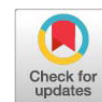


Research Article



Probiotic-Supplemented Refeeding Enhances Biochemical, Antioxidant and Immunomodulatory Responses in Nile Tilapia Following Short-Term Fasting

MOHSEN A. KHORMI*

Department of Biology, College of Science, Jazan University, P.O. Box. 114, Jazan 45142, Saudi Arabia.

Abstract | Understanding physiological responses to fasting and refeeding is essential for optimizing resilience in aquaculture. Probiotics have emerged as promising immunonutritional tools capable of modulating antioxidant defences and inflammatory signalling. Therefore, this study aimed to investigate the effects of probiotic-supplemented refeeding on physiological, biochemical, and immunological responses in Nile tilapia following short-term fasting, with a focus on oxidative stress and gene expression in liver and spleen tissues. In this study, Nile tilapia was allocated into three groups: a non-fasted control group, a group subjected to 5 days of fasting followed by 15 days of refeeding with a basal diet, and a group subjected to the same fasting period followed by refeeding with a probiotic-supplemented diet. Biochemical markers and transcriptional profiles of antioxidant, pro-inflammatory, and anti-inflammatory genes were evaluated across these groups. The probiotic-supplemented diet was prepared by incorporating a lyophilized mixture containing *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus pumilus* (1×10^9 CFU/g) at an inclusion level of 1 g/kg feed. Fasting induced pronounced metabolic and biochemical alterations, including reductions in glucose, cholesterol, and serum protein, alongside elevated hepatic enzymes (ALT, AST), uric acid, creatinine, and malondialdehyde (MDA), reflecting energy depletion, hepatic stress, and oxidative imbalance. Refeeding with the basal diet restored most parameters to control, indicating recovery of metabolic and physiological functions. Notably, probiotic supplementation conferred superior outcomes, with significantly lower ALT, AST, uric acid, creatinine, and MDA compared to both control and basal diet groups, suggesting enhanced oxidative protection and improved hepatic and renal function. Gene expression analysis revealed distinct regulatory patterns. After fasting, antioxidant genes (*Cat*, *SOD-2*) and pro-inflammatory cytokines (*IL-1 β* , *TNF- α*) were significantly upregulated ($p < 0.05$). While anti-inflammatory genes (*TGF- β* , *IL-10*) were significantly downregulated ($p < 0.05$). Basal diet refeeding normalized these responses, whereas probiotic supplementation further enhanced antioxidant and anti-inflammatory gene expression and elevated *IL-1 β* beyond control and basal diet levels. These findings suggest that fasting imposes metabolic stress and oxidative imbalance in Nile tilapia, while Probiotic refeeding restores homeostasis with additional benefits by strengthening immune and antioxidant defences, and improving hepatic and renal protection, underscoring its potential as a dietary strategy to promote resilience and recovery in aquaculture.

Keywords | *Oreochromis niloticus*, Fasting and refeeding, Probiotics, Aquaculture resilience, Cytokine modulation, Oxidative stress

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***Correspondence** | Mohsen A. Khormi, Department of Biology, College of Science, Jazan University, P.O. Box. 114, Jazan 45142, Saudi Arabia; **Email:** makhormi@jazanu.edu.sa

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Aquaculture systems are frequently exposed to multiple stressors, temperature fluctuations, disease outbreaks, and handling procedures, all of which impose stress on farmed fish. To mitigate these impacts, a wide range of studies have focused on optimizing rearing practices through restricted feeding strategies, to develop practical feeding protocols for aquaculture systems (Assis *et al.*, 2020; Bolivar *et al.*, 2006; Morshedi *et al.*, 2017; Oh *et al.*, 2013; Oh and Park, 2019; Silva *et al.*, 2019). Among these strategies, short-term fasting is sometimes employed for management purposes; however, its physiological consequences remain a concern. The severity of fasting-induced stress largely depends on the duration of feed deprivation (Kiron, 2012).

Biochemical indicators are widely recognized as reliable tools for assessing the physiological status of fish (Morshedi *et al.*, 2017). These parameters are sensitive to both biotic and abiotic factors, including food intake (Rehulka *et al.*, 2004). Fasting exerts profound effects on fish physiological by altering metabolic processes (Sakyi *et al.*, 2020) and disrupts the balance between reactive oxygen species (ROS) generation and the antioxidant defences, leading to oxidative stress (Jia *et al.*, 2016). Antioxidant enzymes such as superoxide dismutase (SOD) and catalase (Cat) constitute essential components of the non-specific immune system, functioning to mitigate oxidative damage (Peter *et al.*, 2020). In contrast, MDA, a lipid peroxidation byproduct, is widely recognized as a reliable biomarker of oxidative stress-induced cellular injury (Zhao *et al.*, 2014).

Typically, oxidative stress triggers cellular protective mechanisms and is closely associated with inflammatory responses mediated by cytokines including interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), transforming growth factor- β (TGF- β), and interleukin-10 (IL-10) (Chen *et al.*, 2015). These physiological disturbances compromise fish welfare and productivity, thereby highlighting the importance of nutritional interventions to alleviate fasting-induced stress. These interconnected physiological disturbances highlight the need for targeted nutritional strategies capable of mitigating oxidative stress and modulating immune responses, particularly during the recovery phase following fasting.

Given these physiological disruptions, dietary probiotics, particularly strains belonging to *Bacillus* spp. and *Lactobacillus* spp., have been widely investigated as functional feed additives to enhance resilience in aquaculture species. In Nile tilapia (*Oreochromis niloticus*), several studies indicate that probiotic supplementation mitigates the detrimental impacts of stress on physiological and immunological functions. Shija *et al.* (2023) demonstrated that *Bacillus*-

based probiotics significantly improved immune responses and conferred protection against stress-induced immunosuppression. More recently, Elbahnaswy *et al.* (2024) reported that supplementation with mixed *Bacillus* strains attenuated cortisol secretion, enhanced antioxidant defense mechanisms, and modulated the expression of heat shock proteins in tilapia subjected to acute stress. Overall, these results suggest that probiotic supplementation may represent a promising approach for enhancing stress tolerance and supporting the general health of Nile tilapia in aquaculture systems.

Despite these advances, most previous studies have focused either on the independent effects of fasting or on probiotic supplementation under normal feeding conditions. However, limited information is available regarding their combined effects during the post-fasting refeeding phase, particularly at the transcriptional level of antioxidant and immune-related genes in Nile tilapia. Furthermore, the extent to which probiotic supplementation can modulate recovery responses following nutritional stress remains unclear. Therefore, the present study aimed to evaluate the effects of refeeding with either a basal or probiotic-supplemented diet following short-term fasting in Nile tilapia. Particular emphasis was placed on biochemical parameters, oxidative stress indices, and the expression of key antioxidant and immune-related genes in liver and spleen tissues. It is hypothesized that probiotic-supplemented refeeding enhances antioxidant defenses, modulates cytokine expression, and promotes a more efficient recovery compared to the basal diet.

