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Research article

Influence of Dietary Fermented Mulberry (*Morus alba*) Leaf Inclusion on Growth Performance and Immune Responses in Nile Tilapia (*Oreochromis niloticus*)

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Abstract: This study investigated the effects of dietary fermented mulberry leaves (FML) on growth performance, carcass traits, muscle composition, and innate immune responses of juvenile Nile tilapia (*Oreochromis niloticus*). The use of functional feed additives is increasingly emphasized in aquaculture to reduce antibiotic dependence, particularly during early life stages when fish are highly susceptible to disease. Juvenile tilapias were fed diets containing 0% (control), 1%, 2%, or 4% FML for 60 days. Dietary supplementation with FML significantly improved weight gain, length gain, average daily gain, feed conversion ratio, and survival ($P < 0.05$). The 2% FML diet produced the most favorable outcomes in muscle nutritional quality, including elevated crude protein and reduced lipid and fiber contents ($P < 0.05$). Immune parameters were also enhanced in fish receiving FML. Agglutination titers, lysozyme activity, and phagocytic efficiency increased significantly across FML-treated groups ($P < 0.05$). Moreover, the 2% inclusion level resulted in higher concentrations of the pro-inflammatory cytokines TNF-α and IL-1, indicating activation of innate immune pathways ($P < 0.05$). These findings demonstrate that FML, particularly at a 2% dietary inclusion, is an effective and environmentally sustainable feed supplement capable of improving growth performance, muscle composition, and immune competence in juvenile Nile tilapia. The incorporation of FML may therefore contribute to reduced reliance on antibiotics and support more sustainable aquaculture practices.

Keywords: Fermented mulberry leaves; nile tilapia; immunity

1. Introduction

The global aquaculture of Nile tilapia (*Oreochromis niloticus*) is continually driven by several key attributes: its rapid growth rate, significant tolerance to environmental uncertainty stressors, and relatively low production costs [1, 2]. This high-yield freshwater fish holds substantial economic importance, with its world market valuation projected to reach between USD 9.8 billion and USD 15.1 billion by 2025 [3, 4]. Despite these advantages, high fry mortality during the nursery phase remains a major constraint, largely due to the immature immune systems of juvenile fish. In recent years, the emergence and re-emergence of pathogenic threats, including *Streptococcus* spp., *Aeromonas hydrophila*, *Flavobacterium columnare*, and Tilapia

Lake Virus (TiLV) have significantly disrupted production by increasing disease incidence and mortality [5, 6]. Consequently, there is a growing trend towards seeking safe and environmentally friendly alternative products, particularly those derived from herbal plants and probiotics.

Among these, Mulberry (*Morus alba*) is recognized for its wide array of pharmacological properties, including antidiabetic, antimicrobial, antimutagenic, antioxidant, anticancer, anti-anxiety, antiparasitic, anti-stress, immunostimulating, cholesterol-lowering, kidney-protective, and liver-protective effects. Its beneficial composition includes active compounds such as carotene, vitamin B1, folic acid, isoquercetin, quercetin, tannins, flavonoids, and saponins [7-9]. These bioactive compounds are known to enhance immune function, antioxidant capacity, and disease resistance in fish species, potentially reducing mortality rates. In largemouth bass (*Micropterus salmoides*), incorporating dried mulberry leaves into the diet has been reported to enhance growth performance, improve blood lipid parameters, increase antioxidant enzyme activities, and alleviate liver inflammation [10]. Similarly, research on Indian minor carp (*Labeo bata*) demonstrated that substituting up to half of the fishmeal with fermented mulberry leaves, while keeping dietary fiber content under 5.63%, led to improved growth rates and elevated digestive enzyme activities [11]. Nevertheless, the direct use of raw mulberry leaves in animal feed has been limited by their high dietary fiber content and the presence of anti-nutritional factors (ANFs) like tannins and phytic acid, which can impede nutrient digestion and absorption in livestock [12, 13]. Biological fermentation presents a powerful solution for mitigating these adverse effects of ANFs in food and feed. This transformative process not only degrades these undesirable compounds but also significantly enhances the nutritional value of the product by releasing more digestible nutrients and fostering the growth of beneficial microorganisms [14, 15]. Fermentation by beneficial microbes such as lactic acid bacteria can reduce anti-nutritional factors while increasing the bioavailability of nutrients. The inclusion of fermented mulberry leaf meal has been demonstrated to enhance growth, boost antioxidant activity, and reduce lipid levels in largemouth bass and Indian minor carp [10, 11]. However, limited studies have investigated the effects of fermented mulberry leaf inclusion on juvenile Nile tilapia growth, immune status, and survival under controlled aquaculture conditions.

