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Probiotic supplementation improved the growth, haematology and immune response of Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758)

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Abstract

The present study investigated the effects of probiotic supplementation on the growth performance and immunity of Nile tilapia (*Oreochromis niloticus*). A commercial probiotic was incorporated into the feed at 10 g/kg and administered for one month. After this period, significantly higher weight gain and specific growth rate were found in fish receiving the probiotic supplemented diet than fish fed with control diet ($p < 0.05$). Haematological parameters were also examined, whereby day 7, significantly increased concentration of haemoglobin was found in fish given probiotic supplemented diet than that of fish given control diet ($p < 0.05$). Moreover, white blood cell (WBC), red blood cell (RBC), and platelet counts were also increased significantly in fish given probiotics in their diet than fish fed diet without probiotics ($p < 0.05$). However, at day 30, significantly higher count of WBC was found in probiotic supplemented fish than fish fed diet without probiotic ($p < 0.05$). Furthermore, the probiotic supplemented group of Nile tilapia exhibited significantly elevated expression levels of immune genes including receptor molecules (TLR2, TLR3, TLR8 and TLR9) and effector molecules including IRF3, IL-1β, IL-10, and viperin in the spleen, liver, gill, and brain at 24, 48, and 72 hours of probiotic supplementation ($p < 0.05$). The findings suggest that dietary probiotics can effectively enhance both growth and immunity in Nile tilapia.

Introduction

Raising concerns over leftover antibiotic traces have highlighted their potential risks to both ecosystems and public health, emphasizing the need for alternatives. In the

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aquaculture sector, providing additional nutrients to the diet might have a significant impact on immunity, antioxidant production, and growth of fish[1]. Therefore, researchers' curiosity has been attracted by the discovery of potential substances for enhanced growth and disease resistance in the form of nutritional supplements that can improve fish health and yield. Many substances, including organic acids[2], and probiotics[3], and immunostimulants are utilized in the form of feed additives to accomplish these objectives. Approaches like vaccines and probiotics could help guard against stress and potentially prevent diseases[4]. Probiotics offer simpler solutions that can prevent diseases in animals without the risk of becoming drug-resistant[5]. They provide various benefits, such as better growth and stronger immunity, helping fish stay healthy while also being good for the environment[6]. When administered to animals, probiotics can improve or maintain their gut flora, which can boost growth and immunological responses against harmful bacteria and improve the quality of the water[7]. Probiotics have been utilized to improve Nile tilapia health, resistance to disease, immune response, and growth due to their direct impact on the innate immune system[8].

The formation of anti-bacteria, enhanced adherence to the gastrointestinal mucosa, competitive eradication of harmful microorganisms, and enhanced epithelial barrier are some of the main mechanisms underlie the positive effects of probiotics[9]. The majority of these processes require controlling gene expression in certain tissues, such as the liver and intestines[10]. Accordingly, it is imperative to address the probiotic-mediated gene expression modulation. Fish's innate immunity is boosted by probiotics, which increase their resistance to disease. They boost immunological responses by inducing the production of pro-inflammatory cytokines[11] and chemokines associated with the signaling pathway of nuclear factor NF-kB[12]. Thus, the use of probiotics has been encouraged in aquaculture for improved growth and disease resistance.

This study focused on four immune organs including spleen, gill, liver and brain of Nile tilapia to analyze how innate immunity related selected genes respond to probiotics exposure. Spleen was selected because of its immune function. In addition to the exchange of gas, regulation of ion, acid-base equilibrium, osmoregulation, excreting ammonia and hormone generation, gill performs immunological defense as well[13]. It also serves as a vital organ for the generation of cells that secrete antibodies after direct immersion vaccination[14]. The liver play a crucial role in bile production, toxin elimination and carbohydrates, proteins as well as lipid processing. However, the role of the liver in immunological actions is often ignored. The role of liver in immune response has been demonstrated in a previous study[15]. When it comes to the systemic immune response mechanism, the spleen and kidney are the primary organs of immunity[16]. The spleen and head kidney often secrete cytokines and antimicrobials in response to inflammation[17]. For wide use and adaptability, tilapia is commonly used as a model organism for the study of immune responses against mostly bacterial pathogens and to a lesser extent against viral pathogens. In this study, a commercial probiotic, previously untested for its impact on the growth and immune function of Nile

tilapia, was used. Hence, this study was performed to assess the effects of commercial probiotics on the growth, haematology and immunity of Nile tilapia.