MATERIALS AND METHODS

FISH SOURCE AND ACCLIMATION

Nile tilapia (*O. niloticus*) were sourced from a commercial fish farm. Upon arrival, fish were transferred to the wet laboratory where they were acclimated for two weeks under controlled conditions in 500-L tanks. Water quality was routinely monitored throughout the acclimation and experimental periods. The recorded parameters included dissolved oxygen (5.7 ± 0.7 mg/L), water temperature (25.5 ± 0.6 °C), total ammonia nitrogen (< 0.07 mg/L), and pH (7.2 ± 0.3).

During acclimation, fish were fed a commercial basal diet formulated for Nile tilapia, Skretting (Nutreco, Egypt), containing crude protein (30%), crude lipid (6%), and crude fiber (5.22%).

EXPERIMENTAL DESIGN AND FEEDING REGIMEN

The experimental design overview is summarized in Table 1. Throughout the experiment, two dietary treatments were administered to Nile tilapia (37 ± 1.8 g) with a density of

Table 1: Overview of the experimental design showing treatment groups and feeding regimens.

Group	Treatment description	Feeding regimen	Replicates	Fish per tank
Group 1	Control: Continuous feeding with basal diet	Basal diet for 20-days	3	20
Group 2	Fasting + Refeeding: Fasting followed by refeeding with basal diet	5-days fasting → 15-days basal diet	3	20
Group 3	Probiotic-Treated: Fasting followed by refeeding with probiotic-supplemented diet	5-days fasting → 15-days probiotic diet	3	20

20 fish/replicate/200 L water. The first treatment consisted of a commercial diet for the species Skretting (Nutreco, Egypt) was used as the basal diet.

The second dietary treatment consisted of a probiotic-enriched feed prepared by incorporating a commercially available lyophilized probiotic mixture containing *B. subtilis*, *B. licheniformis*, and *B. pumilus* (1×10^9 CFU/g). The probiotic preparation was added to the basal diet at an inclusion level of 1 g/kg feed following [El-Son et al. \(2022\)](#). To prepare the supplemented diet, the lyophilized probiotic powder was first suspended in sterile distilled water and thoroughly mixed to obtain a homogeneous solution. This suspension was then sprayed onto the feed pellets while mixing continuously to ensure uniform coating. The treated pellets were subsequently air-dried at room temperature ($25 \pm 2^\circ\text{C}$) to eliminate excess moisture and maintain the stability of the probiotic layer prior to storage and experimental use. Before incorporating the probiotic into the diet, cell viability was assessed by plating on nutrient agar to confirm that the number of living bacteria matched the manufacturer's declared counts. After the feed was coated and dried, representative samples were collected and examined to ensure that viable cells were evenly distributed and remain stable within the supplemented diet.

A fixed feeding ration (3% of body weight) was implemented during the refeeding period to maintain consistent nutritional conditions across all experimental groups. Fish were hand-fed twice daily, once in the morning and once in the afternoon.

Fish mortalities were monitored and recorded daily throughout the experimental period. At the end of the trial, the number of surviving fish in each tank was counted, and survival was calculated as a percentage of the initial stocking density.

BLOOD AND TISSUE SAMPLING

At the beginning of the experiment (day 0), and again at the end of both the 5-day fasting phase and the subsequent 15-day refeeding phase, six fish from each tank were randomly chosen and anesthetized with clove oil. This procedure yielded a total of 18 fish per treatment group for each sampling interval.

Immediately following anesthesia, blood samples were collected from the caudal vessels using sterile syringes and transferred into clean Eppendorf tubes. Blood samples ($n = 18/\text{group}$) were then centrifuged at $1500 \times g$ for 15 minutes at 4°C to separate the serum. The resulting serum samples were carefully harvested and stored at -20°C until further biochemical and immunological analyses.

At the same time, fish were dissected using an over dose of clove oil, liver and spleen tissues from one fish per replicate ($n = 3/\text{group}$) were preserved in RNAlater to maintain RNA integrity, refrigerated overnight, and subsequently frozen at -80°C for gene expression analysis.

ANALYSIS OF SERUM BIOCHEMICAL PARAMETERS

Serum samples from three fish per replicate were pooled to obtain sufficient volume for biochemical assays. Each pooled sample represented one biological replicate corresponding to the tank, which was considered the experimental unit for statistical analysis, resulting in six composite samples per group ($n = 6/\text{group}$). This approach minimized handling variability while ensuring adequate sample volume for reliable measurements.

Biochemical parameters were analyzed using commercial assay kits (Bio-Diagnostic, Worcestershire, UK) in accordance with the manufacturer's protocols. The measured indices included glucose (CAT. NO. GL 13 20), cholesterol (CAT. NO. CH 12 20), total protein (CAT. NO. TP 20 20), albumin (CAT. NO. AB 10 10), globulin, alanine aminotransferase (ALT) (CAT. NO. AL 10 31 (45)) (CAT. NO. UA 21 20), and aspartate aminotransferase (AST) (CAT. NO. GL 13 20), uric acid (CAT. NO. UA 21 20), creatinine (CAT. NO. AS 10 61 (45)), and MDA (CAT. NO. MD 25 29) levels. All analyses were conducted using a T80 spectrophotometer (PG Instruments, Leicestershire, UK).

Glucose concentration was determined according to [Trinder \(1969\)](#), cholesterol level as per [Richmond \(1973\)](#) and [Allain et al. \(1974\)](#). Total protein and albumin concentrations were determined using the methods of [Gornal et al. \(1949\)](#) and [Dumas et al. \(1971\)](#), respectively, while globulin levels were calculated following [Busher \(1990\)](#). Enzymatic activities of AST and ALT were evaluated using the method described by [Reitman and](#)

Frankel (1957). Creatinine and uric acid were quantified according to Bartels *et al.* (1972) and Barham and Trinder (1972), respectively. The MDA level was measured based on the protocol of Ohkawa *et al.* (1979).

GENE EXPRESSION ANALYSIS

RNA EXTRACTION AND cDNA SYNTHESIS

Total RNA was extracted from 30 mg of each tissue sample (n= 3 per group) using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA concentration and purity were assessed with a NanoDrop spectrophotometer, and RNA integrity was evaluated by measuring the A260/A280 ratio. Subsequently, 1 µg of total RNA was reverse-transcribed into first-strand cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) in accordance with the manufacturer's protocol. The synthesized cDNA was stored at -20 °C until use.

QUANTITATIVE REAL-TIME PCR (qRT-PCR)

Gene-specific primers for Nile tilapia were selected as listed in Table 2. β -actin served as the reference gene for normalization. Primer efficiency ranged from 90 to 100%, confirming reliable amplification. qRT-PCR reactions were carried out using the HERAPLUS SYBR Green qPCR Kit (Willowfort, UK), following the manufacturer's guidelines.

Thermal cycling parameters were optimized individually for each target gene. For *IL-1 β* , *TGF- β* , *IL-10*, catalase (*Cat*), superoxide dismutase-2 (*SOD-2*), and beta-actin (β -actin), the PCR program consisted of an initial denaturation at 95 °C for 3 min, followed by 40 amplification cycles of 95 °C for 10 s and 60 °C for 1 min. For *TNF- α* , the cycling protocol included an initial denaturation at 95 °C

for 3 min, followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s.