Consequently, this research aimed to explore the impact of incorporating fermented mulberry leaves into the diets of juvenile tilapia. We evaluated various inclusion ratios to assess their effects on growth performance, survival rates, and overall health of the fish. Ultimately, this study sought to minimize reliance on antibiotics and synthetic additives while promoting safer, more sustainable aquaculture practices.

2. Materials and Methods

2.1 Ethical statement

All experimental protocols, methods, and feeding regimes were approved on 30 July 2025 by the Institutional Animal Care and Use Committee of the Faculty of Fisheries Technology and Aquatic Resources, Maejo University (Chiangmai, Thailand).

2.2 Feed preparation

Experimental feeds were formulated using commercial ready-to-use pellets (Betagro 831) as the basal feed. Fermented mulberry leaves were prepared by combining 250 g of mulberry leaves, 150 g of banana shoots, and 150 g of molasses with 1 L of water and a yogurt inoculum containing 10^8 CFU/mL *Lactobacillus casei*, followed by anaerobic fermentation for 30 days. The prepared fermented mulberry leaf powder was incorporated at inclusion levels of 0% (control), 1%, 2%, and 4% per kilogram of feed. Each diet was thoroughly mixed to ensure homogeneity, coated with 3% gelatin to enhance palatability and bind the ingredients, and then redried in a hot air oven at 55 °C for 24 h to achieve a stable moisture content before storage. The finalized feed samples were packaged in airtight bags and stored at 4°C until use. Before the feeding trial, all experimental diets underwent proximate analysis to determine their nutritional composition, including moisture, ash, crude protein, crude fat, and crude fiber content, following standard methods [16]. The nitrogen-free extract (NFE), representing carbohydrate content, was determined by difference. Fish were fed twice daily, specifically between 08:30-09:30 h and 16:30-17:30 h. To ensure optimal nutrition relative to growth, the daily feed ration was adjusted every 14 days based on the incremental increase in fish biomass.

The feeding trial was conducted for a total of 60 days, with fish growth performance monitored at 30-day intervals until the experiment's conclusion. (Table 1).

Table 1. The nutrient profile in mulberry leaves, fermented mulberry leaves, and experimental feeds.

	fermented mulberry leaves (MBL)	mulberry leaves (ML)	control (MBL0)	1% (MBL1)	2% (MBL2)	4% (MBL4)
Moisture (%)	4.26 ± 0.21^{d}	6.59 ± 0.02^{c}	3.25 ± 0.02^{b}	3.22 ± 0.03^{b}	3.01 ± 0.02^{b}	2.38 ± 0.05^{a}
Ash (%)	23.91 ± 0.06^{c}	12.90 ± 0.02^{b}	10.80 ± 0.01^{a}	11.13 ± 0.02^{a}	11.27 ± 0.36^{a}	11.45 ± 0.07^{a}
Protein (%)	24.08 ± 1.32^{a}	18.98 ± 0.74^{a}	34.46 ± 0.28^{b}	34.62 ± 1.02^{b}	35.45 ± 2.58^{b}	36.13 ± 0.00^{b}
Lipid (%)	9.49 ± 0.29^{d}	6.52 ± 0.07^{c}	5.17 ± 0.04^{a}	5.51 ± 0.09^{ab}	6.09 ± 0.05^{bc}	6.34 ± 0.03^{c}
Fiber (%)	11.53 ± 0.45^{b}	16.59 ± 0.26^{c}	9.86 ± 0.06^{a}	9.93 ± 0.09^{a}	10.25 ± 0.15^{a}	10.81 ± 0.12^{ab}
NFE (%)	26.72 ± 1.54^{a}	38.42 ± 0.78^{b}	36.46 ± 0.24^{b}	35.58 ± 0.91^{b}	33.93 ± 2.50^{b}	32.89 ± 0.22^{ab}

Note:Values in the same row with different superscripts are significantly different ($P < 0.05$)

2.3. Fish and feeding trial

Nile tilapia (*Oreochromis niloticus*) fingerlings were procured from the Faculty of Fisheries Technology and Aquatic Resources, Maejo University. Before the commencement of the experiment, all fingerlings underwent a 14-day acclimatization period, during which they were housed in cages and fed a control diet to apparent satiation twice daily (morning and evening). A total of 360 fingerlings, with an initial mean body weight of 13.06±2.64 grams, were then randomly selected and their individual lengths and weights measured. Subsequently, the fingerlings were evenly distributed into experimental cages measuring 1×1×1 meter (width × length × height). The experiment consisted of five dietary treatments (control and four levels of fermented mulberry leaf meal) with three replicates per treatment, each replicate containing 30 fish per cage. Throughout the experimental duration, fish were fed a daily ration equivalent to 5% of their total body weight, administered in two equal portions per day.