Materials and Methods

Design of experiment: The experiments were conducted at the University of Dhaka in a controlled environment where consistent lighting was used to minimize environmental variability. Two sets of experiments were conducted in triplicate with the acclimated Nile tilapia where first experiment investigated growth and haematology and the second experiment investigated the expression of genes in different organs of Nile tilapia upon feeding probiotic supplemented diet.

Experimental feed, fish and rearing conditions: A commercial floating feed "Nourish tilapia grower feed" was purchased from the companies' outlets of Dhaka, Bangladesh. According to the manufacturer data the feed contained fishmeal, soybean meal, animal protein, amino acids, vitamins, and minerals as well as macronutrient including moisture (10%), crude protein (25%), ash (12%), crude fiber (4.77%) and fat (4%).

Ninety samples of male mono-sex Nile tilapia *O. niloticus* with an average initial weight of 20.87 ± 5.58 g were collected from commercial hatchery situated in Noakhali, Bangladesh. The samples were healthy and no visible clinical symptoms (e.g., hemorrhage, abdominal swelling, sluggish movement or missing scales) were observed.

The fish were conditioned in a 500-liter tank under continuous aeration. Regular feeding was done. Fish were conditioned for seven days. After conditioning, the fish were allocated randomly in 80-L aquarium with 60-L water (15 individuals per aquarium, two treatment conditions, each with three replicates) and allowed to adapt for an additional seven days before the experimental trial started. Daily replacement of 30 % water resulted in the keeping of water quality at maintenance levels of pH 7.50 ± 0.22, average dissolved oxygen content 5.42 ± 0.48 mg/L and temperature 26.46 ± 0.92°C. After the acclimatization phase, the fish were cultured under the specific conditions for a period of 30 days.

Preparation of probiotic supplemented diets and dose: In this study, commercial probiotics composed of *Rhodococcus* sp., *Photopseudomonas* sp., *Bacillus subtilis*, *B. coagulans*, *B. pumilus*, *B. licheniformis*, *B. megaterium*, *B. amyloliquifacians*, *B. polymyxa*, *B. mesentericus*, *Saccharomyces cerevisiae*, and enzyme complex were used (supplied from manufacturer). The commercial tilapia grower feed was blended into powder and re-pelleted with or without probiotic (for control group). To prepare the experimental diet 10 g probiotics was diluted in 50 mL of sterile distill water and mixed with 1 kg feed powder for homogenous mixing to achieve desired probiotic concentration of 10g/kg. The probiotic mixed powder was then re-pelleted and air dried. For control feed, commercial feed was re-pelleted with just adding distill water and air dried. Fish in the control group had a control diet throughout the experiment, whereas fish in the treatment group received probiotic supplemented diet at 10 g/Kg of feed throughout the experimental period (30 days). The dosage calculation for this commercial

probiotic was estimated based on a previous study who reported positive immunological and growth responses in Nile tilapia at same dosage[18].

Fish were provided feed at a daily rate of 10 % of their body weight, split into two equal portions (5 % body weight (BW) each, administered at 9:00 AM and 4.00 PM. The amount of feed was correctly adjusted based on the water temperature and feeding behavior to assure satiation and the elimination of any uneaten feed within 10 minutes after feeding. Water quality of the rearing tanks was monitored, and the parameters were recorded every alternative day using a multi parameter (U-50 multi-parameter water quality checker; HORIBA Advanced Techno Co. Ltd., Japan). The values were within the range of optimum levels for aquaculture (data not shown).

Fish growth analysis: The initial weight and length of the fish from each experimental unit were measured. After one month experiment, the final weight and length of individual fish were measured. To determine the feed conversion ratio (FCR), The total amount of feed provided to both the treatment and control groups was recorded. Growth parameters including weight gain (WG), FCR and specific growth rate (SGR) were then calculated using standard formula.

$$WG = \text{Final body weight (g)} - \text{Initial body weight (g)}$$

$$FCR = \frac{\text{Feed consumed (g)}}{\text{Weight gain (g)}}$$

$$SGR\ (\%) = \frac{\text{Ln(Final weight)} - \text{Ln(Initial weight)}}{\text{Days}} \times 100$$

Blood sample collection and haematological analysis: For haematological analysis, one fish from each replicate of each treatment was caught using a small scoop net. Before collection of blood, fish were anesthetized. The sedation was performed with 2-phenoxyethanol (using 0.1% v/v). Blood was sampled from each fish from the caudal vein. Heparinized syringe and needle were used for the collection of blood. Blood was collected in heparinized tubes. Blood was analysed from Dhaka Medical College Hospital, Bangladesh. The hematological analysis included hemoglobin, WBC, RBC, and platelets. The blood parameter data were then calculated. Blood samples were taken at day 7 and 30 of experiment.

