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Enhancement of innate immune responses in Nile tilapia (*Oreochromis niloticus)* by dietary green-synthesized nanoparticles

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Abstract

This study evaluated the impact of dietary green-synthesized nanoparticles on innate immune responses in Nile tilapia (*Oreochromis niloticus*). Plant-based Zinc, selenium, copper, and manganese nanoparticles were incorporated into diets at 30 mg/kg and 60 mg/kg concentrations and administered over a 28-day feeding trial. The expression of some selected important immune genes, including IL-1β, IL-10, TLR3, IRF3, viperin, and TGF-β, was assessed in gill and liver tissues using quantitative real-time PCR. Fish fed nanoparticle supplemented diets exhibited significant upregulation of pro-inflammatory (IL-1β), anti-inflammatory (IL-10, TGF-β), and antiviral (TLR3, IRF3, viperin) genes compared to the control group ($p < 0.05$). Gene expression exhibited dose and time dependent patterns showing significant upregulation as early as day 7 and sustained until days 14, 21, and 28, particularly at the higher supplementation dose (60 mg/kg). These results suggest that green-synthesized nanoparticles can modulate innate immunity by enhancing both inflammatory and regulatory pathways, promoting immune preparedness without apparent adverse effects. The findings support the potential application of plant-based nanoparticles as eco-friendly immunostimulants in aquaculture, offering a promising alternative to antibiotic use.

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Introduction

Worldwide, aquaculture has become one of the fastest-growing food production sectors, addressing the growing demand for sustainable protein. In Bangladesh, the fisheries sector plays a critical socio-economic role, contributing 3.57% to GDP and supporting over 17 million people through employment and livelihoods[1]. Nile tilapia (*Oreochromis niloticus*), known for its rapid growth, environmental tolerance, and consumer acceptance, has emerged as a dominant species in freshwater aquaculture systems[2]. However, intensification of tilapia farming has increased the vulnerability of fish to bacterial and viral diseases, which are now one of the major constraints to productivity. Traditional reliance on antibiotics for disease management has led to growing concerns about antimicrobial resistance (AMR), environmental contamination, and food safety[3,4]. Thus, there is an urgent need to explore alternative, eco-friendly strategies to promote fish health and enhance innate immune defenses.

Nanotechnology offers promising applications in aquaculture, particularly in improving feed efficiency, enhancing immune responses, and reducing pathogen loads. Nanoparticles, less then 100 nm in diameter, possess unique physicochemical properties including increased surface reactivity and enhanced cellular interaction[5]. These features allow them to function as efficient carriers of nutrients and immunostimulants[6,7]. Among these, green-synthesized nanoparticles produced from biological materials like plant extracts, stand out due to their biocompatibility, reduced toxicity, and environmental sustainability[8,9]. Trace elements such as zinc (Zn), selenium (Se), copper (Cu), and manganese (Mn) are essential micronutrients known to support growth, antioxidant defense, and immune modulation in fish[10,11].

Like other vertebrates, in fish, the innate immune system is the primary defense mechanism, comprising physical barriers and immune mediators like cytokines and toll-like receptors. Nanoparticles have demonstrated the ability to modulate the expression of key immune genes, thereby enhancing resistance to pathogens. Interleukin-1β (IL-1β) acts as a pro-inflammatory cytokine, initiating immune signaling and leukocyte activation[12]. Interleukin-10 (IL-10) is an anti-inflammatory cytokine that regulates excessive immune responses[13]. IRF3 and viperin are crucial in antiviral defense, promoting interferon production and viral clearance[14,15]. Transforming growth factor-beta (TGF-β) contributes to immune regulation and tissue repair[16]. Toll-like receptor 3 (TLR3) detects double-stranded RNA of viral origin, activating downstream immune responses[17].

Despite these promising findings, most existing studies utilized chemically synthesized nanoparticles, and limited research has been conducted using plant-based, green-synthesized nanoparticles in tilapia, a major gap in aquaculture immunological research. Therefore, the current study aimed to investigate the immune response of Nile tilapia upon feeding diet supplemented with green-synthesized nanoparticles (Zn, Se, Cu, Mn).

Materials and Methods

Experimental animals and conditions: Nile tilapia (*Oreochromis niloticus*) with an average initial body weight of 12.99 ± 2.78 g were obtained from a hatchery in Noakhali, Bangladesh. The experiment was conducted at Bangladesh Council for Scientific and Industrial Research, Dhaka, Bangladesh. Before starting the experiment, fish were conditioned for one week. The experimental design included three dietary treatment groups: a control group (no nanoparticle supplementation), a low-dose group (30 mg/kg feed), and a high-dose group (60 mg/kg feed). The doses and combinations (Table 1) were selected based on the study conducted Khan *et al*.[18]. A total of 370 fishes were allocated in the treatment units. Each treatment was conducted in triplicate in cemented tanks with a volume of 4.5325 m^3 and 30 fish per replicate were allocated. Fish were given control and nanoparticle supplemented diet at 8% of body weight over a period of 28 days. The diet was divided into two ration and provided twice at 09:00 am and 04:00 pm.

Green nanoparticle synthesis: The synthesis of CuO, ZnO, MnO_2 and selenium (Se) nanoparticles was carried out using eco-friendly techniques. CuO NPs were prepared from 0.05M $CuSO_4 \cdot 5H_2O$ and 0.5% chitosan solution. ZnO NPs were synthesized using soluble starch and $Zn(NO_3)_2$. Selenium nanoparticles (SeNPs) were synthesized via a green synthesis approach using *Emblica officinalis* fruit extract and 10 mM sodium selenite (Na_2SeO_3) while MnO_2 was synthesized from pineapple peels and $KMnO_4$.

Experimental diets: A commercial starter feed was powdered and divided into three portions. One portion served as the control diet, while the others were supplemented with nanoparticles at 30 mg/kg (Dose 1) and 60 mg/kg (Dose 2) (Table 1). The ingredients were mixed with purified water, re-pelletized, and dried at 60°C. Formulated diets were stored at room temperature in well ventilation condition.

Table 1. Doses of nanoparticles used in the present study

NPs	% in feed	Dose 1(mg/kg)	Dose 2(mg/kg)
Zn	30	9	18
Se	25	7.5	15
Cu	9	2.7	5.4
Mn	36	10.8	21.6
	Total	30	60

Sampling for immune gene expression: Fish from each treatment were sacrificed at 7, 14, 21, and 28 days post-feeding. Tissue samples (gill, liver, and muscle) were collected aseptically, immediately immersed in RNA later, and stored at –20°C until RNA extraction. Three fish per treatment (one from each replicate) per time point were sampled. Before sampling fish were anesthetized using 2-phenoxyethanol at 600 μL/L according to Sajan *et al*.[19].

Extraction of total RNA and synthesis of complementary DNA (cDNA): TRIzol reagent was used for total RNA extraction. The extraction was performed following the manufacturer instructions. Briefly, tissue homogenization was done using a mortar and pestle followed by chloroform phase separation and isopropanol precipitation were conducted to isolate RNA. RNA was washed with 75% ethanol and air-dried before being resuspended in nuclease-free water. RNA quantity and purity were measured using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific). Finally, complementary DNA (cDNA) was synthesized using Takara PrimeScript™ 1st Strand cDNA Synthesis Kit via a two-step RT protocol involving primer annealing and extension.