2.4 Samples collection

Upon the conclusion of the feeding trial, all experimental fish underwent a 24-hour fasting period to facilitate complete gut evacuation. To minimize stress during sampling, fish from each replicate cage were first anaesthetized in a 50 ppm clove oil solution. Following anesthesia, the final total count and body weight of fish in each cage were measured to determine growth performance indices. Subsequently, six fish were randomly selected per treatment for blood collection to analyse immunological responses (bacterial agglutination, lysozyme activity, and respiratory burst assays). Blood was drawn from the caudal vein of these selected fish using a sterile 1 mL syringe and immediately transferred into Eppendorf tubes. These samples were left to coagulate for 4 hours at room temperature. The coagulated samples were then subjected to centrifugation at 3,500 × g for 5 minutes. The resulting supernatant (serum) was carefully harvested and immediately frozen at -80 °C for later biochemical and immunological analyses.

2.5 Growth Performance Evaluation

Throughout the experimental period, fish growth was periodically monitored. A random sub-sample comprising 30% of the fish from each cage was weighed (g) and measured for total length (cm). To ensure consistent nutritional intake, feed rations were meticulously adjusted biweekly based on the observed weight gain, thereby maintaining the prescribed feeding rate at 5% of the total fish biomass. At the conclusion of the 60-day feeding trial, all fish within each experimental unit were enumerated and weighed to assess final growth performance. The following parameters were subsequently calculated: average weight gain (WG, g), average length gain (LG, cm), average daily gain (ADG), feed conversion ratio (FCR), and survival rate (SR).

2.6 Carcass quality and Body composition analysis

At the end of the feeding trial, five fish were randomly sampled from each experimental replicate. To assess yield and body composition, each specimen was meticulously dissected. The key bodily components—

including the edible muscle, fins, bones, and gonads were separated and individually weighed. The weight data obtained from this detailed physical analysis were subsequently used to calculate critical morphometric and yield indicators. These parameters included the edible flesh percentage, dress-out percentage, carcass waste percentage, and the hepatosomatic index (HSI), determined according to the established methodology described by Hasan et al. [17]. To determine the nutritional profile of the fish, muscle samples were initially minced and then subjected to drying in a hot air oven until a constant weight was achieved. The dried tissue was subsequently ground into a homogeneous powder for analysis. Proximate analysis was performed to quantify moisture, ash, crude protein, crude fat, and crude fiber content. The carbohydrate fraction, reported as Nitrogen-Free Extract (NFE), was calculated using the formula: NFE (%) = 100 - (% Moisture + % Ash + % Crude Fiber + % Crude Fat + % Crude Protein). All procedures adhered to the methods outlined by AOAC [16].

2.7 Bacterial agglutination activity

Antibody levels, specifically agglutination titers, were quantified using U-bottom microtiter plates. The assay was conducted following the modification of the protocols by [18, 19]. Briefly, serum samples were subjected to two-fold serial dilutions (ranging from 1:2 to 1:128) in phosphate-buffered saline (PBS). Subsequently, 50 µL of an *Aeromonas hydrophila* bacterial suspension (optical density at 540 nm [OD_{540nm}] = 1.0; equivalent to 10^7 cells/mL) was added to each well containing the diluted serum. The plates were then incubated at room temperature for 24 hours, after which the agglutination titer of the antibody was determined. The agglutination titer was defined as the reciprocal of the highest dilution of serum that resulted in complete agglutination of the bacterial cells.

2.8 Lysozyme determination

Serum lysozyme activity was determined spectrophotometrically, based on the lysis of *Micrococcus lysodeikticus*, following the method described by [20]. Briefly, 25 µL of serum was combined with 175 µL of a *Micrococcus lysodeikticus* bacterial suspension in a microtiter plate well. The decrease in turbidity, indicating bacterial lysis, was subsequently measured at an optical density of 540 nm (OD_{540nm}) using a microplate reader. Readings were recorded every 30 seconds for a total duration of 10 minutes.