Gene expression study: The expression innate immune genes in different organs of tilapia was studied to investigate the effects of probiotics on immune response of tilapia. For this purpose, one fish from each treatment group was sampled and anesthetized. Fish was sampled at 24 h, 48 h, and 72 h of experiment. Gill, spleen, liver and brain were collected from the anesthetized fish. The tissues were preserved in RNAlater solution until RNA extraction was performed.

RNA isolation, synthesis of cDNA and quantitative real-time PCR (qRT-PCR): Extraction of RNA was performed from 50 mg of tissue samples (gill, liver, spleen and brain) from

treatment groups using TRIzol™ Reagent (Thermo Fisher Scientific Inc., USA) following the supplier's protocol with a few adjustments. RNA quantification was performed using NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA). RNA integrity was also assessed by absorbance ratio of 260/280. RNA with the ratio between 1.8-2.0 was used for subsequent analysis. Synthesis of cDNA was performed using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara, Japan) following the instructions of the manufacturer.

The expression levels of four Toll-Like Receptors (TLRs) including TLR2, TLR3, TLR8 and TLR9, and effector molecules including IRF3, IL-1β, IL-10, and viperin genes (Table 1) in different tissues (gill, liver, spleen and brain) of *Oreochromis niloticus* were determined using qRT-PCR following the method explained by Ferdoush *et al.*[19]. SYBR green based mastermix was used for qRT-PCR. In 20 µL reaction mixture, 10 µL of master mix (2x) (Luminous Color HiGreen, Themo Fisher Scientific), 0.5 µL (10 pM) of each of forward and reverse primers, 2 µL (diluted 1:10) of cDNA and 7 µL of nuclease free water were included. PCR was conducted in a qPCR machine (qTower^3, Analytik Jena, Germany). The temperature profile of the reaction was as follow: initial denaturation step for 2 mi at 50°C followed by 40 cycles of denaturation for 10s at 95°C, primer specific annealing for 30s at 56-60°C and elongation for 15s at 72°C. A melt curve analysis was performed to verify specificity of amplification which had the following temperature- time profile: 95°C for 10s, 55°C for 5s and 95°C for 30s. A cooling step at 40°C for 30s was also included in the PCR program. The $2^{-\Delta\Delta Ct}$ method as described previously[20], was employed to determine fold expression of target gene in each immune organ of tilapia of treatment group relative to the expression of fish in control group. The expression was normalized by the expression of ß-actin of respective sample.

Statistical analysis: The growth parameter and hematological data were analyzed using the software GraphPad Prism version 9 (San Diego, CA, USA). The differences in growth between the probiotic supplemented group and the control group were determined by t-test at $p < 0.05$. Microsoft Excel (2013) was used to calculate $2^{-\Delta\Delta Ct}$. To identity the differences in the expression of target genes at different time points, multiple t-test was employed at $p < 0.05$ using the same software.

Table 1. The primers for PCR of target genes of Nile tilapia. Size of PCR product, melting temperature and accession numbers are provided

Gene	Sequence (5′-3′)	Amplicon size (bp)	Annealing temperature (°C)	Accession number
TLR2	F-TCTGGGCTATCCTTCCCCAA R-TCGCAGATGTAGCTGTCCAC	221	60	XM_013264298.3
TLR3	F-CTGTCCGTCACTCCGAAACA R-CCGGGATTGATCTGCGCTAT	108	59	XM_003449728.4
TLR8	F-TCTGAGTGGGTGATGAGCA R-TGTACTGGATGCTCTGGGTG	137	60	XM_019352831
TLR9	F-ACCTTCCTGGACCTCAGTCA R-TGGCATGCAGGGTGAGATTT	178	60	XM_005477981.4
IRF3	F-GGTACGACACATCAGCGTGC R-CTGGCAACATAGAGCAGCAGTA	183	60	XM_005448320.3
IL-1β	F-TGAGAGCCTACTTTAGGATTCTGC R-GCGGCTATTACAACCAATGCT	150	59	XM_005457887.2
IL-10	F-CTCAGATGGAGAGCAGAGGTC R-CTTGATTTGGGTCAGCAGGT	134	60	KP645180.1
Viperin	F-ATCAACTTCTCTGGCGGA R-AGATAGACACCATATTTCTGGAC	161	56	XM_003453237.3
ß-actin	F-GCTACTCCTTCACCACCACAG R-CGTCAGGCAGCTCGTAACTC	144	60	KJ126772.1