Fluorescence signals were captured during the extension phase of each PCR cycle. Contamination was controlled through no-template controls in all reactions to detect potential contamination and ensure specificity. Primer specificity was confirmed by agarose gel electrophoresis in addition to melt curve analysis, which was conducted post-amplification from 65 °C to 95 °C, with temperature increasing in 0.5 °C increments every 0.05 seconds. Single peak profiles confirmed specific amplification of target genes. For each biological sample, qRT-PCR reactions were performed in duplicate wells within the same run, and the mean cycle threshold (Ct) value was used for subsequent analysis. Relative expression levels of target genes were determined using the $2^{-\Delta\Delta C_t}$ method, as described by Livak and Schmittgen (2001).

DATA ANALYSIS

All statistical analyses and visualizations were performed using GraphPad Prism (version 9.3.0; San Diego, CA, USA). Data are presented as mean $\pm$ standard error of the mean (SEM). Data were analyzed using two-way analysis of variance (ANOVA), treating diet as the main factor, and analyses were conducted separately at each time point (fasting vs. refeeding). Thus, comparisons focused on diet related differences within each time interval. When significant interactions were detected, analyses were stratified by time point to evaluate diet-related differences within fasting and refeeding phases. Significance was set at $p < 0.05$, and post hoc testing was applied via Tukey's post hoc test to identify significant pairwise differences between treatment groups.

Table 2: Primers employed for quantitative real-time PCR analysis.

Primer	Sequences (5'-3')	Amplified size (bp)	GenBank accession number	Reference
β -actin F	CAGGATGCAGAAGGAGATCACA	92	KJ126772.1	(He <i>et al.</i> , 2014)
β -actin R	CGATCCAGACGGAGTATTTACG			
<i>Cat</i> F	TCCTGGAGCCTCAGCCAT	79	JF801726	(Varela-Valencia <i>et al.</i> , 2014)
<i>Cat</i> R	ACAGTTATCACACAGGTGCATCTTT			
<i>SOD-2</i> F	CTCCAGCCTGCCCTCAA	58	JF801727.1	(Elashry <i>et al.</i> , 2024)
<i>SOD-2</i> R	TCCAGAAGATGGTGTGGTTAATGTG			
<i>IL-1β</i> F	TGCACTGTCACTGACAGCCAA	113	DQ061114	(Choi <i>et al.</i> , 2007)
<i>IL-1β</i> R	ATGTTTCAGGTGCACTATGCGG			
<i>TNF-α</i> F	GGAAGCAGCTCCACTCTGATGA	137	JF957373.1	(Qiang <i>et al.</i> , 2016)
<i>TNF-α</i> R	CACAGCGTGTCTCCTTCGTTCA			
<i>TGF-β</i> F	GTTTGAACCTTCGGCGGTACTG	80	NM_001311325.1	(Standen <i>et al.</i> , 2016)
<i>TGF-β</i> R	TCCTGCTCATAGTCCCAGAGA			
<i>IL-10</i> F	CTCAGATGGAGAGCAGAGGTC	134	KP645180.1	(Ferdoush <i>et al.</i> , 2024)
<i>IL-10</i> R	CTTGATTTGGGTCAGCAGGT			

SERUM BIOCHEMICAL ANALYSIS

In the present study, no mortality was observed during either the fasting or refeeding phases, survival rate was 100%. After the 5-day fasting period, serum glucose and cholesterol concentrations (Figure 1A,B) were significantly reduced. After refeeding for 15 days with the basal diet, serum glucose concentrations returned to levels similar to those of the control group. However, fish that received the probiotic-enriched diet during the refeeding period showed a further significant decrease in glucose levels compared with both the control and basal-refed groups. Cholesterol levels, however, returned to values similar to the control group irrespective of diet type.

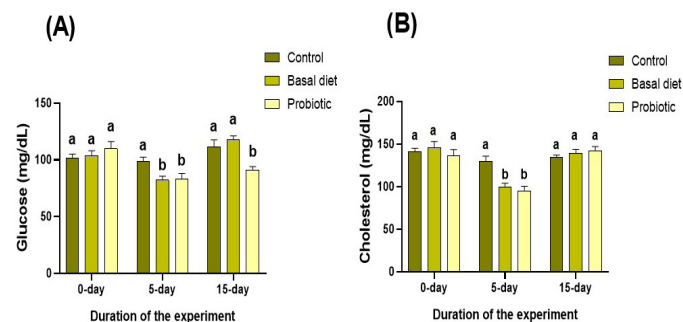


Figure 1: Changes in serum glucose (A) and cholesterol (B) concentrations in Nile tilapia measured at the beginning of the experiment, after a 5-day fasting phase, and after 15 days of refeeding with either a basal diet or a probiotic-enriched diet. Data are presented as mean $\pm$ SEM (n= 6). Differences among groups at each sampling point were analyzed using two-way ANOVA ($p < 0.05$), with distinct lowercase letters indicating statistically significant differences.

Serum total protein, albumin, and globulin concentrations (Figure 2A-C) declined significantly during the fasting period. After refeeding with the basal diet, these parameters returned to values similar to those observed in the control group. While fish refed with the probiotic-supplemented diet exhibited the concentrations exceeded both control and basal diet levels.

Serum ALT and AST (Figure 3A, B) activities increased significantly after fasting, but both enzymes returned to control values after refeeding with the basal diet. Fish refed with the probiotic-supplemented diet exhibited significantly lower levels than the control and the basal diet.

Uric acid and creatinine concentrations (Figure 3C, D) rose markedly during the fasting period. Upon refeeding with the basal diet, both parameters returned to levels similar to the control group. Fish refed with the probiotic-supplemented diet displayed significantly lower creatinine

concentrations relative to both the control and basal diet groups. In contrast, the reduction in uric acid concentration was significant only when compared with the basal diet group.

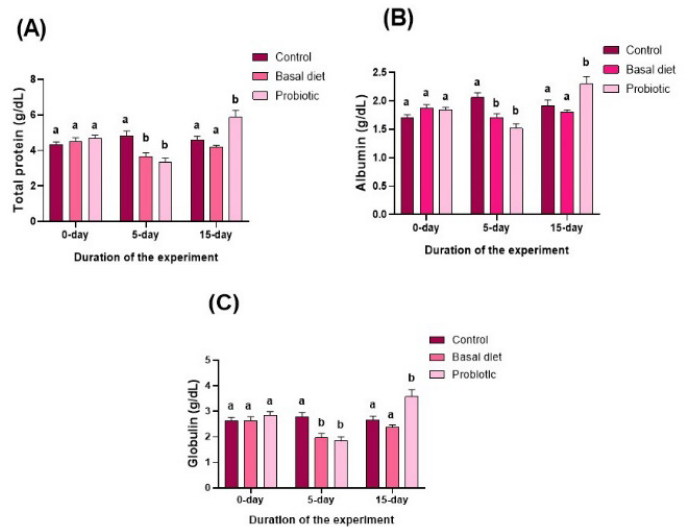


Figure 2: Changes in serum total protein (A), albumin (B), and globulin (C) concentrations in Nile tilapia measured at the beginning of the experiment, after a 5-day fasting phase, and after 15 days of refeeding with either a basal diet or a probiotic-enriched diet. Data are presented as mean $\pm$ SEM (n= 6). Differences among groups at each sampling point were analyzed using two-way ANOVA ($p < 0.05$), with distinct lowercase letters indicating statistically significant differences.