2.9 Respiratory burst activity

The respiratory burst activity of leukocytes, serving as an indicator of their oxidative killing capacity, was assessed following the method described by Secombes [21]. Briefly, isolated leukocytes were seeded into a 96-well microtiter plate and mixed with 100 µL of 0.2 % Nitro blue tetrazolium chloride (NBT) solution. The plate was then incubated at room temperature for 2 hours. Upon completion of the incubation, the supernatant was carefully discarded. The wells were then thoroughly washed with methanol, followed by an additional wash with 70% methanol, and allowed to air-dry at room temperature for 2 minutes. To solubilize the formazan product, dimethyl sulfoxide (DMSO) and 2N KOH were sequentially added to the original wells. The entire well contents were then transferred to a new 96-well microtiter plate, and the optical density at 655 nm (OD_{655nm}) was measured using a Microplate reader.

2.10 RNA extraction, cDNA synthesis, and quantitative real-time PCR (qPCR)

At the end of the feeding trial, liver samples (~0.2 g) were excised from three randomly selected fish per treatment group. Samples were immediately preserved in RNAlater® (Amnion, Cambridgeshire, UK) and stored at –80 °C until RNA extraction. Total RNA was isolated using 1 mL of QIAzol® Lysis Reagent (Qiagen, USA) and further purified using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Residual genomic DNA was eliminated by DNase I treatment (Thermo Fisher Scientific, USA). RNA purity and concentration were assessed via absorbance at 260 and 280 nm using a Synergy™ H1 Multi-Mode Microplate Reader (BioTek Instruments Inc., USA), while RNA integrity was confirmed by electrophoresis on a 2% agarose gel. First-strand cDNA synthesis was performed using 1 µg of total RNA and the RevertAid™ First-Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the manufacturer's protocol. Synthesized cDNA was treated with RNase H (Invitrogen, USA) at 37 °C for 20 min and stored at –20 °C until use.

Quantitative real-time PCR was carried out on a PCRmax ECO 48 Real-Time PCR System (PCRmax, UK) using Maxima™ SYBR Green/ROX qPCR Master Mix (2×) (Thermo Fisher Scientific, Lithuania). Primer sequences used for amplification are provided in Table 2. β-actin was selected as the reference gene for normalization. Primer efficiency was validated via standard curve analysis using serially diluted cDNA, ensuring correlation coefficients (R^2) > 0.99. A melt curve analysis was performed post-amplification to confirm product specificity. Each qPCR reaction was performed in triplicate in a 20 μL volume, comprising 2 μL of 1:100 diluted cDNA, 10 μL of SYBR Green Master Mix, 1 μL of each primer (1 pmol), and 6 μL of nuclease-free water. The thermal cycling protocol included an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 s, and annealing/extension at 60 °C for 30 s. Relative gene expression was quantified using the 2^−ΔΔCt method.

Table 2. Primers used to quantify the relative gene expression

Genes	Primer sequences (5′ - 3′)	References
TNF-α	F-GCTGGAGGCCAATAAAATCA R-CCTTCGTCAGTCTCCAGCTC	Kayansamruaj et al. [22]
IL1-β	F-AAGATGAATTGTGGAGCTGTGTT R-AAAAGCATCGACAGTATGTGAAAT	Chadzinska et al. [23]
Actin	F-TGGTGGGTATGGGTCAGAAAG R- TGTTGGCTTTGGGGTTCA	XM_003443127

2.11 Statistical Analysis

All experimental data were subjected to one-way analysis of variance (ANOVA) to determine significant differences among treatment groups. When statistical significance was detected, Tukey's Honest Significant Difference (HSD) post hoc test was employed for multiple comparisons of treatment means. All statistical analyses were conducted using SPSS for Windows (Version 30.0), with a significance level established at $p < 0.05$.