Results and Discussion

Probiotic induced growth of Nile tilapia: At the end of the experiment, probiotics were found to increase weight gain as well as specific growth rate significantly than that of fish fed diet without probiotics (Table 2; $p < 0.05$). FCR in fish provided with probiotic supplementation diet, on the other hand, significantly decreased ($p < 0.05$). These results supported by previous studies indicating the growth promoting effects of probiotics, possibly through improved nutrient assimilation and gut microbiota modulation. Similar to the findings of the current study, previous studies have shown that the beneficial bacteria introduced into the water or fed to the fish or shrimp, improved their growth and survival rates[21]. These results validated the fact that probiotic diets may have growth-promoting effects on tilapia. Fish growth depends on different factors such as nutrient absorption, feed assimilation, digestive enzyme activity, gut microbiota composition, and immune function. By enhancing the activity of digestive enzymes and as a result, the generation of micronutrients for absorption, altering the gut microbiota can enhance growth performance and resulted in a reduced FCR[22]. Enhanced digestive enzyme activity and better nutrient availability may account for the improved growth metrics observed.

Table 2. Growth of Nile tilapia fed diets with and without probiotics for a period of one month. Significant differences are indicated by asterisk (*)

	Control (Mean ± SD)	Probiotic (Mean ± SD)	p value
Initial average weight (g)	20.87 ± 5.58	21.42 ± 7.91	0.7026 ($p > 0.05$)
Final average weight (g)	24.82 ± 6.57	28.55 ± 9.65*	0.0363 ($p < 0.05$)
Weight gain (g)	3.98 ± 1.88	7.13 ± 2.91**	0.0033 ($p < 0.01$)
SGR (%)	0.58 ± 0.25	0.99 ± 0.37*	0.0116 ($p < 0.05$)
FCR	2.13 ± 0.10	1.50 ± 0.16*	0.0219 ($p < 0.05$)

Effects of probiotics on haematology of Nile tilapia: Haematology of Nile tilapia was investigated in the present study where at day 7, the hemoglobin (Hb), white blood cell (WBC) and red blood cells (RBC) counts were significantly increased in fish fed probiotic supplemented diet ($p < 0.05$; Fig. 1). In a previous study, probiotic *B. cereus* has been shown to increase hemoglobin amounts in Nile tilapia[23]. The hemoglobin concentration of the blood is essential because it serves as an oxygen delivery element to the bloodstream. Rises in level reflect a greater oxygen supply to the fish, which improves fish health. Moreover, in a previous investigation, supplementing with *L. plantarum* significantly raised RBC levels in Nile tilapia when compared to a control diet[24]. The probiotic supplemented diet increased the supply of oxygen and many of the nutrients needed for the production of red blood cells (RBCs), including iron, vitamin A, and vitamin B12[22].

However, after one month of feeding probiotic supplemented diet, WBC count in fish fed probiotic supplemented diet was significantly higher than fish fed control diet (Fig. 1b; $p < 0.0001$). Elevated hemoglobin and RBC levels suggest improved oxygen carrying capacity, while increased WBC and platelet levels indicate a strengthened innate immune response. The significant increase in WBC count even after 30 days highlights the sustained immunomodulatory effect of probiotics. These findings align with studies showing improved haematological health in fish supplemented with probiotic strains such as Bacillus cereus and Lactobacillus plantarum. WBC constitutes one of the initial lines of defense against diseases, an increase in WBC in the bloodstream of fish provided *Bacillus* enriched feeds could represent modulation of the innate immunity, perhaps leading to increase protection and tolerance to stressful conditions or infections[25]. Thus, it might have been said that after feeding probiotics, Nile tilapia could respond better to stressors. Hematological parameters provide an accurate view of fish well-being and environmental condition as these are subjected to a variety of factors such as animal shape, culture conditions, biological role, nutrition, and as a whole environmental factors[26].