Quantitative Real-Time PCR (qPCR): Expression of immune-related genes including IL-1β, IL-10, IRF3, viperin, TGF-β, and TLR3was quantified using qPCR (Table 2). The β-actin gene served as the endogenous control. Each 20 μL qPCR reaction contained:10 μL HiGreen qPCR Master Mix, 0.5 μL of each forward and reverse primer (10 pM), 2 μL diluted (1:10) cDNA template and 7 μL nuclease free water. The temperature profile included an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15s, annealing at 56–60°C (varied among primers) for 30s, and extension at 72°C for 30s. A melt curve analysis was performed at 95°C for 10s, 55°C for 5s, and 95°C for 30s, with a final cooling step at 40°C for 30s to verify the generation of a single specific amplicon. For real-time PCR (qPCR) analysis, three samples from each treatment (one from each replicate) were used with 2 technical replicates of each sample. Thus, a total of 12 fish from each treatment was used for real-time PCR.

Table 2. List of target primers with amplicon size and accession number used in the study

Gene	Sequence (5′---3′)	Amplicon size (bp)	Accession number
IL-1 β	F-TGAGAGCCTACTTTAGGATTCTGC	150	XM_005457887.2
	R-GCGGCTATTACAACCAATGCT		
IRF3	F- GGTACGACACATCAGCGTGC	183	XM_005448320.3
	R- CTGGCAACATAGAGCAGCAGTA		
Viperin	F- ATCAACTTCTCTGGCGGA	161	XM_003453237.3
	R- AGATAGACACCATATTTCTGGAC		
TGF-β	F- GAGATCCCTGCCAACTTGCT	230	NM_001311325.1
	R- TCCCCGACGTTACTCCGTA		
TLR3	F-CTGTCCGTCACTCCGAAACA	108	XM_003449728.4
	R-CCGGGATTGATCTGCGCTAT		
IL-10	F- CTCAGATGGAGAGCAGAGGTC	134	KP645180.1
	R- CTTGATTTGGGTCAGCAGGT		

Data analysis: Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. Data were presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 9. One-way ANOVA followed by Tukey's multiple comparisons test was employed to evaluate significant differences in gene expression between treatments at $p < 0.05$.

Results and Discussion

Expression of different genes was evaluated at 7, 14, 21, and 28 days after the fish were given different dosages of a nanoparticle supplemented diet. The expression of key immune molecules, including three effector molecules IL-1β, IL-10, and viperin, the transforming growth factor TGF-β, the integrator protein IRF3, and the receptor protein TLR3, was examined using RT-qPCR.

Expression of IL-1β (pro-inflammatory cytokine): In the gill of tilapia, at day 7, the expression of IL-1β was significantly upregulated in both dose 1 ($p < 0.01$) and dose 2 ($p<0.05$) compared to the control (Fig. 1a). No significant difference was found between the two doses. However, at day 14, no significant difference was observed across experimental groups ($p > 0.05$; Fig. 1b) while at day 21, both doses resulted in significantly elevated IL-1β expression ($p < 0.05$; Fig. 1c), with similar expression between doses. At day 28, dose 1 and dose 2 showed significant upregulation ($p < 0.001$; Fig. 1d), with dose 1 showing a significantly higher response than dose 2 ($p < 0.01$). The lack of IL-1β upregulation on day 14 may be due to a temporary immune tolerance or regulatory phase following the initial activation observed on day 7. This transient suppression could result from homeostatic mechanisms that control inflammation to prevent tissue damage. IL-1β expression could be influenced by cytokine feedback loops or delayed activation of immune cells that do not peak at day 14 but rather reappear by day 21 or 28[20].

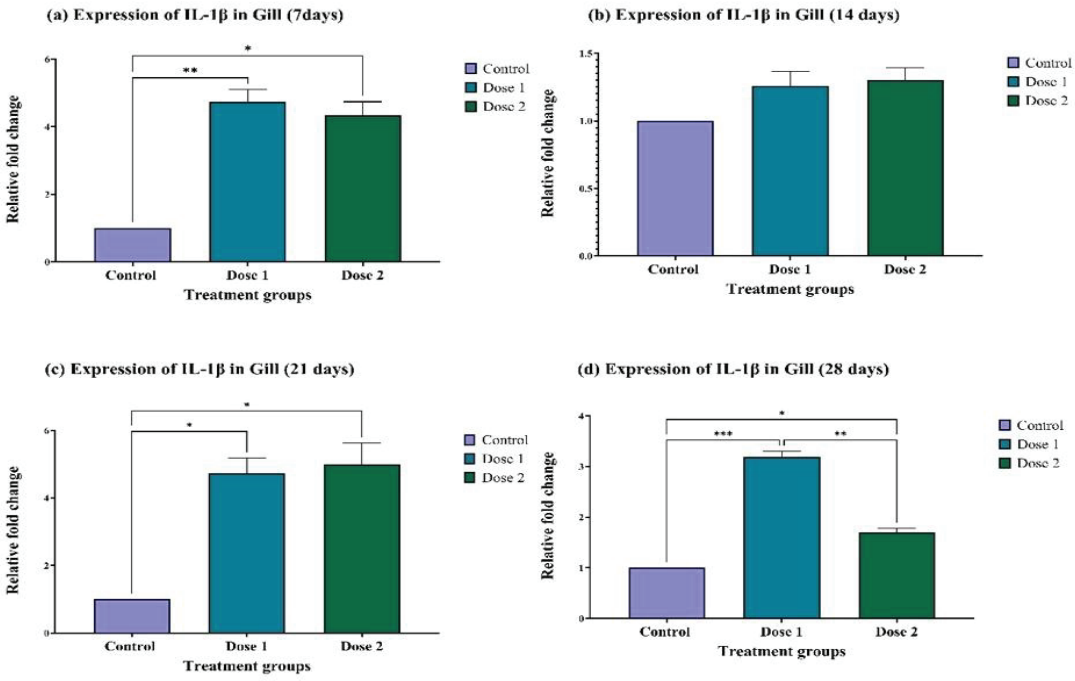

Fig. 1. IL-1β expression in the gill of Nile tilapia following green-synthesized nanoparticle supplementation for a period of 28 days. The expression of IL-1β in treatment groups was presented as fold change compared to control. Bar with different colors with asterisk (*) are significantly different at $p < 0.05$, $p < 0.01$, and $p < 0.001$which are represented as *, **, and ***, respectively.

In liver, IL-1β expression increased significantly at all time points (7, 14, 21, and 28 days) in both treated groups ($p < 0.05$; Fig. 2a–d), with no significant difference between doses at any time point.