3. Results and Discussion

3.1 Growth Performances

Table 3 presents the effects of dietary fermented mulberry leaf (FML) inclusion on the growth performance of juvenile Nile tilapia. Fish received FML at inclusion levels of 1%, 2%, and 4% (MBL1, MBL2, and MBL4, respectively) exhibited significantly greater ($P < 0.05$) weight gain (WG), length gain (LG), and average daily gain (ADG) compared to the control group (MBL0). Specifically, WG increased from 45.25 ± 2.79 g in the control to 78.52 ± 1.93 g (MBL1), 81.30 ± 1.81 g (MBL2), and 77.33 ± 1.90 g (MBL4). LG rose from 7.07 ± 0.17 cm to a range of 10.09 ± 0.16 to 10.66 ± 0.22 cm, while ADG improved from 0.75 ± 0.09 g/day to 1.29 ± 0.06 to 2.71 ± 0.06 g/day across FML-treated groups. No significant differences ($P > 0.05$) were observed among the FML-treated groups, indicating that increasing inclusion beyond 1% did not yield further statistical improvement.

Feed conversion ratio (FCR) was significantly reduced ($P < 0.05$) in MBL1 (1.25 ± 0.01) and MBL2 (1.24 ± 0.04) compared to MBL0 (1.43 ± 0.03), indicating improved feed efficiency. Although MBL4 (1.34 ± 0.02) showed numerically better FCR than the control, the difference was not statistically significant ($P > 0.05$). Survival rates (SR) were also significantly higher ($P < 0.05$) in all FML-supplemented groups compared to the control, with no statistical differences among the FML treatments.

These improvements in growth performances and feed efficiency align with previous findings on the use of fermented plant-based additives in aquaculture diets [24, 25]. The enhanced outcomes are likely linked to the fermentation process, which degrades anti-nutritional factors (ANFs) such as phytic acid and tannins—compounds known to inhibit nutrient digestibility and mineral bioavailability [26–28]. Fermentation not only mitigates these limitations but also promotes the release of bioactive compounds and beneficial microbial metabolites, which may further enhance digestive enzyme activity and nutrient assimilation [29-30]. Moreover, improved survival rates observed in FML-fed groups may be attributed to strengthened physiological resilience, potentially resulting from enhanced immunity and gut health, as reported in other

studies using fermented herbal additives [31-33]. However, no dose-dependent relationship was observed beyond the 2% inclusion level, suggesting a possible plateau effect in nutrient uptake or microbial stimulation. Future work may focus on investigating the optimal dosage window and long-term physiological impacts.

Table 3. Growth performances of juvenile tiapla fed with the experimental diets for 60 days.

	control (MBL0)	**1% (MBL1)**	**2% (MBL2)**	**4% (MBL4)**
WG (g)	45.25± 2.79^{a}	78.52 ± 1.93^{b}	81.30 ± 1.81^{b}	77.33 ± 1.90^{b}
LG (cm)	7.07 ± 0.17^{a}	10.11 ± 0.26^{b}	10.66 ± 0.22^{b}	10.09 ± 0.16^{b}
ADG (g/day)	0.75 ± 0.09^{a}	1.31 ± 0.06^{b}	1.35 ± 0.06^{b}	1.29 ± 0.06^{b}
FCR	1.43 ± 0.03^{b}	1.25 ± 0.01^{a}	1.24 ± 0.04^{a}	1.34 ± 0.02^{ab}
SR (%)	94.44 ±1.92^{a}	98.89 ±0.83^{b}	100 ± 0.00^{b}	98.89 ±0.63^{b}

Note: Values in the same row with different superscripts are significantly different ($P < 0.05$). WG = Weight Gain, LG = Length Gain, ADG = Average daily gain, FCR = Food Conversion Rate and SR = Survival Rate

3.2. Carcass Quality and Muscle Composition

In addition to growth performance, body composition is a critical parameter in nutritional studies, serving as a reliable indicator of feed quality, carcass yield, and the overall nutritive value of fish muscle [33-34]. The effects of dietary fermented mulberry leaf (FML) supplementation on carcass traits of juvenile *Oreochromis niloticus* are summarized in Table 4. No significant differences ($P > 0.05$) were observed among treatments in edible flesh percentage (EF: 32.96 ± 0.76% to 34.65 ± 0.62%), dress-out percentage (Do: 0.43 ± 0.01% to 0.44 ± 0.00%), or carcass waste (CW: 56.37 ± 0.26% to 57.41 ± 0.97%), indicating that FML supplementation did not adversely affect marketable yield. However, the hepatosomatic index (HSI) showed a significant increase ($P < 0.05$) in fish fed 4% FML (MBL4: 1.34 ± 0.16%) compared to the control group (MBL0: 0.61 ± 0.00%). Intermediate HSI values were recorded in the 1% and 2% FML groups (MBL1: 1.05 ± 0.09% and MBL2: 1.17 ± 0.23%), though these did not differ statistically from either the control or MBL4. Given the liver's central role in metabolism and detoxification, this elevation in HSI at higher inclusion levels may reflect hepatic metabolic stress or lipid accumulation, despite the absence of histological evidence of damage. Further studies employing liver histopathology and biochemical profiling are warranted to clarify this response.