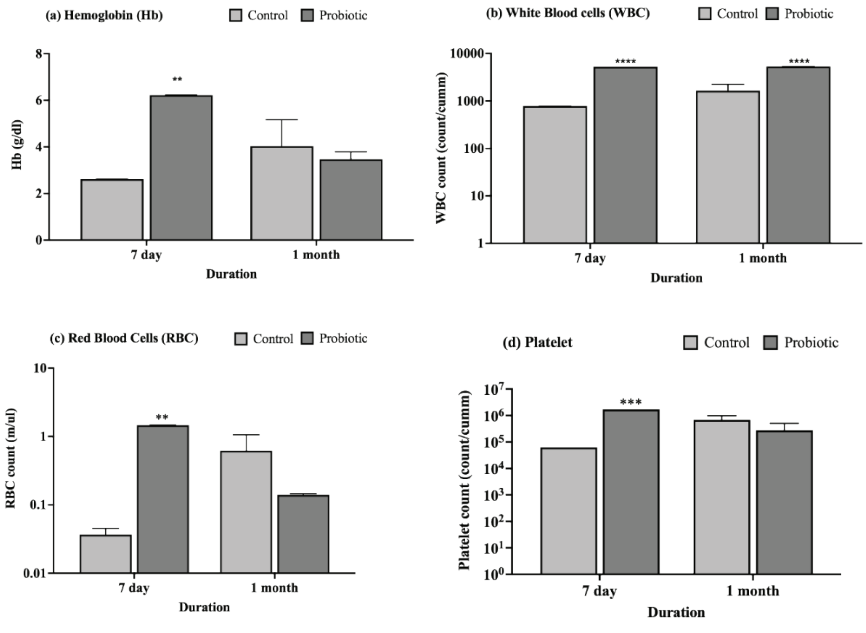

Fig. 1. Hematological parameters including (a) hemoglobin (Hb) concentration, (b) WBC count, (c) RBC count and (d) platelet count (mean ± SD) of Nile tilapia fed control diet and diet with probiotics. Bar with asterisk **, *** and **** denote significant difference $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively.

Effects of probiotics on the expression of innate immune molecules in Nile tilapia

Expression of receptor molecules: Amon the TLRs, TLR2 and TLR3 expression was significantly higher in the spleen of probiotic supplemented fish than that of control fish at 48 h after feeding probiotic supplemented diet (Fig. 2a & Fig. 2e; $p < 0.05$). However, in liver of fish fed probiotic supplemented diet, TLR2 expression was significantly upregulated (Fig. 2b; $p < 0.05$) while expression of TLR3 & TLR8 was significantly downregulated at 72h of feeding probiotic supplemented diet (Fig. 2f & Fig. 2j; $p < 0.05$). Previously, probiotics have been found to show immunomodulatory effect on TLR2 and TLR3 which are consistent with the present findings[27,28]. Moreover, in the gill of tilapia upon feeding probiotic supplemented diet, significantly higher expression of TLR2 was found at 72 h (Fig. 2c; $p < 0.05$) whereas in gill significantly downregulation of TLR9 expression was observed at 24 h (Fig. 2o; $p < 0.05$). Furthermore, the expression of all the TLRs investigated in this study was significantly upregulated in the brain of tilapia at 72 h of feeding probiotic added diet (Fig. 2d, 2h, 2l, 2p; $p < 0.05$), which corroborates to the findings of Qian *et al*.[29] who found TLR8 and TLR9 expression at higher level in *P. crocea*. This may suggest that the fish have mounted an effective immune response by the probiotics, and the down regulation of TLR may indicate a decrease in inflammation.

