is used individually.

Hematological indicators, which might change depending on the type of stressor, are commonly employed to assess the health state of the index fish [61]. The present investigation found that the experimental fish's RBCs, Hb, HCT, MCV, MCH, MCHC, and WBC were significantly affected by the addition of CNP or/and VE supplements to their meals. The capacity of CNP to improve the absorption of macro and micronutrients, which predict good health, may be the reason why fish fed on CNP-VE diet showed the best values [21, 54]. The reduction of MCH and MCHC in fish supplemented with CNP and VE might stimulate erythropoiesis and antioxidant protection, leading to a larger pool of RBCs with relatively less haemoglobin per cell [54, 62]. This is not necessarily a sign of deficiency but an adaptive physiological response, reflecting improved haematological efficiency and better overall health in aquaculture systems [63]. CNP and VE possess strong antioxidant activity, which protects haemoglobin from oxidative damage [64]. As the cellular component of innate immunity and the release of humoral chemicals such lectins, cytokines, complement system components, and cationic antimicrobial peptides, WBCs are essential to fish immunological function [65]. The immunostimulatory effect and antibacterial capabilities of CNP may have contributed to the study's finding that WBC counts increased significantly in the CNP-VE combo group [54]. Platelets play a critical role in fish health by mediating essential physiological processes, for example, hemostasis, wound healing, and immune response [66]. Their stability in count or function often reflects a balanced physiological state, suggesting that the diet improvement condition using nanoparticles and/or VE did not induce stress or severe enough pathology to disrupt platelet activity [67].

There is a close physiological link between blood biochemical parameters and growth in fish [53, 68]. While not always a direct linear correlation, a healthier biochemical status, for example, glucose, protein, lipid, and antioxidants supports improved growth by ensuring efficient nutrient utilization, energy allocation, and reduced disease stress [52]. In the current investigation, dietary conditions directly affect blood glucose. The blood glucose level of the pabda in T4 group was lower than that of the other supplemented diet groups. While CNP and VE supplementation had a considerable influence on glucose levels in the current investigation. Consistent with our findings, Acar et al. [62] observed that feeding Mozambique tilapia essential oil decreased its serum glucose level. Prior research has demonstrated that the glucose level decreases in farm conditions when fish under less stress [53, 62]. The decrease in glucose in the fish fed CNPVE supplementation in T4 might be due to the antioxidant effect that minimize oxidative

stress [69]. Plying enzymes, stimulating macrophages, and releasing antimicrobial peptides, total protein is essential to fish non-specific immune responses [70]. In our investigation, the CNP-VE supplemented groups showed a small rise in serum total protein concentration. Nevertheless, employing dietary CNP resulted in a considerable increase in the serum TP level of Nile tilapia [54] and silver carp (*Hypophthalmichthys molitrix*) [71]. Literature on different fish species indicates that dietary CNP and VE can both enhance protein metabolism [53]. Elevated globulin levels in diet supplementation indicate enhanced humoral immune response. CNP act as immunostimulants, binding to immune cells and stimulating antibody production [62] while VE enhances B-cell function and antibody synthesis [72]. Hence, fish in T4 had stronger immune protection, contributing to higher disease resistance.

Like other vertebrates, fish's liver plays a crucial role in numerous metabolic processes, including the synthesis of defense components, detoxification, lipid metabolism, and food metabolism [73]. The liver is also a major site for the production of complement proteins, which play critical roles in pathogen lysis and modulation of antibody-mediated immunity [74]. Beyond systemic immunity, nutrient absorption and microbial metabolites from the intestine reach the liver via the portal circulation, directly influencing hepatic metabolism and immune signalling [75]. Bilirubin is a breakdown product of haemoglobin catabolism, formed during the degradation of heme in RBCs. Elevated bilirubin levels are often associated with liver dysfunction, hemolysis or oxidative damage, where hepatocytes fail to process bilirubin efficiently [76]. Hepatotoxicity can be measured specifically using ALT and AST. In this investigation, the addition of CNP resulted in a linear decrease in the serum activities of ALT and AST when compared to the control. Similarly, Mehrpak et al. [77] observed that *Cyprinus carpio* fish fed on vitamin C in addition to chitosan had changed AST and ALT activity. Chitosan's antioxidant activity, which protects hepatocytes from oxidative damage, may be responsible for this benefit [78]. Since the liver synthesizes the majority of serum proteins, the improved hepatic functioning seen in this study may account for the elevated serum protein level [79]. Cell membranes, steroid hormones, myelin sheaths, bile acids, and some fat-soluble vitamins are all significantly influenced by cholesterol [80]. In the current investigation, the groups receiving CNP/VE had significantly lower serum cholesterol levels, with the control group obtaining the highest values. Notably, the outcomes show that *O. pabda* fed CNP-VE increased AP, and decreased cholesterol and triglyceride values. In the current study, reduced cholesterol in T4 could be interpreted as a metabolic adaptation toward better nutrient utilization and improved health status, which ultimately supports enhanced growth and disease resistance [81]. Parallel to this, Abd El-Naby et al. [82] and Younus et al. [71] revealed that biochemical values of Nile tilapia and silver carp that were fed chitosan nanoparticles increased. They attributed this influence to chitosan's potential role in enhancing antibacterial, immune, and antioxidant conditions.