The expression patterns of IL-1β are consistent with recent reports on selenium nanoparticles (SeNPs) in tilapia and other species. For example, In a study by Tawfik *et al.*[21] higher doses (15-60 mg/kg diet) of nanoZnO elevated the expression of IL-1β in tilapia. However, Husseiny *et al.*[22] demonstrated that Nile tilapia fed diets containing 1.0 mg/kg SeNPs exhibited significantly enhanced immunological indices relative to controls. Similarly, microalga-fabricated SeNPs (0.75–1.5 mg/kg) significantly increased pro-inflammatory IL-1β in tilapia intestines, without elevating TNF-α or TGF-β1, indicating an immune-priming effect without pathological inflammation[23]. In European seabass, dietary SeNPs (0.25–0.5 mg/kg) also upregulated IL-1β gene expression in muscle[24]. This suggests that the gill, as a frontline mucosal organ, mounted a localized pro-inflammatory response to the dietary nanoparticles. In contrast, hepatic IL-1β remained relatively controlled, indicating no systemic inflammatory overload at either 30 or 60 mg/kg. Importantly, the localized IL-1β induction in gills aligns with findings from other studies where nanoparticle

exposure stimulated innate immune genes without causing generalized inflammation. For instance, Ni *et al.*[25] observed that zebrafish exposed to biogenic silver nanoparticles had significant upregulation of IL-1β (as well as other cytokines) with higher nanoparticle concentrations and longer exposure times. Similarly, dietary selenium nanoparticles at 1 mg/kg fed to European seabass elevated IL-1β expression in the liver, indicating an immunostimulatory effect of nanoparticle supplementation on pro-inflammatory cytokines[24]. Our results are comparable in that the nanoparticle diet prompted IL-1β expression, particularly in gill tissue, but notably did not trigger uncontrolled inflammation in systemic organs. This organ specific containment of IL-1β is a favorable outcome, as excessive systemic IL-1β could be detrimental. In fact, Al-Wakeel *et al.*[26] reported that green algae-synthesized selenium nanoparticles fed to tilapia did not induce any significant increase in hepatic pro-inflammatory cytokine genes, consistent with an absence of overt inflammatory responses. The ability of our green-synthesized nanoparticles to provoke a mucosal immune alert (in gills) while avoiding liver inflammation mirrors these observations and highlights a balanced immune activation.

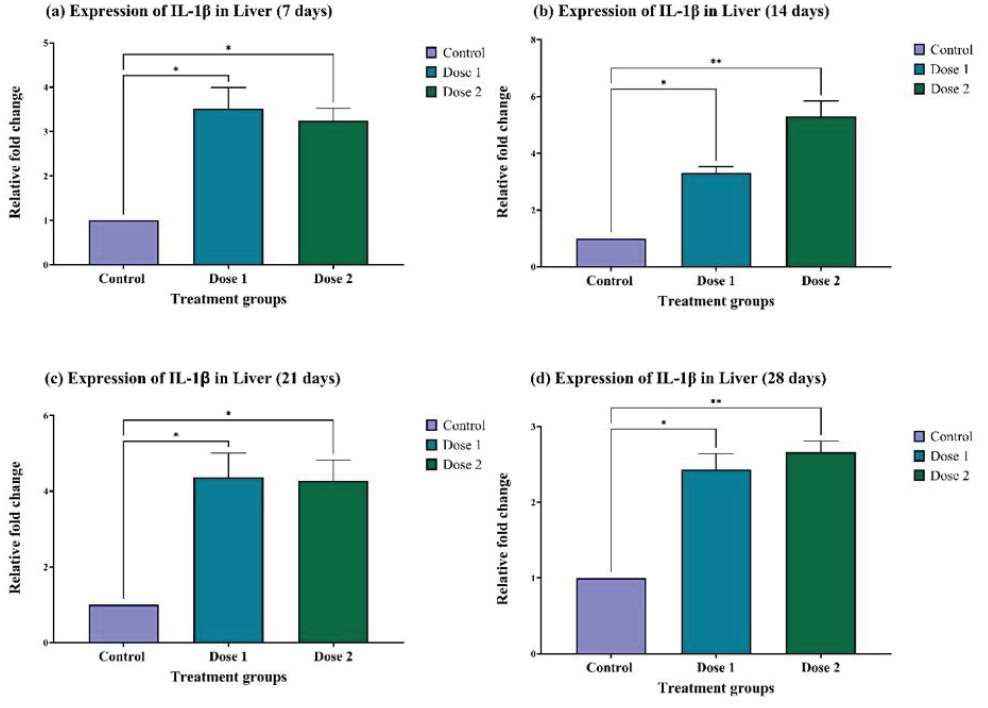

Fig. 2. IL-1β expression in the liver of Nile tilapia following green synthesized nanoparticle supplementation for a period of 28 days. The expression of IL-1β in treatment groups was presented as fold change compared to control. Bar with different colors with asterisk (*) are significantly different at $p < 0.05$, $p < 0.01$, and $p < 0.001$which are represented as *, **, and ***, respectively.

It is also noteworthy that in stress-related scenarios, nanoparticle intervention can modulate IL-1β dynamics: for example, a chitosan-vitamin C/E nanoparticle diet given to tilapia prior to osmotic stress was shown to hasten the decline of stress-elevated IL-1β in head-kidney, helping fish recover faster from the inflammatory surge[27].

Expression of IL-10 and TGF-β (anti-inflammatory cytokines): IL-10 expression in the gill was significantly upregulated at all time points in both treatment groups compared to the control ($p < 0.05$; Fig. 3a–d) in the gill of tilapia. However, no significant difference was observed between dose 1 and dose 2 at any time.

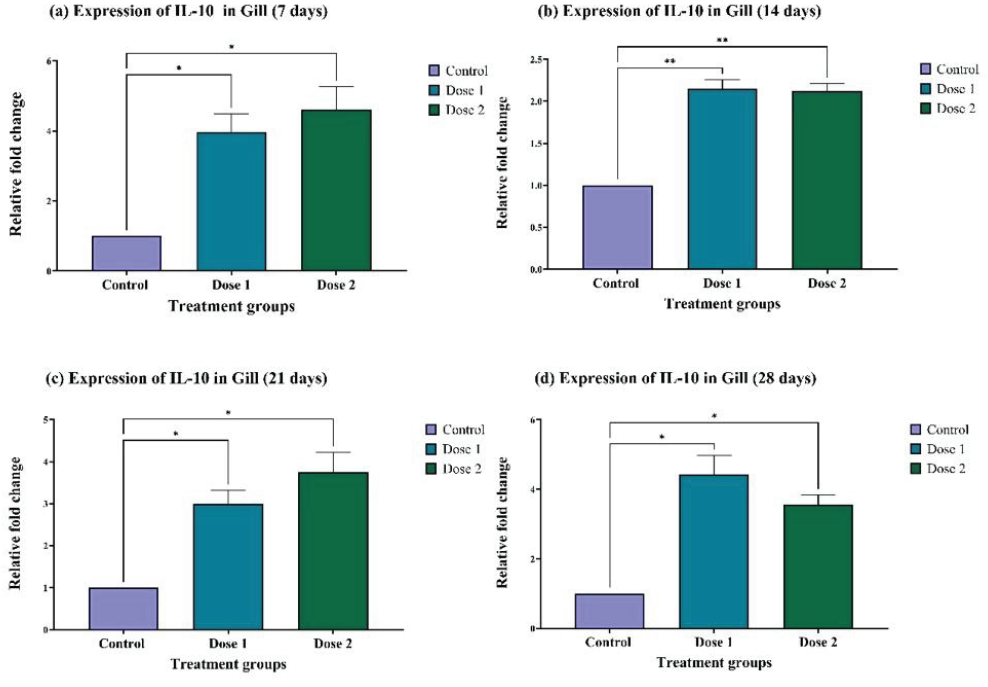

Fig. 3. Expression of IL-10 in the gill of Nile tilapia at different time durations. Data were presented as fold change compared to control. Bar with different colors with asterisk (*) are significantly different at $p < 0.05$ and $p < 0.01$ which are represented as * and **, respectively.