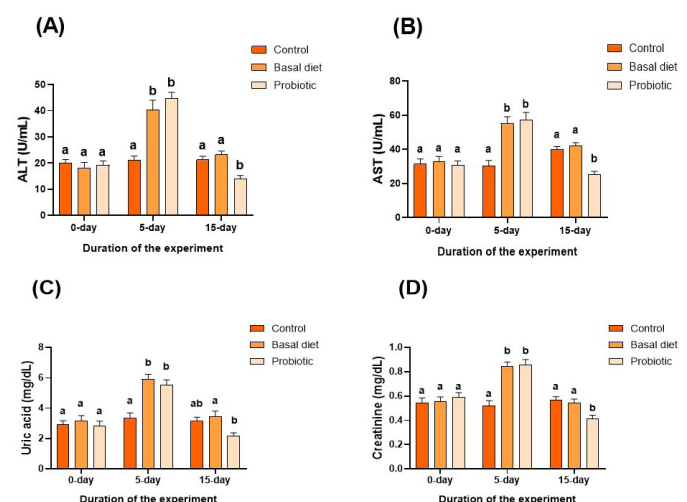


Figure 3: Changes in serum alanine aminotransferase (ALT) (A), and aspartate aminotransferase (AST) (B), uric acid (C) and creatinine (D) concentrations in Nile tilapia measured at the beginning of the experiment, after a 5-day fasting phase, and after 15 days of refeeding with either a basal diet or a probiotic-enriched diet. Data are presented as mean $\pm$ SEM (n= 6). Differences among groups at each sampling point were analyzed using two-way ANOVA ($p < 0.05$), with distinct lowercase letters indicating statistically significant differences.

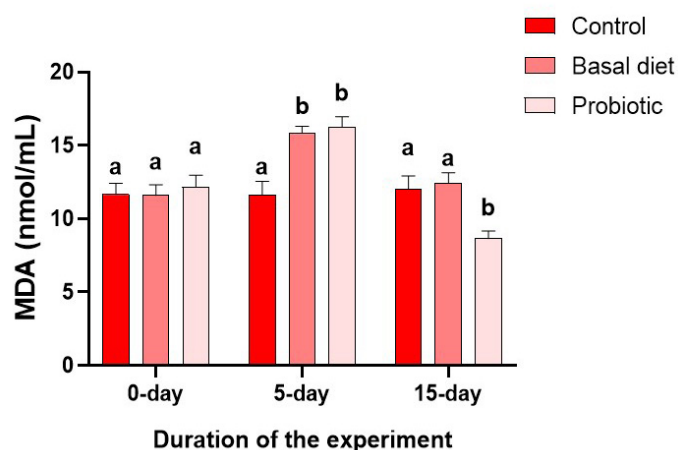


Figure 4: Changes in serum malondialdehyde (MDA) concentrations in Nile tilapia measured at the beginning of the experiment, after a 5-day fasting phase, and after 15 days of refeeding with either a basal diet or a probiotic-enriched diet. Data are presented as mean $\pm$ SEM ($n = 6$). Differences among groups at each sampling point were analyzed using two-way ANOVA ($p < 0.05$), with distinct lowercase letters indicating statistically significant differences.

MDA levels (Figure 4) increased markedly following the fasting period. Upon refeeding with the basal diet, MDA concentrations returned to values similar to the control group. Notably, fish refeed with the probiotic-supplemented diet exhibited significantly lower MDA levels than the control group.

GENE EXPRESSION ANALYSIS

The expression of the studied genes in liver and spleen tissues revealed distinct regulatory responses to fasting and subsequent refeeding. At the onset of the experiment (Figures 5, 6, 7), all groups displayed comparable expression levels, with no statistically significant differences detected, confirming a uniform baseline prior to dietary intervention.

Following fasting for 5-days, both antioxidant genes (*Cat* and *SOD-2*) exhibited a marked and statistically significant upregulation in the liver and spleen tissues of Nile tilapia, relative to the control group ($p < 0.05$). Upon refeeding with the basal diet, the expression levels of these genes normalized, returning to levels similar to those observed in the control group. Strikingly, refeeding with the probiotic-enriched diet induced a further significant upregulation of both genes, surpassing the expression levels in both the control and basal diet-refed groups (Figure 5A, B).

The pro-inflammatory cytokine genes *IL-1 β* and *TNF- α* showed a significant upregulated in the liver and spleen tissues of Nile tilapia following 5-days of fasting, in comparison with the control ($p < 0.05$). Refeeding with the basal diet returned the expression of these genes to levels

similar to those of the control group. While, refeeding with the probiotic-enriched diet resulted in a further significant upregulation of *IL-1 β* , surpassing the expression levels in both the control and basal diet-refed groups (Figure 6A, B).

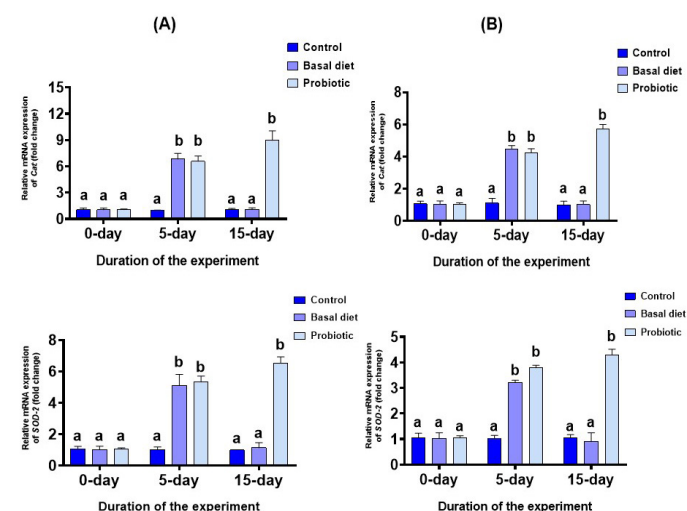


Figure 5: Expression levels of antioxidant genes catalase (*Cat*), superoxide dismutase-2 (*SOD-2*) in the liver (A) and spleen (B) tissues in Nile tilapia measured at the beginning of the experiment, after a 5-day fasting phase, and after 15 days of refeeding with either a basal diet or a probiotic-enriched diet. Data are presented as mean $\pm$ SEM ($n = 3$). Differences among groups at each sampling point were analyzed using two-way ANOVA ($p < 0.05$), with distinct lowercase letters indicating statistically significant differences.