Table 4. Effect of mulberry leaves supplemented in the Nile tilapia diet on carcass quality.

	control (MBL0)	**1% (MBL1)**	**2% (MBL2)**	**4% (MBL4)**
EF (%)	32.96 ± 0.76^{a}	34.65 ± 0.62^{a}	34.14 ± 0.29^{a}	33.86 ± 0.24^{a}
Do (%)	0.43 ± 0.01^{a}	0.43 ± 0.01^{a}	0.43 ± 0.00^{a}	0.44 ± 0.00^{a}
CW (%)	57.41 ± 0.97^{a}	56.91 ± 0.69^{a}	57.1 ± 0.21^{a}	56.37 ± 0.26^{a}
HSI (%)	0.61 ± 0.00.^{a}	1.05 ± 0.09^{ab}	1.17 ± 0.23^{ab}	1.34 ± 0.16^{b}

Note: Values in the same row with different superscripts are significantly different ($P < 0.05$)
EF = Edible flesh, Do = Dress-out percentage, CW = Carcass waste and HSI = Hepatasomatic index

Muscle proximate composition results are presented in Table 5. No significant differences ($P > 0.05$) were observed in muscle moisture or ash content across the groups. However, fish receiving 2% FML (MBL2) demonstrated a significantly higher crude protein content (53.61 ± 0.32%) compared to the control (47.05 ± 0.23%) and the 4% group (47.00 ± 0.09%) ($P < 0.05$). This enhancement in muscle protein is likely due to improved protein digestibility and assimilation, facilitated by fermentation processes that reduce anti-nutritional factors such as tannins and phytic acid, known to inhibit protein utilization. Simultaneously, the MBL2 group exhibited a marked reduction in muscle lipid content (6.61 ± 0.03%) compared to MBL0 (8.35 ± 0.09%) and MBL4 (7.85 ± 0.06%) ($P < 0.05$). These lipid-lowering effects may stem from the presence of polyphenols and flavonoids in FML, which have been shown to modulate lipid metabolism and enhance

hepatic lipid regulation in fish. A corresponding decrease in muscle crude fiber content in MBL2 (4.14 ± 0.02%) further suggests improved feed fiber utilization post-fermentation.

Additionally, the nitrogen-free extract (NFE), an indicator of carbohydrate reserves, was significantly reduced ($P < 0.05$) in MBL1 (26.45 ± 0.84%) and MBL2 (26.09 ± 0.23%) compared to MBL0 (30.89 ± 0.44%) and MBL4 (31.06 ± 0.63%). This reduction implies a shift in energy partitioning, favoring protein deposition over carbohydrate storage, supporting the observed enhancement in lean tissue accretion. These findings align with previous reports demonstrating that mulberry leaf supplementation can enhance muscle protein content in *Oreochromis niloticus* [36] and improve protein-to-fat ratios in *Megalobrama amblycephala* [29]. Overall, dietary inclusion of FML at 2% appears optimal for improving muscle nutritional quality by increasing protein and reducing lipid and carbohydrate content without compromising carcass yield. Nevertheless, the increase in HSI at higher inclusion levels suggests that caution is needed when exceeding optimal dosages. Future investigations incorporating liver enzyme assays, histological evaluation, and gut microbiome analysis could provide further insights into the metabolic and immunological responses elicited by FML supplementation.

Table 5. Muscle composition of juvenile Nile tilapia fed with the experimental diets.

	control (MBL0)	**1% (MBL1)**	**2% (MBL2)**	**4% (MBL4)**
Moisture (%)	6.84 ± 0.22^{a}	7.33 ± 0.76^{a}	6.98 ± 0.34^{a}	7.57 ± 0.77^{a}
Ash (%)	2.60 ± 0.32^{a}	2.29 ± 0.07^{a}	2.57 ± 0.04^{a}	2.35 ± 0.04^{a}
Lipid (%)	8.35 ± 0.09^{c}	7.90 ± 0.03^{b}	6.61 ± 0.03^{a}	7.85 ± 0.06^{b}
Fiber (%)	4.27 ± 0.01^{b}	4.21 ± 0.02^{ab}	4.14 ± 0.02^{a}	4.16 ± 0.02^{a}
Protein (%)	47.05 ± 0.23^{a}	51.82 ± 0.35^{b}	53.61 ± 0.32^{c}	47.00 ± 0.09^{a}
NFE (%)	30.89 ± 0.44^{b}	26.45 ± 0.84^{a}	26.09 ± 0.23^{a}	31.06 ± 0.63^{b}