In case of liver, at day 7, 14 and 28 IL-10 expression was significantly higher in both treatment groups compared to control ($p < 0.05$; Fig. 4a, b and d). However, at day 21, only dose 1 showed significant upregulation ($p < 0.05$), while dose 2 did not differ from control (Fig. 4c).

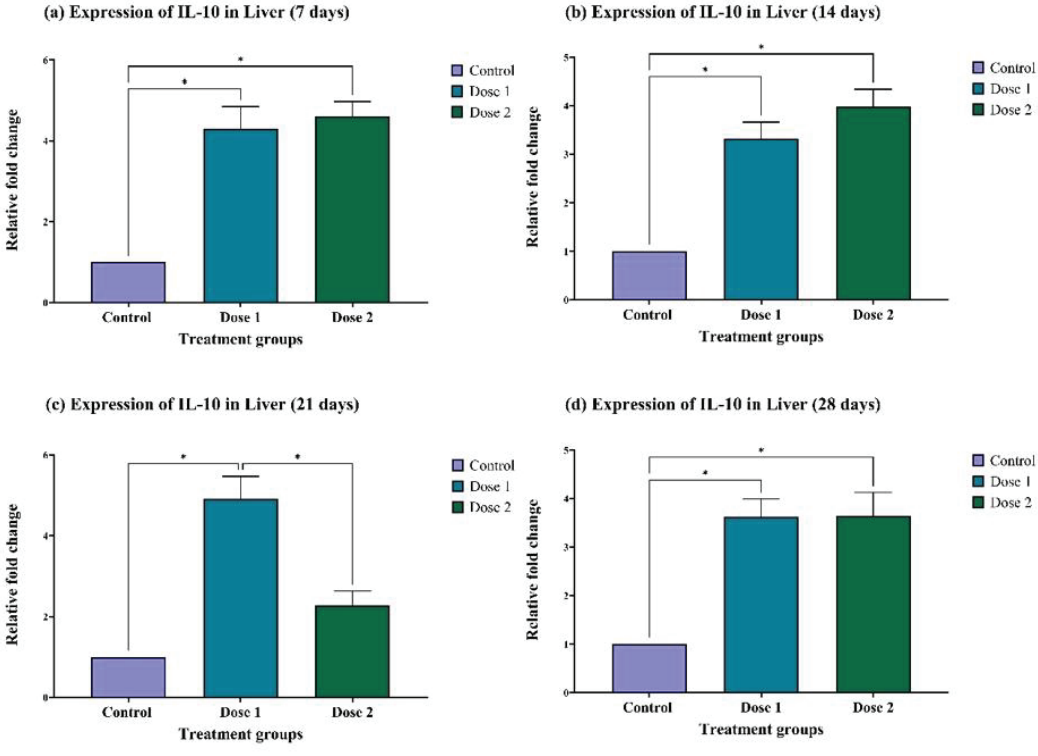

Fig. 4. Expression of IL-10 in the liver of Nile tilapia at different time durations. Data were presented as fold change compared to control. Bar with different colors with asterisk (*) is significantly different at $p < 0.05$ which is represented as *.

The expression of TGF-β in the gill was significantly upregulated at all time points ($p < 0.05$; Fig. 5a–d) in both doses, with no significant difference between them.

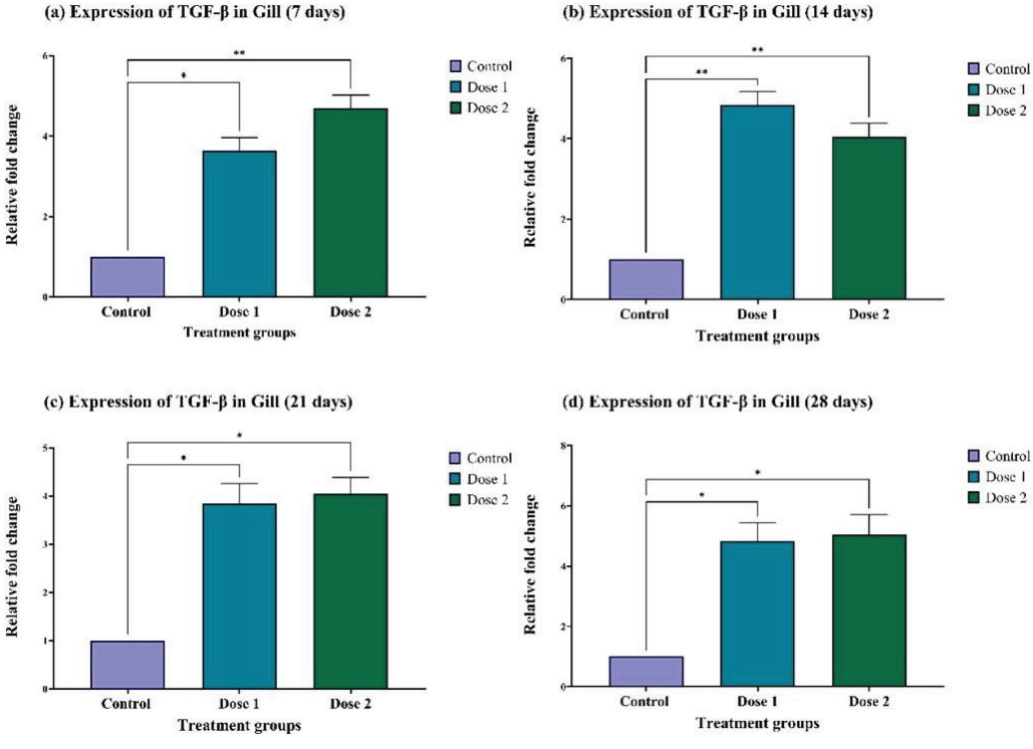

Fig. 5. Expression of TGF-β in the gill of Nile tilapia at different time durations. Data were presented as fold change compared to control. Bar with different colors with asterisk (*) are significantly different at $p < 0.05$ and $p < 0.01$ which are represented as * and **, respectively.

TGF-β expression in liver showed no significant difference was observed at day 7 ($p > 0.05$; Fig. 6a). From day 14 to 28, both doses showed significant upregulation ($p < 0.05$; Fig. 6b–d), with no difference between the treatment groups.