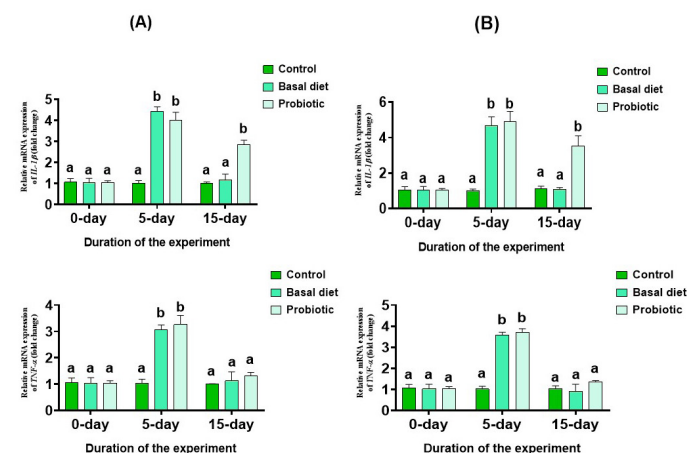


Figure 6: Expression levels of pro-inflammatory cytokine genes interleukin-1 β (*IL-1 β*), tumor necrosis factor- α (*TNF- α*) in the liver (A) and spleen (B) tissues in Nile tilapia measured at the beginning of the experiment, after a 5-day fasting phase, and after 15 days of refeeding with either a basal diet or a probiotic-enriched diet. Data are presented as mean $\pm$ SEM ($n = 3$). Differences among groups at each sampling point were analyzed using two-way ANOVA ($p < 0.05$), with distinct lowercase letters indicating statistically significant differences.

Conversely, the anti-inflammatory genes *TGF-β* and *IL-10* displayed an opposite response. Their expression was significantly downregulated in both liver and spleen tissues after fasting. While refeeding with the basal diet returned their expression to baseline levels similar to the control group. Probiotic supplementation during refeeding led to a marked upregulation of *TGF-β* and *IL-10*, surpassing levels observed in both the control and basal diet-refed groups (Figure 7A, B).

on antioxidant and immune pathways during the refeeding recovery phase offers a novel and practical perspective for aquaculture management. This study addresses this gap by providing novel evidence that probiotic-supplemented refeeding not only restores homeostasis but also suggests additional beneficial effects, strengthening antioxidant defenses and modulating inflammatory responses beyond the effects of basal diets. These findings highlight the innovative potential of integrating refeeding strategies with probiotic supplementation to support recovery and adaptive responses in aquaculture systems.

The observed shifts in serum parameters during fasting and refeeding primarily reflect hepatic metabolic adjustments (Liu *et al.*, 2022; Ntanti *et al.*, 2023; Peng *et al.*, 2026). Additionally, the liver exhibits the highest mRNA expression levels of antioxidant genes such as SOD and CAT following fasting, indicating its central role in counteracting oxidative stress (Metón *et al.*, 2003; Peter *et al.*, 2020; Rios *et al.*, 2006). While the spleen contributes more specifically to immune-related gene expression (Dimitroglou *et al.*, 2011; Nayak, 2010). This highlights the complementary roles of these organs in mediating recovery and immune modulation during nutritional stress.

In the present study, a significant decline in glucose was observed after 5-days of fasting. This reduction reflects the mobilization of endogenous energy reserves, primarily through glycogenolysis and lipolysis. Under conditions of food deprivation, glucose homeostasis is generally maintained via glycogen depletion (glycogenolysis) and/or gluconeogenesis (Furné *et al.*, 2012; Polakof *et al.*, 2012). However, the decrease in glucose observed here suggests that glycogenolysis alone was insufficient to sustain normal glycemia under nutrient-limited conditions, indicating a metabolic imbalance and possible limitations in compensatory gluconeogenic pathways.

Several studies corroborate the present findings, in Nile tilapia (Mishra *et al.*, 2024) demonstrated that a 3-day fasting period significantly reduced blood glucose levels. Similarly, Peng *et al.* (2026) reported in *Megalobrama amblycephala* that a 2–3 day fast enhanced glycolysis and redirected amino acid catabolism toward energy maintenance. In golden pompano (*Trachinotus ovatus*), Liu *et al.* (2022) observed that 7-days of starvation induced hypoglycemia and markedly altered the activities of glycolysis-related enzymes, including glucokinase (GK), pyruvate kinase (PK), and phosphofructokinase (PFK). At the molecular level, Ntanti *et al.* (2023) found that fasting in sea bass modulated the expression of glycolytic genes, highlighting that the metabolic response to food deprivation extends beyond blood biochemical parameters to transcriptional regulation.

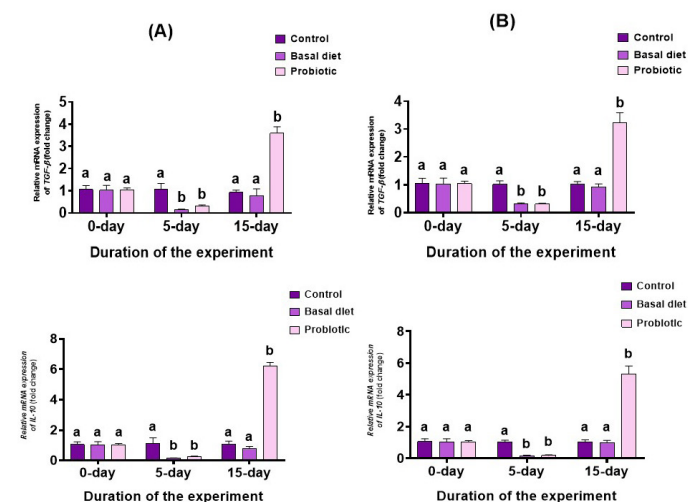


Figure 7: Expression levels of anti-inflammatory cytokine genes transforming growth factor- β (*TGF-β*), and interleukin-10 (*IL-10*) in the liver (A) and spleen (B) tissues in Nile tilapia measured at the beginning of the experiment, after a 5-day fasting phase, and after 15 days of refeeding with either a basal diet or a probiotic-enriched diet. Data are presented as mean $\pm$ SEM (n = 3). Differences among groups at each sampling point were analyzed using two-way ANOVA ($p < 0.05$), with distinct lowercase letters indicating statistically significant differences.

DISCUSSION

Aquaculture practices frequently involve periods of feed restriction, either for management purposes or due to environmental constraints, which can significantly affect fish physiology. Short-term fasting has been reported to disrupt metabolic homeostasis, increase oxidative stress, and alter immune responses in fish species (Yu *et al.*, 2025). To mitigate these adverse effects, functional diets supplemented with probiotics have gained attention as a promising strategy to enhance resilience.

Previous studies have demonstrated that probiotic supplementation improves feed utilization, growth performance, and water quality, while also supporting immune function and reducing oxidative stress in tilapia (Ahmed *et al.*, 2024; Mathew *et al.*, 2025; Yu *et al.*, 2025). However, specifically analyzing their transcriptional impact

Following refeeding with the basal diet, glucose concentrations in the present study returned to control values, confirming the reversibility of fasting-induced metabolic suppression and restoration of glycemic status. This recovery aligns with earlier findings that physiological functions can be fully restored after short-term starvation once feeding resumes (Metón *et al.*, 2003; Mishra *et al.*, 2024; Rios *et al.*, 2006). Overall, the glucose response observed here supports the conclusion that tilapia experience measurable metabolic stress during fasting but exhibit a rapid normalization of carbohydrate metabolism upon refeeding. While refeeding with the probiotic-supplemented diet decreased glucose levels, probiotic supplementation has been reported to enhance intestinal microbial balance and improve carbohydrate metabolism by increasing the efficiency of glucose utilization (Dimitroglou *et al.*, 2011; Nayak, 2010). This finding is in line with previous studies in tilapia fish fed on diet supplemented with different types of probiotics (Eissa *et al.*, 2024; Falcinelli *et al.*, 2016; Fernandes *et al.*, 2015; Metwaly *et al.*, 2018).