Note: Values in the same row with different superscripts are significantly different ($P < 0.05$)

3.3 Immune Responses

Figures 1 and 2 illustrate the effects of dietary fermented mulberry leaf (FML) supplementation on various immune parameters in Nile tilapia, including humoral and cellular immune markers. Key immune indices analyzed included agglutination titer, lysozyme activity, bacterial phagocytic efficiency, and the expression levels of pro-inflammatory cytokines—Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-1 (IL-1). Fish that received FML-supplemented diets exhibited significantly higher agglutination titers than those in the control group ($P < 0.05$), as shown in Figure 1A. Consistent titers of 6 were recorded across all treatment groups (1%, 2%, and 4% FML), compared to a value of 5 in the control, suggesting an enhancement in humoral immunity associated with FML inclusion. A dose-dependent increase in serum lysozyme activity was observed with FML supplementation ($P < 0.05$), as shown in Figure 1B. The highest activity was detected in the 2% FML group (4.73 U/ml), followed by 4% (4.59 U/ml) and 1% (4.22 U/ml), in contrast to the control group (2.84 U/ml). Given lysozyme's role in degrading bacterial cell walls, this elevation underscores the potentiation of non-specific immune defense mechanisms. Phagocytic activity of leukocytes was significantly enhanced in all FML-treated groups compared to the control ($P < 0.05$), as depicted in Figure 1C. Efficiency rose from 0.236 μg/ml in the control to 0.287 μg/ml, 0.310 μg/ml, and 0.309 μg/ml in the 1%, 2%, and 4% FML groups, respectively. This indicates that FML supports the cellular immune response by promoting more effective pathogen clearance via phagocytosis. The immunostimulatory effect of dietary mulberry leaf supplementation was confirmed by changes in TNF-α and IL-1expression (Figures 2A and 2B, respectively). Specifically, the 2% group (MBL2) exhibited the highest TNF-α mRNA level, significantly exceeding all other treatments ($P < 0.05$), suggesting a robust inflammatory response at this concentration. In contrast, IL-1 expression was significantly induced across all inclusion levels 1% (MBL1), 2% (MBL2), 4% (MBL4), with these elevated values statistically comparable to one another but significantly greater than the control group ($P < 0.05$). Collectively, these cytokine profiles indicate that the bioactive components in mulberry leaves successfully activate inflammatory signaling pathways when supplemented at appropriate dietary concentrations.