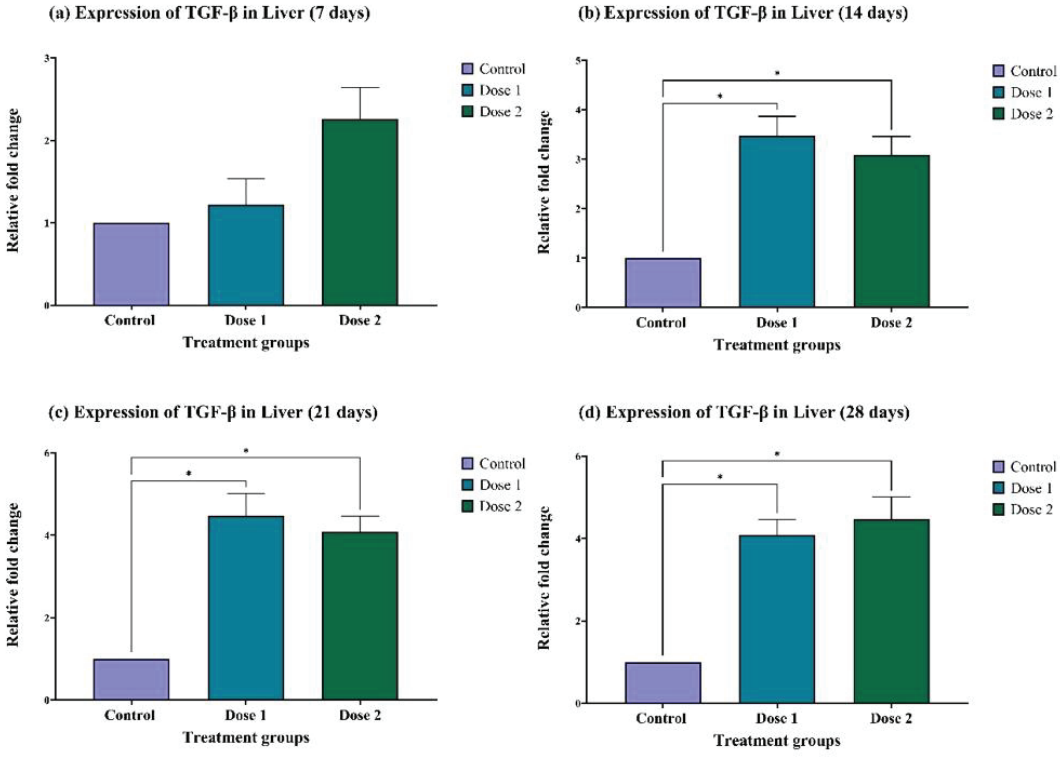

Fig. 6. Expression of TGF-β in the liver of Nile tilapia at different time durations. Data were presented as fold change compared to control. Bar with different colors with asterisk (*) are significantly different at $p < 0.05$ which is represented as *.

The expression pattern of T IL-10 suggests that the green-synthesized nanoparticle diet not only triggered pro-inflammatory signals at mucosal surfaces but also engaged anti-inflammatory regulatory mechanisms in internal organs to maintain immune homeostasis. Elevated hepatic IL-10 could serve to counterbalance the pro-inflammatory signals emanating from peripheral tissues, preventing systemic inflammation. Such a balancing act is supported by recent studies where Popoola and Behera[27] observed that in rohu carp (*Labeo rohita*) fed dietary silver nanoparticles and challenged with bacteria, IL-10 was significantly upregulated in immune organs (liver, kidney, spleen) of NP-fed fish compared to controls. Interestingly, their work demonstrated a dose-dependent IL-10 response across organs – moderate NP inclusion led to the highest IL-10 levels in gills and kidney, whereas an excessive dose (higher inclusion) somewhat reduced IL-10 expression in gills. We see a parallel in tilapia where the 60 mg/kg dose (analogous to a high inclusion) yielded strong IL-10 induction in liver but did not further increase (and possibly leveled off) IL-10 in gills, implying an optimal dose range for maximizing anti-inflammatory gene induction. Our findings also resonate with the Ni *et al*.[25] zebrafish study, which reported IL-10 gene

upregulation alongside IL-1β in fish exposed to biogenic AgNPs, denoting a concurrent activation of pro- and anti-inflammatory pathways. This concurrent IL-10 elevation is biologically meaningful as IL-10 is known to prevent excessive inflammation and tissue damage. The fact that our nanoparticle supplemented fish had increased IL-10 (and TGF-β) in the liver suggests the fish mounted an anti-inflammatory response to modulate any nanoparticle-induced inflammatory signals. This matches the pattern in other nanoparticle trials where immunostimulant is accompanied by compensatory anti-inflammatory signals. For example, Elnagar *et al.*[28] found that tilapia fed a diet enriched with chitosan-based vitamin C/E nanoparticles showed higher baseline immune gene expression (including IL-10) and were more resilient to stress, indicating that such supplements can fortify the anti-inflammatory capacity of fish before or after a stressor.

Expression of TLR3 and IRF3 (viral recognition and signaling):

Expression of TLR3 was significantly upregulated in both doses at all time points ($p < 0.05$ to $p < 0.01$; Fig. 7a–d). Moreover, at day 21, dose 2 expression was significantly higher than dose 1 ($p < 0.05$).

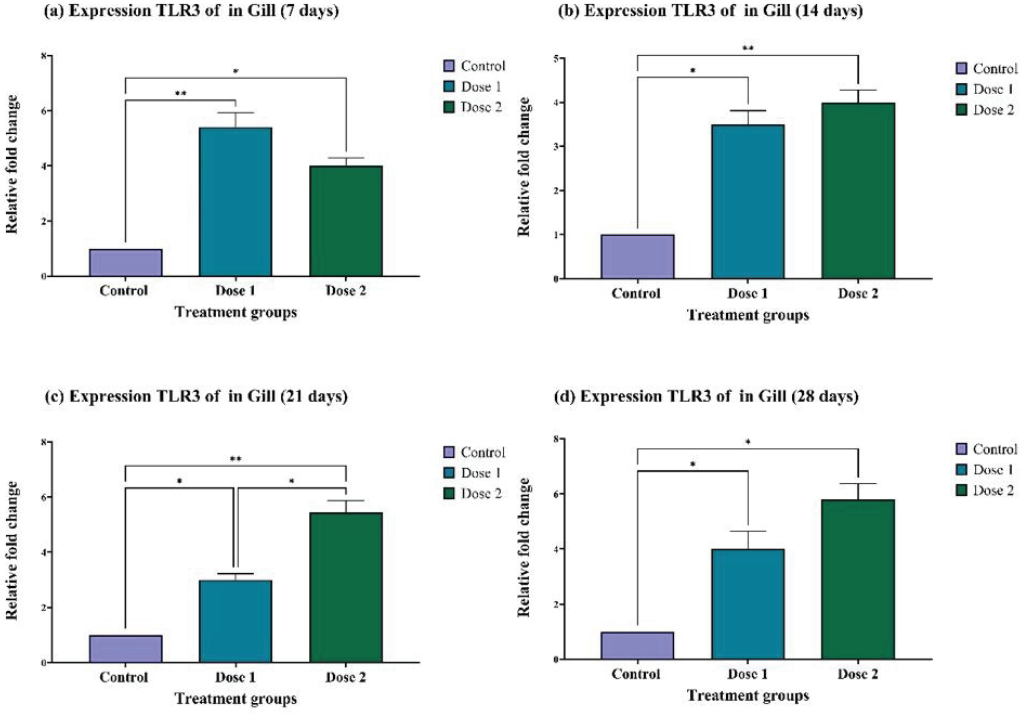

Fig. 7. Expression of TLR3 in gill of Nile tilapia at different time durations. Data were presented as fold change compared to control. Bar with different colors with asterisk (*) are significantly different at $p < 0.05$ and $p < 0.01$ which are represented as * and **, respectively.