In the present study, cholesterol levels decreased markedly after 5-days of fasting, suggesting that Nile tilapia utilize lipid reserves as an energy source during feed deprivation (Wang *et al.*, 2017). This response is consistent with findings in several fish species, where fasting similarly reduced plasma cholesterol, including *Oncorhynchus mykiss* (Heming and Paleczny, 1987), *Salmo trutta* (Regost *et al.*, 2001), *Acipenser naccarii* (Furné *et al.*, 2012), and Nile tilapia subjected to 3–7 days of fasting (Wang *et al.*, 2019). Cholesterol serves as a structural lipid and a precursor for steroid hormones such as cortisol, which regulates multiple metabolic processes (Mommsen *et al.*, 1999); therefore, its reduction during fasting may reflect both decreased dietary supply and its utilization in metabolic and hormonal responses to nutrient limitation.

However, cholesterol responses to fasting appear highly species-specific, as no change was reported in *Piaractus brachypomus*, pirapitinga (Favero *et al.*, 2021), *Colossoma macropomum*, Tambaqui (Assis *et al.*, 2020), and *Lophiosilurus alexandri*, pacamã (Silva *et al.*, 2019), while increases were documented in *Piaractus mesopotamicus*, pacu (Favero *et al.*, 2018) and *Anabas testudineus*, climbing perch (Godavarthy *et al.*, 2012). These contrasting results highlight the variability in lipid mobilization strategies across taxa. Furné *et al.* (2012) further suggested that plasma cholesterol is more strongly influenced by dietary intake than by mobilization of endogenous reserves, which aligns with the present study, where cholesterol levels returned to control values after refeeding. Although reduced cholesterol during fasting may represent a disadvantageous physiological state due to its essential structural and endocrine roles, the normalization observed

post-refeeding confirms that this effect is reversible and that short-term fasting does not impose lasting disruption to lipid homeostasis and demonstrating that probiotic supplementation did not alter lipid recovery dynamics.

In the present study, the significant decline in total protein, albumin, and globulin levels following the fasting indicates a clear suppression of protein metabolism under nutrient-limited conditions. Similar reductions have been documented in fish subjected to starvation, reflecting decreased dietary amino acid availability and increased mobilization of endogenous proteins to support essential metabolic processes (Navarro and Gutiérrez, 1995; Polakof *et al.*, 2012).

Following refeeding with the basal diet, protein fractions in the current study returned to control levels, suggesting that normal dietary input rapidly restores hepatic protein synthesis and immune-related globulin production. Notably, fish refed with the probiotic-supplemented diet exhibited protein, albumin, and globulin levels that exceeded those of the other groups. The observed increase in serum protein, albumin, and globulin levels in fish refed with the probiotic-supplemented diet may be hypothesized to result from improved digestive efficiency or enhanced amino acid absorption or immune stimulation (Yilmaz *et al.*, 2016). Because most serum proteins are synthesized and secreted by hepatocytes, while some globulins are produced in the liver and others by immune cells such as mononuclear phagocytes (Asadi *et al.*, 2012; Tothova *et al.*, 2016), serum protein levels are closely correlated with hepatic protein synthesis (Kanani *et al.*, 2014). Thus, the observed increases likely reflect enhanced hepatic activity. These findings are consistent with previous studies in Nile tilapia, which reported higher total serum protein and globulin levels in fish fed probiotic-supplemented diets (Kumar *et al.*, 2006; Nayak *et al.*, 2007), and with results showing elevated serum albumin when fed a three-bacteria cocktail (*Lactobacillus acidophilus*, *Streptococcus thermophilus*, and *Bifidobacterium bifidum*) (Ayyat *et al.*, 2014).

The AST is a liver-specific enzyme that facilitates gluconeogenesis from amino acids in conjunction with ALT (Marie, 1994). ALT, in turn, plays a key role in amino acid synthesis and deamination under stressful conditions, helping to meet the organism's elevated energy demands (Samanta *et al.*, 2014; Van Waarde and De Wilde-Van, 1982). Both enzymes are present in several tissues, including the liver, heart, and kidney. During periods of stress, such as starvation, these tissues may become damaged, leading to the release of additional AST and ALT into the bloodstream (Cholestech, 2004). Consequently, blood levels of AST and ALT are directly proportional to the degree of tissue damage (Cholestech, 2004). The present study found an increase in serum ALT

and AST during starvation periods. Similar results were reported by Park *et al.* (2012) on *Paralichthys olivaceus*, Yarmohammadi *et al.* (2015) on *Acipenser persicus* and Dar *et al.* (2019) on *Labeo rohita* and Sakyi *et al.* (2020) on Nile tilapia, indicating that serum ALT and AST function as a major role in connecting carbohydrate and protein metabolism as it shows the important roles in protein utilization as a substrate for gluconeogenesis in Nile tilapia. Also, the increase of these enzymes in the starved group suggests that there could damage tissues/organs since the starved fish utilize the protein and carbohydrate. However, it is important to note that the magnitude and pattern of these responses may vary among species due to differences in metabolic strategies, feeding habits, and physiological adaptability to starvation (Ashouri *et al.*, 2020; Navarro and Gutiérrez, 1995; Polakof *et al.*, 2012; Sakyi *et al.*, 2020). But the fed group reveals lower levels of AST and ALT because the organs/tissues seem normal and healthy. Similar to the study in *Acipenser persicus* (Yarmohammadi *et al.*, 2015), *Acipenser baerii* (Ashouri *et al.*, 2020), and Nile tilapia (Sakyi *et al.*, 2020), the levels of serum ALT and AST were attained normal levels after refeeding. This recovery may likewise differ across species depending on their capacity for compensatory growth and metabolic restoration.

A significant decrease in the serum levels of ALT, and AST were noticed in probiotics-supplemented group. Since AST and ALT are well-established indicators of hepatic integrity, their reduction in the probiotic-supplemented group suggests improved hepatic status based on biochemical indicators and reduced hepatocellular damage (Saryono and Proverawati, 2019). Nile tilapia also revealed the same decrease of transaminases (*e.g.* ALT, and AST) under the effect of dead *Saccharomyces cerevisiae* yeast (Abdel-Tawwab *et al.*, 2008) and both of live *B. subtilis* and *S. cerevisiae* (Marzouk *et al.*, 2008), *B. licheniformis* (Soltan and El-Laithy, 2008) and *E. faecium* and *B. coagulans* (El-Moghazy *et al.*, 2015). Talib (2004) indicated that probiotics have no hepatotoxic or nephrotoxic effects in *O. niloticus* and *Mugil cephalus*.