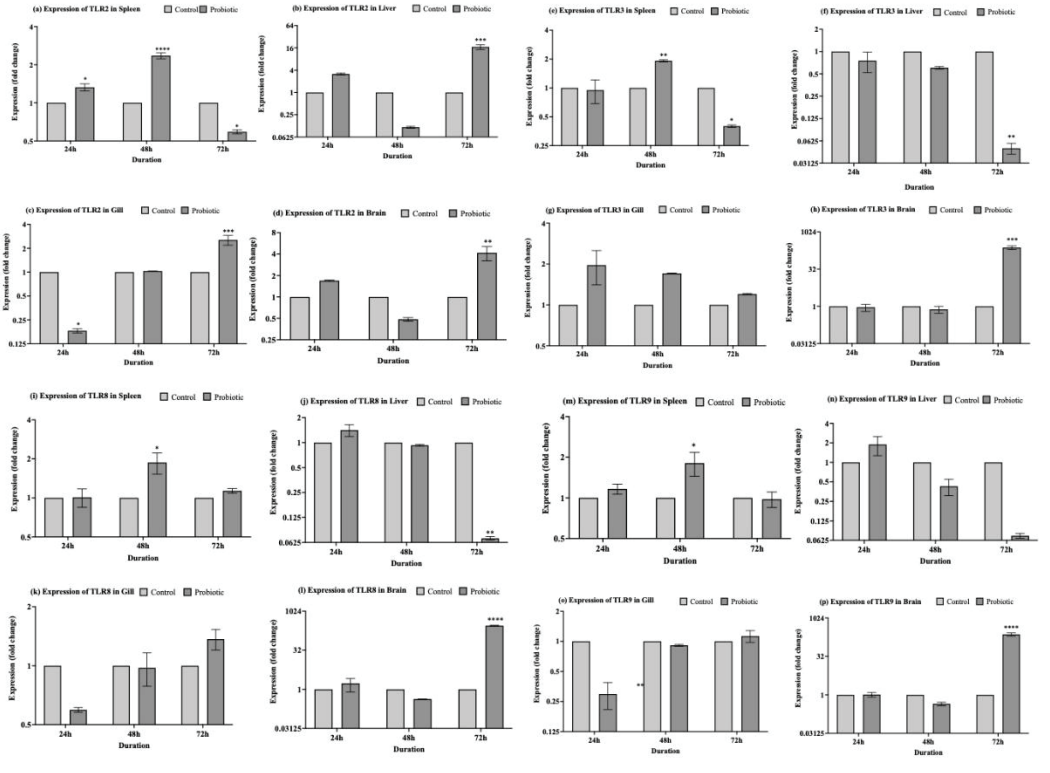

Fig. 2. Expression of Toll-Like Receptors (TLR2, TLR3, TLR8 and TLR9) in different organs of Nile tilapia fed control and probiotic supplemented diet. Data were expressed as fold change (mean ± SD) relative to control group. Bar with asterisk *, **, *** and **** denote significant difference at $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively.

Expression levels of IRF3, IL-1β, IL-10, and viperin across various tissues: In the present study, significant increased expression of IRF3 was detected in the spleen after 24 and 48 hours of probiotic supplementation (Fig. 3a; $p < 0.05$). In the same way, viperin expression was also increased significantly in both the spleen and brain at 24 h (Fig. 3m; $p < 0.05$). At 72 hours, IRF3 expression was notably increased in the liver and brain (Fig. 3b & 3d; $p < 0.05$), while viperin expression peaked in the liver at 48 hours (Fig. 3n; $p < 0.05$). In contrast, IRF3 expression was significantly downregulated in the same organ at 24 hours (Fig. 3c; $p < 0.05$), whereas expression of viperin was significantly upregulated in the gill at 72 hours (Fig. 3o; $p < 0.05$). Overall, probiotic supplementation resulted in increased viperin expression in the spleen (24 h, 48 h), liver (48 h), gill (72 h), and brain (24 h). Earlier study supports the findings of the current study where probiotic-induced upregulation of IRF3 and viperin were reported[30]. The enhanced expression of viperin suggests a potential antiviral mechanism, possibly through interference with amino acid metabolism and inhibition of pathogen respiration, thereby contributing to viral defense in fish[31].

Overexpression of IL-1β indicates the progression of activation of immune cells. It has been found to operate as an immunological biomarker, activating immune cells such as lymphocytes, natural killer cells, and macrophages[32]. In the current investigation, an upregulation of IL-1β expression was detected in the liver, gill, and brain tissues after 72 h of feeding probiotic enriched feed compared to the respective organs in fish fed control diet. From this result, it can be demonstrated that probiotics have the potential to increase IL-1β which enhances immune response against potential pathogens.

IL-10 is a key cytokine involved in regulating immune responses, known for its strong anti-inflammatory effects on various immune cells[33]. It plays a crucial role in modulating metabolic pathways that control inflammation[34]. In this study, IL-10 expression was significantly upregulated in the spleen at 48 h following probiotic supplementation (Fig. 3i; $p < 0.05$), while a notable downregulation was observed in the liver at the same time point (Fig. 3j; $p < 0.05$). At 72 h, IL-10 levels decreased significantly in both the spleen and liver but were markedly upregulated in the brain (Fig. 3i, 3j & 3l; $p < 0.05$). Additionally, IL-1β, a pro-inflammatory cytokine, showed significantly elevated expression in the spleen and brain at 72 h (Fig. 3e & 3h; $p < 0.05$), aligning with earlier studies that reported probiotic-induced modulation of immune signaling pathways through IL-10 regulation[35]. These findings suggest a complex, tissue-specific role of probiotics in balancing pro- and anti-inflammatory immune responses.