In the liver of tilapia, at day 7, dose 2 showed significantly higher expression than both control and dose 1 ($p < 0.01$ and $p < 0.05$, respectively; Fig. 8a). On the other hand, at day 14, only dose 2 was significantly upregulated ($p < 0.05$). At days 21 and 28, both doses showed significant upregulation ($p < 0.05$), with no difference between them (Fig. 8c–d).

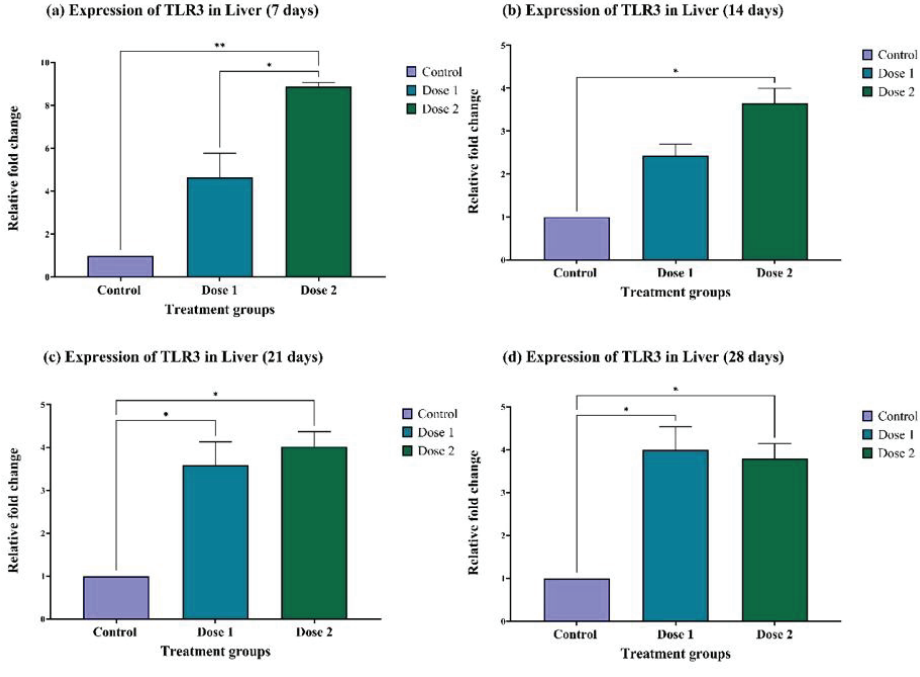

Fig. 8. Expression of TLR3 in the liver of Nile tilapia at different time durations. Data were presented as fold change compared to control. Bar with different colors with asterisk (*) are significantly different at $p < 0.05$, $p < 0.01$ which are represented as * and **, respectively.

In the gill of tilapia, at day 7, IRF3 expression was significantly higher in dose 2 ($p < 0.01$), and higher than dose 1 ($p < 0.05$; Fig. 9a) while at day 14, no significant changes were observed ($p > 0.05$; Fig. 9b). However, at day 21, both doses significantly upregulated IRF3 expression ($p < 0.05$; Fig. 9c), with dose 2 higher than dose 1 ($p < 0.01$). At day 28, only dose 2 showed a significant increase ($p < 0.01$; Fig. 9d).

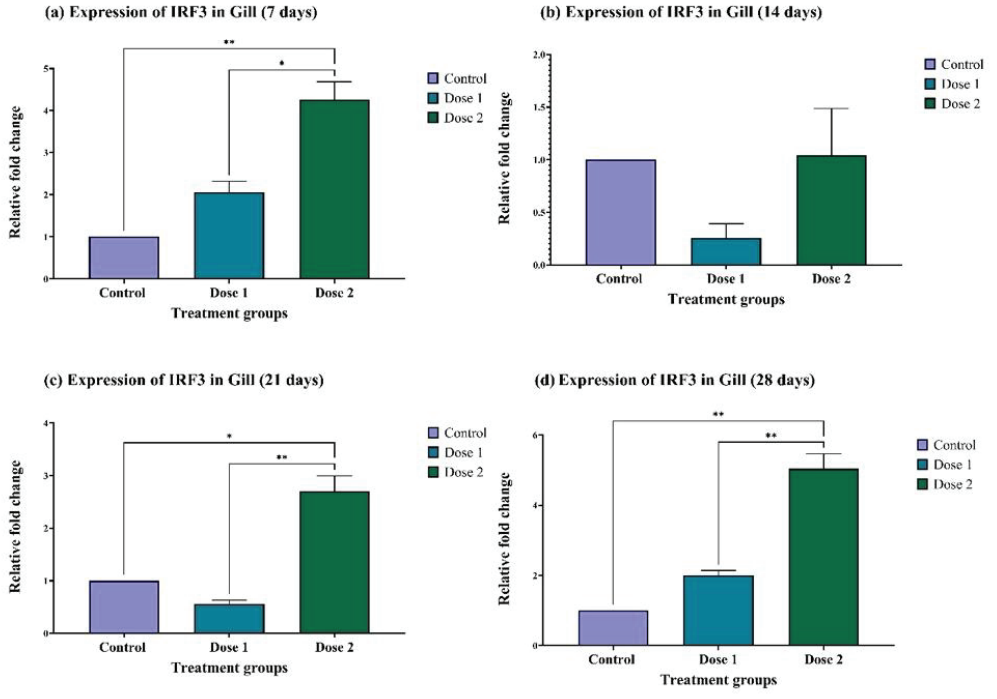

Fig. 9. Expression of IRF3 in the gill of Nile tilapia at different time durations. Data were presented as fold change compared to control. Bar with different colors with asterisk (*) are significantly different at $p < 0.05$ and $p < 0.01$ which are represented as * and **, respectively.