In the present study, fasting cause a significant elevation in uric acid and creatinine levels, indicating a state of metabolic stress and enhanced catabolism of endogenous proteins. In teleost fish, creatinine and uric acid are widely recognized as indicators of muscle protein degradation and renal function, and their increase during starvation reflects a shift toward protein mobilization for energy production when lipid and carbohydrate reserves become insufficient (Navarro and Gutiérrez, 1995; Polakof *et al.*, 2012). Following refeeding in the current study, uric acid and creatinine returned to control values in fish receiving the basal diet, confirming that the fasting-induced catabolic state is reversible once adequate nutrients are restored.

Notably, fish refed with the probiotic-supplemented diet showed even lower levels than the control group, the decrease in uric acid and creatinine, markers of renal health, suggesting potential beneficial effects on renal biochemical indicators (Ali and Abdelaziz, 2014; Baothman *et al.*, 2023), suggesting improved renal clearance, reduced endogenous protein breakdown, or enhanced overall metabolic efficiency. Probiotics are known to improve intestinal microbial balance and nutrient utilization, which may reduce excessive protein catabolism and the subsequent production of nitrogenous waste products (Dawood *et al.*, 2018; Ringø *et al.*, 2018). Improved digestive efficiency and amino acid utilization can decrease the breakdown of endogenous proteins, thereby limiting the formation of metabolic by-products such as creatinine and uric acid (Hai, 2015). Additionally, probiotics may influence nitrogen metabolism and purine catabolism through microbial assimilation of nitrogenous compounds within the gut, which may reduce uric acid formation derived from purine degradation (Balcázar *et al.*, 2006). Another possible mechanism involves the enhancement of antioxidant defense systems and the reduction of oxidative stress, which protects renal and hepatic tissues from cellular damage and helps maintain normal filtration and metabolic functions (Xie *et al.*, 2024). Collectively, these physiological effects may explain the lower circulating concentrations of creatinine and uric acid observed in fish receiving probiotic-supplemented diets during the refeeding period. Similar findings have been reported in probiotic-supplemented Red tilapia (Eissa *et al.*, 2024), in which *B. subtilis* and *B. Licheniformis* decrease the levels of uric acid, urea, and creatinine, ALT, AST, ALP.

The MDA levels were significantly elevated during the 5-day fasting period compared to the refeeding phase, indicating that fasting-induced stress amplifies oxidative damage in fish, as reflected by increased MDA accumulation after fasting and recovery after refeeding. This trend aligns with previous observations in *Pseudosciaena crocea* (Zhang *et al.*, 2008) and golden pompano (*Trachinotus ovatus*) Liu *et al.* (2022). Moreover (Cheng *et al.*, 2024) reported that a 3-day fasting in Rice Flower Carp (*Cyprinus carpio*) resulted in elevated ROS and MDA levels, and after a subsequent 7-day refeeding period, these oxidative markers returned to levels statistically comparable to those of the control group. Notably, fish refed with the probiotic-supplemented diet showed a significant reduction in serum MDA, an important indicator of oxidative stress and lipid peroxidation. Lower MDA levels suggest that probiotic attenuated oxidative damage, thereby preserving hepatic and renal integrity (Ahmed *et al.*, 2015; Baothman *et al.*, 2023). Together, these results indicate that while fasting imposes temporary metabolic stress, refeeding particularly with probiotics can accelerate recovery and improve metabolic homeostasis beyond baseline levels.

The current study revealed that short-term fasting followed by refeeding significantly influences the expression of key antioxidant genes, *Cat* and *SOD-2*, in Nile tilapia. Notably, a pronounced upregulation of *Cat* and *SOD-2* in liver and spleen tissues after 5-days of fasting indicates a physiological adaptation to oxidative stress induced by nutritional deprivation. Fasting triggers metabolic changes that elevate the production of ROS, thereby necessitating a robust antioxidant response. As central components of the non-specific immune system, SOD and CAT play crucial roles in neutralizing ROS and safeguarding cellular structures such as membranes and DNA from oxidative damage (Campa-Córdova *et al.*, 2002; Castellanos-González *et al.*, 2002), so here, Nile tilapia appear to counteract oxidative damage by enhancing the activity of antioxidant enzymes.

Upon refeeding with the basal diet, the normalization of *Cat* and *SOD-2* expression to control levels reflects the restoration of metabolic homeostasis. This suggests that the oxidative stress induced by fasting is transient and reversible with adequate nutritional replenishment. The return to baseline levels also implies that the basal diet is sufficient to support recovery without further stimulating antioxidant gene expression. Interestingly, refeeding with the probiotic-supplemented diet was associated with a sustained and significant upregulation of *SOD-2* and *Cat* compared with the basal-refed and control groups, suggesting a stronger antioxidant response in fish receiving the probiotic diet. Previous studies have reported that probiotic supplementation can be associated with reduced cortisol levels and increased antioxidant enzyme activity, which may help fish better cope with oxidative stress (Elsegeny *et al.*, 2025). Therefore, the enhanced expression of antioxidant genes observed in the present study may reflect an improved physiological response during the recovery phase. Although the exact mechanisms were not investigated here, earlier research suggests that probiotics may influence host antioxidant status through several indirect pathways, including interactions with gut microbiota, improved nutrient utilization, or modulation of immune and stress-related signaling pathways (Shija *et al.*, 2023). Consequently, the elevated expression levels observed in the probiotic-refed group may indicate a beneficial association between refeeding and probiotic supplementation that supports cellular protection against oxidative damage. In addition, the liver exhibited the highest mRNA expression levels of the antioxidant enzymes *Cat* and *SOD-2* after the 5-day fasting period, highlighting its central role in regulating oxidative balance during nutritional deprivation.

The findings of the present study are consistent with prior research that demonstrated that elevated ROS levels during early fasting are primarily driven by intensified

lipid and fatty acid oxidation (Morales *et al.*, 2004). In Rice Flower Carp (*C. carpio*), Cheng *et al.* (2024) demonstrated that 3-day fasting period led to elevated levels of ROS, with a significant upregulation of antioxidant markers including GSH, SOD, CAT, GST, GR, and GPX. However, following a 7-day refeeding period, only GSH levels declined to values statistically indistinguishable from the control. In *Sparus macrocephalus*, Zhang *et al.* (2007) reported significant increase of SOD activity in the liver of fasted fish from day 3. Also, Wang *et al.* (2019) reported that Nile tilapia fasted for 3-days show an activation of antioxidant enzymes. Furné *et al.* (2009) observed increased SOD and CAT activities during periods of food restriction followed by a decline after refeeding in *Acipenser naccarii* and *Oncorhynchus mykiss* and similar results were noted by Zhao *et al.* (2024) in Largemouth Bass (*Micropterus salmoides*).