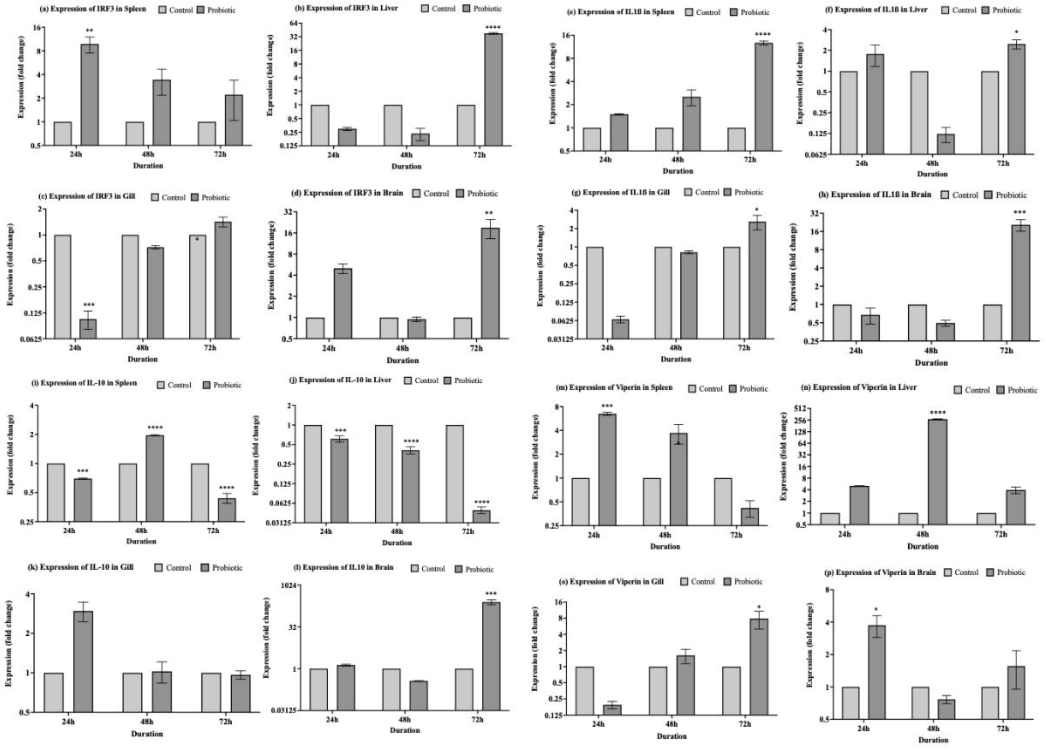

Fig. 3. Expression of effector molecules (IRF3, IL-1β, IL-10 and viperin) in different organs of Nile tilapia fed control and probiotic supplemented diet. Data were expressed as fold change (mean ± SD) relative to control group. Bar with asterisk *, **, *** and **** denote significant difference at $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively.

The observed gene expression patterns confirm that probiotics enhance the innate immunity through both stimulation and regulation of immune genes. The spleen and brain appeared to be the most responsive tissues, emphasizing their importance in systemic immunity. These findings demonstrate that probiotic supplementation not only boosts growth and physiological health but also primes the immune system for better pathogen defense.

Conclusion

Nile tilapia showed an increased growth and feed efficiency upon feeding probiotic supplemented diet. The haematological parameters including haemoglobin, WBC, RBC and platelet counts were increased in response to probiotic supplementation. The innate immune genes in tilapia were modulated by the addition of probiotics in feed. The study demonstrates how probiotics affect growth and immune function in Nile tilapia. The study confirms their potential to replace or reduce the use of chemotherapeutics and antibiotics,

aligning with global efforts to promote environmentally friendly and health conscious aquaculture practices. However, further large-scale investigations should be performed to fully evaluate the sustained impacts of probiotic supplementation on fish growth, gut microbiome and immunity under commercial aquaculture conditions. Moreover, different probiotic strains, different doses of probiotics and method of probiotic application should be tested to obtain maximum outcomes.

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