The expression of IRF3 in the liver increased significantly in tilapia fed diet supplemented nanoparticles at 60mg/kg (dose 2) and 30mg/kg(dose 1) at day 7 than fish fed control diet ($p < 0.05$; Fig. 10a). However, at day 14, both doses showed significant upregulation ($p < 0.05$; Fig. 10b). At day 21, only dose 2 was significantly elevated ($p < 0.01$), and it was also significantly higher than dose 1 ($p < 0.05$; Fig. 10c). However, at day 28, both doses showed upregulation ($p < 0.01$), with dose 2 remaining significantly higher ($p < 0.05$; Fig. 10d). These findings indicate that green-synthesized nanoparticles might prime the antiviral defenses in the gill to recognize viral intruders more readily (via higher TLR3), and the liver is prepared to amplify antiviral signaling cascades (via IRF3) if needed. Comparisons to literature reveal that few studies have directly measured such genes in nanoparticle trials, making our observations relatively novel. However, indirect evidence from related research supports our results. In the zebrafish study by Ni *et al.*[25] with abalone-viscera-synthesized AgNPs, while TLR3/IRF3 were not measured, other antiviral cytokines like IL-12 and an interferon-stimulated gene (defensin, DEFB1) were significantly upregulated, especially at higher NP doses. This implies activation of the interferon pathway, consistent with what an IRF3 increase would suggest. Moreover, González-Fernández and Cuesta[29] investigated how nanomaterials affect antiviral responses in fish

and found that toxic nanoplastics had the opposite effect when European seabass were exposed to functionalized nanoplastic particles, the normal upregulation of TLRs and IRF-related genes during a viral infection was markedly suppressed, leading to a blunted interferon response. In that study, high concentrations of nanoplastics down-regulated TLR3 and IRF3-associated signaling, correlating with increased viral susceptibility (nodavirus replication) in the fish. Our findings stand in stark contrast to those results. Instead of suppressing antiviral pathways, the green-synthesized nanoparticles in our trial appear to enhance them or keep them primed. The elevated gill TLR3 in NP-fed tilapia may improve early detection of viruses at entry points, while increased IRF3 in the liver could facilitate a swift interferon-mediated response, should a viral challenge occur. This proactive antiviral posture is a novel finding for fish nanoparticle nutrition, highlighting a potential benefit beyond general immunity. To our knowledge, this is one of the first demonstrations that a nanoparticle supplement can modulate genes like IRF3, viperin, and TLRs in fish. It opens a discussion on using such functional feeds to bolster resistance against viral diseases in aquaculture.

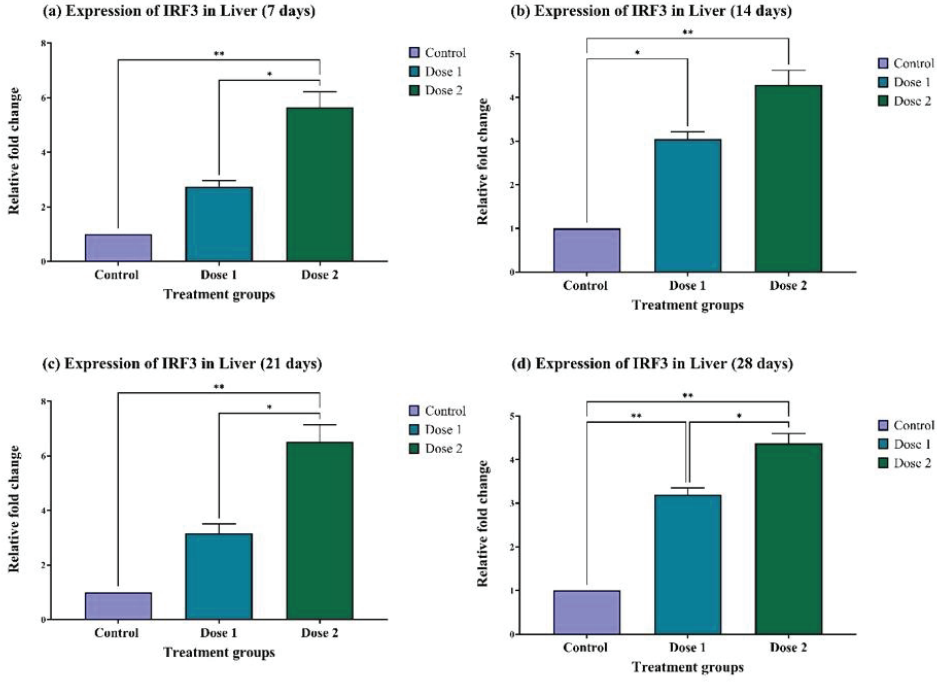

Fig. 10. Expression of IRF3 in the liver of Nile at different time durations. Data were presented as fold change compared to control. Bar with different colors with asterisk (*) are significantly different at $p < 0.05$ and $p < 0.01$ which are represented as * and **, respectively.

Viperin (interferon stimulated gene) expression: In the gill of tilapia, at day 7, both doses significantly increased viperin expression ($p < 0.05$), with dose 2 higher than dose 1 ($p < 0.05$; Fig. 11a). However, at days 14 and 21, both doses maintained significantly elevated levels ($p < 0.05$; Fig. 11b–c), with no difference between doses. On the other hand, at day 28, both doses showed significant upregulation ($p < 0.01$), and dose 1 had a higher response ($p < 0.05$; Fig. 11d).

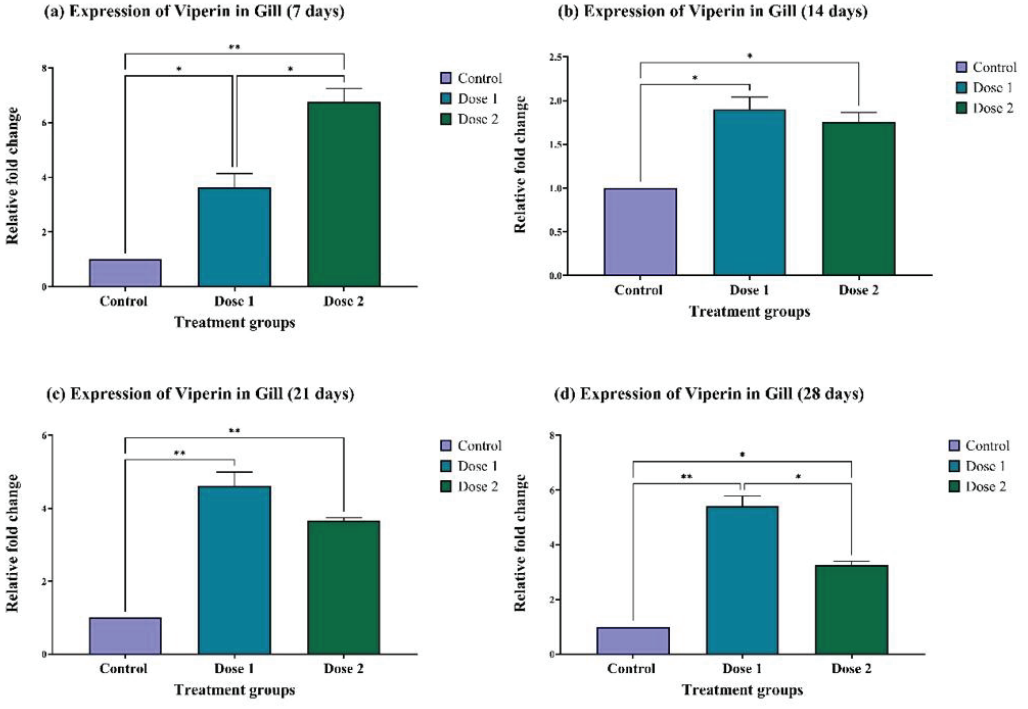

Fig. 11. Expression of viperin in the gill of Nile tilapia at different time durations. Data were presented as fold change compared to control. Bar with different colors with asterisk (*) are significantly different at $p < 0.05$ and $p < 0.01$which are represented as * and **, respectively.