In this study, 5-days fasting markedly impaired the immune status of fish and induced inflammation in Nile tilapia which is triggered by a strong pro-inflammatory response and suppressed anti-inflammatory mediators. For instance, upregulated the levels of *IL-1 β* and *TNF- α* genes in the liver and spleen and downregulated *IL-10* and *TGF- β* genes expression. The observed upregulation of *IL-1 β* and *TNF- α* after five days of fasting indicates that nutrient deprivation acts as a physiological stressor, activating innate immune pathways. *IL-1 β* , and *TNF- α* are key mediators of inflammation and considered typical pro-inflammatory cytokines that induce host defense through an inflammatory reaction (Scapigliati *et al.*, 2001) often elevated during stress to mobilize immune defenses. In contrast, *IL-10* and *TGF- β* are key anti-inflammatory cytokines that help regulate the immune response by limiting the release of pro-inflammatory mediators, thereby preventing excessive immune activation (Koj 1998). Consistent with this, Zhao *et al.* (2024) reported that fasting in largemouth bass led to elevated levels of *TNF- α* , *IL-1 β* , and *IL-15*, accompanied by reduced *IL-10* and *TGF- β* expression in the gut. Similar patterns have been observed in common carp (*C. carpio* L.) (Zhao *et al.*, 2022) and Nile tilapia, where periods of fasting triggered stress-related gene expression and modulated immune responses (Elbialy *et al.*, 2022). The normalization of *IL-1 β* , *TNF- α* , *IL-10* and *TGF- β* genes expression upon refeeding with the basal diet suggests that nutrient availability rapidly restores homeostasis and recovery of immune balance.

Interestingly, refeeding with probiotics further enhanced *IL-1 β* expression, surpassing basal diet levels. This suggests that probiotics may act as immunostimulants. Similarly, probiotics have been shown to upregulate pro-inflammatory cytokines *IL-1 β* and *TNF- α* in Nile tilapia, enhancing disease resistance by stimulating

innate immunity (Metwaly *et al.*, 2018; Tan *et al.*, 2019). Moreover, probiotic supplementation led to pronounced upregulation of both *TGF-β* and *IL-10*, exceeding control levels as reported by Ferdoush *et al.* (2024). The observed upregulation of *IL-1β* in the probiotic-refed group may reflect activation of innate immune responses rather than a purely detrimental inflammatory state. While *IL-1β* is a key pro-inflammatory cytokine, its elevation can also indicate enhanced immune readiness. Importantly, the concurrent upregulation of anti-inflammatory cytokines (*IL-10* and *TGF-β*) suggests that this response is likely regulated and balanced rather than indicative of excessive or pathological inflammation. Similar outcomes were observed in mixed probiotic-fed Nile tilapia, where *Bacillus*, *Enterococcus*, *Lactobacillus* and *Pediococcus* spp. supplementation improved immune protection through pro-inflammatory genes *TNF-α* and *IL-1β*, and anti-inflammatory genes *IL-10* and *TGF-β* upregulation (Standen *et al.*, 2016). The combined results highlight that fasting induces immune stress, while probiotics modulate cytokine expression in a bidirectional manner, stimulating pro-inflammatory mediators for defense and simultaneously boosting anti-inflammatory regulators to maintain homeostasis. This dual modulation shows that probiotics may contribute to improved adaptive responses to environmental stressors and improve disease resistance in Nile tilapia.

It is important to note that the effects of probiotics can vary widely depending on the specific strains used and their inclusion of dosages. Differences in *Bacillus* spp., combinations of strains, and the concentration of viable cells can influence growth, immune responses, and antioxidant capacity in fish. For instance, *B. subtilis*, *B. licheniformis*, and *B. pumilus* have been reported to exert distinct physiological effects in Nile tilapia depending on the dosage applied (Richmond, 1973; Shija *et al.*, 2023; Yilmaz *et al.*, 2016). Therefore, discrepancies in reported results across studies may reflect these variations rather than inconsistencies in experimental methodology. This highlights the need to carefully consider both strain selection and dosage when interpreting probiotic effects in aquaculture.

Overall, the results indicate that fasting induces metabolic and oxidative stress, while probiotic-supplemented refeeding may enhance recovery through modulation of antioxidant and immune responses. These effects can be mechanistically explained by the activation of gluconeogenesis and protein catabolism during fasting, leading to increased hepatic enzyme activity and oxidative stress due to elevated ROS production. The upregulation of antioxidant genes such as SOD and Cat likely represents a compensatory response to counteract oxidative damage. Probiotic supplementation during refeeding appears to further enhance these protective mechanisms. Probiotics

may stimulate antioxidant defense systems through the production of bioactive compounds and activation of redox-related pathways, while also regulating cytokine expression by enhancing anti-inflammatory mediators and modulating pro-inflammatory responses. These combined effects contribute to improved recovery, reduced tissue damage, and enhanced physiological stability following nutritional stress.

A limitation of the present study is the relatively short experimental duration, which may not fully capture long-term physiological and immunological responses to probiotic supplementation. In addition, although bacterial probiotics were evaluated, the study did not include comprehensive microbiome analysis. Given the growing evidence that host-microbiota interactions play a key role in regulating metabolism and immune responses, future studies incorporating integrated microbiome profiling would provide deeper mechanistic insight into probiotic-mediated effects in aquaculture species.

CONCLUSIONS

This study indicates that short-term fasting in Nile tilapia induces metabolic stress and oxidative imbalance, as reflected by changes in selected biochemical markers and transcriptional regulation of antioxidant and inflammatory genes. Refeeding with a basal diet restored most measured biochemical parameters toward baseline levels, indicating recovery from fasting-induced alterations. Probiotic supplementation during refeeding was associated with more pronounced changes, including reduced biochemical indicators related to hepatic and renal stress, enhanced antioxidant-related responses, and modulation of inflammatory cytokine expression, involving both pro-inflammatory and anti-inflammatory pathways. These findings suggest that probiotics may support recovery following nutritional stress; however, further studies involving longer-term feeding trials and additional functional assessments are required to confirm these effects and their broader applicability in aquaculture practices.

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NOVELTY STATEMENT

This is the first study to evaluate the synergistic effects of short-term fasting and probiotic-assisted refeeding in Nile tilapia at both physiological and molecular levels. The study demonstrates that probiotic supplementation dur-

ing the post-fasting recovery phase effectively improves antioxidant defenses, modulates immune-related cytokine expression, and alleviates hepatic and renal stress. This integrated strategy introduces a novel nutritional approach for enhancing stress resilience and recovery efficiency in aquaculture systems.

AUTHOR'S CONTRIBUTION

The author confirms sole responsibility for manuscript preparation.

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IRB STATEMENT

All experimental procedures were reviewed and approved by the Research Ethics Committee of the Faculty of Science (REC-FSCI-SVU) (Approval No. 002/11/25; Approval date: 11 Nov. 2025) and were conducted in accordance with international guidelines for the ethical treatment of animals in research.

INFORMED CONSENT STATEMENT

Not applicable.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are available in the repository.

ETHICAL STATEMENT

All experimental procedures were approved by the Research Ethics Committee of the Faculty of Science (REC-FSCI-SVU) (Approval No. 002/11/25), and were conducted in accordance with internationally accepted guideline for animal welfare and ethical treatment in scientific experiments.

GENERATIVE AI AND AI ASSISTED TECHNOLOGY STATEMENT

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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