IgM is the primary immunoglobulin found in fish, and serum immunoglobulins are important parts of the humoral immune system [83]. It is the predominant antibody in fish's circulation and reflects the strength of adaptive humoral immunity [84]. Because of its measurable changes after infection, stress, or immunostimulant supplementation, IgM is widely applied as a biomarker for fish health and disease resistance in experimental and farmed conditions. Higher IgM levels in fish blood generally indicate enhanced immune status and improved disease resistance [85]. Niu et al. [86] observed that turbot (*Scophthalmus maximus*)

produced more antibodies when exposed to VE. In northern whiting, *Sillago sihama*, the addition of VE was likewise observed to significantly boost the IgM content [87]. Furthermore, VE was effective in reducing mortality and enhancing specific immunity only in immunocompromised fish, but showed no significant effect in healthy fish [88]. The results of the current study showed that VE's activities were boosted when it was encapsulated in CNPs, in addition to the chitosan's beneficial effects. Fish immune systems were stimulated by CNP-VE in a synergistic manner. The result may be attributed to the immune stimulating properties of CNPs, which likely activated the non-specific immune response in fish [20, 89]. This effect was further enhanced by VE supplementation [90]. The observed increase in IgM levels could be a positive correlation of disease resistance, particularly under stressful or pathogen-challenged conditions [91]. Harikrishnan et al. [89] observed that increased IgM was associated with improved survival rates in Nile tilapia challenged with *Aeromonas hydrophila* following dietary immunostimulant supplementation while Raida and Buchmann [92] observed IgM titers exhibited stronger protection against *Yersinia ruckeri* infection, underscoring IgM's protective role in bacterial defense. Although the elevation of IgM in supplemented groups likely correlates with improved disease resistance, definitive confirmation would require pathogen-challenge trials and functional assays.

Measurement of intestinal histomorphometrical features is an essential tool for assessing how dietary supplements affect the fish body's overall immune system, adjacent intestinal immunity, and intestinal absorption capacity [33, 93]. The gut histology images show a clear progression from poorly developed villi in control (T1) to highly organized, elongated villi with expanded lumen in T4 (CNP-VE). These structural improvements enhance nutrient assimilation and gut health, which aligns with biochemical findings of reduced glucose (efficient energy utilization) and reduced bilirubin that might improve antioxidant status and hepatoprotection [76, 94]. This might be the cause of exhibiting better growth and physiological homeostasis in T4 treatment [69]. In agreement, Dawood et al. [33] found that goblet cells increased in *Liza ramada*. Abd El-Naby et al. [82] reported that Nile tilapia fed CNPs showed improved intestinal villi and histological features. The CNPs' antibacterial action reduced intestinal pathogenicity and inflammation by improving the diversity and integrity of intestinal epithelial cells, which was made possible by dietary CNP. The outcomes are consistent with the findings of [95], who reported that chitosan enhanced the immunity and intestinal integrity of Pacific white shrimp (*Litopenaeus vannamei*) locally. The combined effect of CNP and VE might stimulate intestinal enzyme activity facilitating efficient nutrient utilization and promoting growth [96]. These mechanisms might improve gut structure and functionality under CNP and VE supplementation, suggesting a close link between antioxidant protection, enhanced gut permeability, and enzymatic activity in supporting fish health and performance [97, 98].

5 Conclusion

The present study demonstrates that dietary supplementation with CNP, VE, and their combination significantly enhances growth performance, feed utilization, hematobiochemical parameters, immune responses, and intestinal morphology in *O. pabda*. The synergistic effects observed in the combined supplementation group suggest a promising strategy for improving overall fish health and productivity in aquaculture. These findings highlight the potential of chitosan nanoparticles and α-tocopherol as functional feed additives to promote sustainable and efficient fish farming practices. Further studies

are needed to optimize the doses of CNP and VE for different fish species, and also, longer feeding periods could reveal a true synergistic interaction between CNP and VE on fish immunity and disease resistance.

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Author contributions
S.K.M., M.L.R. and S.K.D. conceived and designed the experiments. M.S.M., S.K.D., M.E.A., M.T.H. and D.P. performed the statistical analysis and prepared the manuscript, the tables and the figures. M.S.M., T.R. and M.N.I. conducted the experiment. M.N.I., T.R., S.K.M., M.T.H. and M.L.R. collected the samples. All authors have read and agreed to the published version of the manuscript.

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Data availability
Data are contained within the article.

Declarations

Consent to participate
Not applicable.

Consent publication
Not applicable.

Competing interests
The authors declare no competing interests.

Ethical approval
All fish experiments in the present study were approved by the Research Management Wing of Gazipur Agricultural University, Bangladesh and was carried out following the Basel Declaration (Approval Code: GAU/RMW/2022/134(KA)6068, Approval date: 22 April 2025).

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