Expression of viperin in the liver of tilapia significantly increased in both doses at all time points ($p < 0.05$; Fig. 12a–d). No differences were observed between doses at any point. The pattern of expression of viperin implies that the systemic interferon response (reflected by liver viperin) is more sensitive to dietary nanoparticle stimulation than mucosal interferon response in gills. It is possible that any interferon produced in response to nanoparticle stimuli predominantly affected internal organs like liver and spleen, thereby inducing viperin there, while the gill, constantly exposed to waterborne microbes and particles, may require a stronger or longer stimulation to significantly elevate viperin. Nevertheless, even a slight increase in gill viperin at 60 mg/kg suggests some enhancement

of local antiviral capacity. The role of viperin has been studied in viral infections and vaccine contexts in fish. Generally, higher viperin indicates an ongoing antiviral state. The fact that our NP-fed fish upregulated viperin without any viral infection indicates an innate immune priming effect. This could be considered analogous to an "immune training" phenomenon where exposure to benign immunostimulants (like plant-capped nanoparticles) readies the immune system for future challenges. Our findings extend the current knowledge of nanoparticle immunomodulation by demonstrating for the first time that a dietary nanoparticle can elevate an ISG (viperin) in a food fish species. By incorporating viperin (and TLR3, IRF3) into our assessments, we reveal an additional layer of immune modulation.

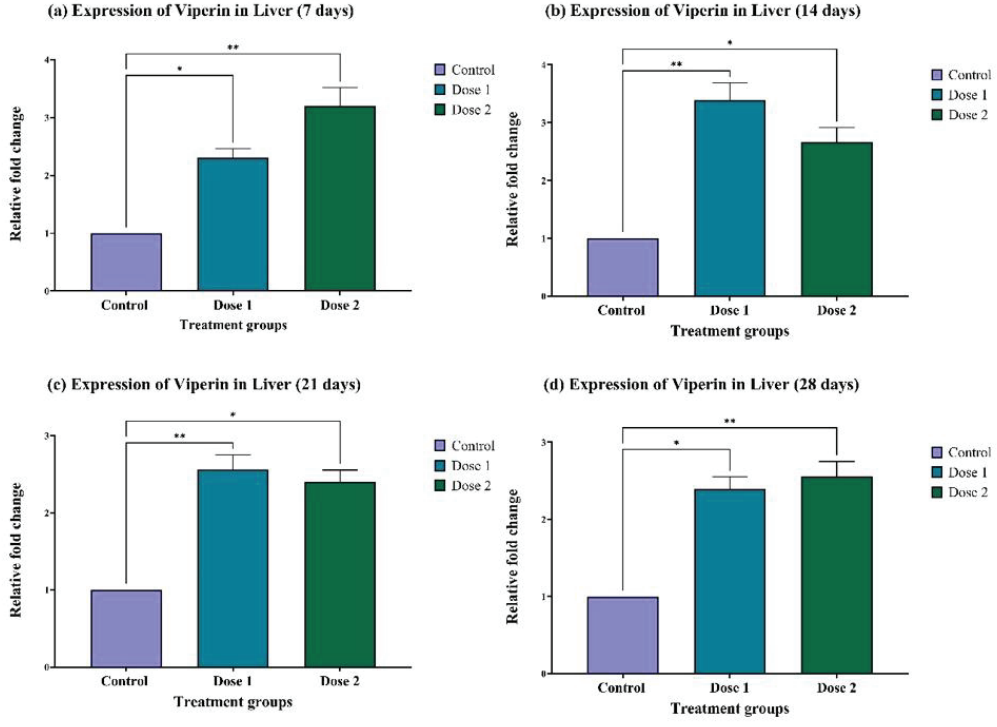

Fig. 12. Expression of viperin in the liver of Nile tilapia at different time durations. Data were presented as fold change compared to control. Bar with different colors with asterisk (*) are significantly different at $p < 0.05$ and $p < 0.01$ which are represented as * and **, respectively.

In the present study, we used green-synthesized nanoparticle formulation, which showed robust activation of both pro- and anti-inflammatory markers. Upregulation of IL-1β and IRF3/viperin suggests enhanced antiviral and general innate defenses, while elevated IL-10 and TGF-β likely provide regulatory feedback to limit excessive inflammation. Such balanced cytokine responses mirror natural antiviral pathways: for

example, viperin is classically induced via TLR3/RIG-I engagement of IRF3[30]. The simultaneous rise in TLR3, IRF3 and viperin transcripts implies that nanoparticle diet may prime the interferon-mediated antiviral system which aligns with the notion that nanoparticles can "re-balance" pro- and anti-inflammatory signaling, enhancing immunity without causing chronic stress[23,26].

Dose-dependence emerged as a clear theme. Higher nanoparticle doses generally induced stronger gene expression. In our study, the greatest upregulation of immune genes was seen at the highest supplementation level. This agrees with the literature: for instance, Zahran *et al*.[23] reported a nine- to ten-fold increase in intestinal IL-1β and IL-8 at 1.5 mg/kg SeNPs and only three- to five-fold at 0.75 mg/kg. Similarly, Abd El-Kader *et al*.[24] found peak immune gene induction in European seabass at 0.5 mg/kg SeNPs, with diminished returns at 1.0 mg/kg. Such non-linear responses suggest that moderate nanoparticle dosing often maximizes benefit. Too low a dose may fail to elicit strong effects, while very high doses could saturate transporters or risk toxicity[31]. In all cases, careful dose optimization is required.

From a sustainability standpoint, these findings are encouraging. Nanoparticle-enriched feeds can contribute to sustainable aquaculture by enhancing immune competence meaning healthier stocks with reduced need for antibiotics or chemotherapeutics, supporting environmentally friendly practices[31,32]. However, as noted in recent reviews by Dube[31], the potential toxicity and long-term impacts of nanoparticles must be carefully assessed. Our study used green synthesis methods, which are thought to yield more biocompatible particles, but rigorous safety evaluation (including impacts on gut microbiota, non-target organisms, and food chain transfer) will be essential before widespread adoption.

Conclusion

The findings of the present study indicate that green-synthesized dietary nanoparticles significantly enhance the innate immune response of Nile tilapia in a dose-dependent manner. Fish receiving nanoparticle-supplemented diets showed marked upregulation of multiple innate immune genes (IL-1β, IL-10, IRF3, viperin, TGF-β, TLR3) in both liver and gill tissues over 28 days. Overall, our results suggest that properly formulated nano-enhanced diets could play a valuable role in sustainable aquaculture. By improving innate disease resistance, green-synthesized nanoparticles help meet production goals while minimizing environmental impact. Future work should focus on refining optimal dosages, understanding the mechanistic basis of the immune modulation, and ensuring the long-term safety of these dietary interventions in tilapia aquaculture.

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Declaration of generative AI

The authors used ChatGPT for language improvement and readability of the manuscript.

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