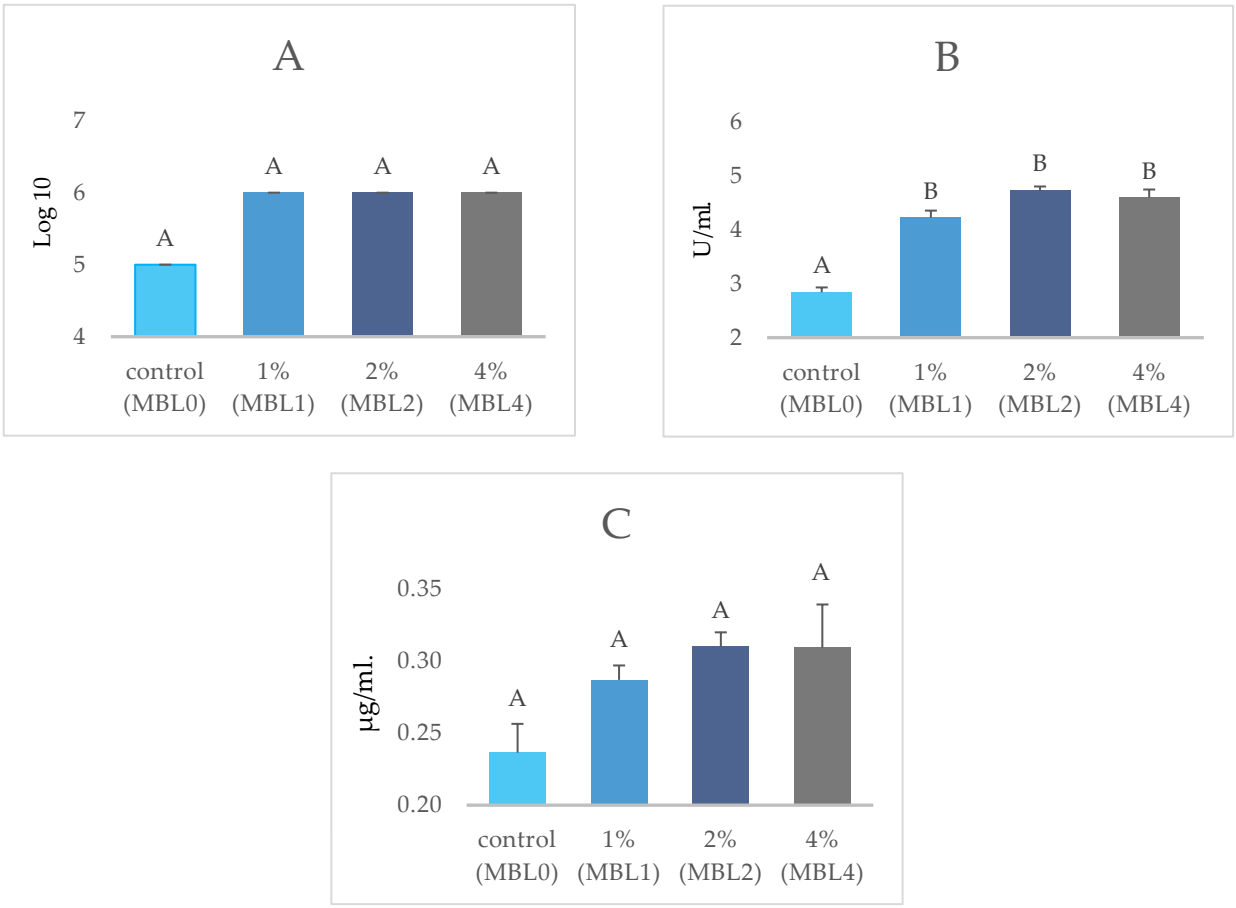

Figure 1. Agglutination titer **(**A**)**, Lysozyme determination **(**B**)**, and testing the efficiency of destroying bacteria of white blood cells (C) of fish fed experimental diets supplemented with different levels of mulberry leaves. Different power signs indicate significant differences between groups ($P < 0.05$).

These results indicate that FML supplementation significantly enhances both innate and adaptive immune responses in juvenile Nile tilapia. Improvements in humoral immunity (lysozyme activity and agglutination titers), innate cellular defense (phagocytic efficiency), and pro-inflammatory cytokine expression reflect a comprehensive upregulation of the immune system. Notably, the 2% inclusion level consistently yielded the most pronounced effects across all parameters, implying a threshold beyond which additional FML does not further enhance immunity and may even result in diminished responses. The inherent immune system is crucial for protecting fish health against microbial invaders [37 - 38]. This defense mechanism operates by inhibiting pathogen entry, mediating inflammatory reactions, and facilitating tissue repair [39]. Research suggests that immune responses in aquatic species can be significantly stimulated by dietary practices [40], gastrointestinal tract health [41], and ecological interactions, leading to increased resistance against disease and environmental stress. This enhancement occurs through the activation of immune cells and the modulation of the immune response towards anti-inflammatory cytokine production [42 - 43]. The resulting improvement in the first line of defense is frequently quantified by measuring parameters such as lysozyme activity, agglutination titers, and the rate of bacterial phagocytosis. Likely mediated by bioactive compounds in mulberry, such as flavonoids and saponins. These compounds, further potentiated through fermentation, may increase bioavailability and functional efficacy [7, 27, 44]. Furthermore, the significant upregulation of TNF-α and IL-1 aligns with previous findings in *Megalobrama amblycephala* and *Oreochromis niloticus* demonstrating that plant-derived polysaccharides can modulate immune gene expression and confer anti-inflammatory effects [29, 36]. Given that juvenile tilapia possess an underdeveloped immune system and are particularly vulnerable during early developmental stages, the

immunostimulatory effect of FML is especially valuable. The enhanced immune capacity may reduce reliance on antibiotics in aquaculture, supporting more sustainable and health-conscious farming practices.

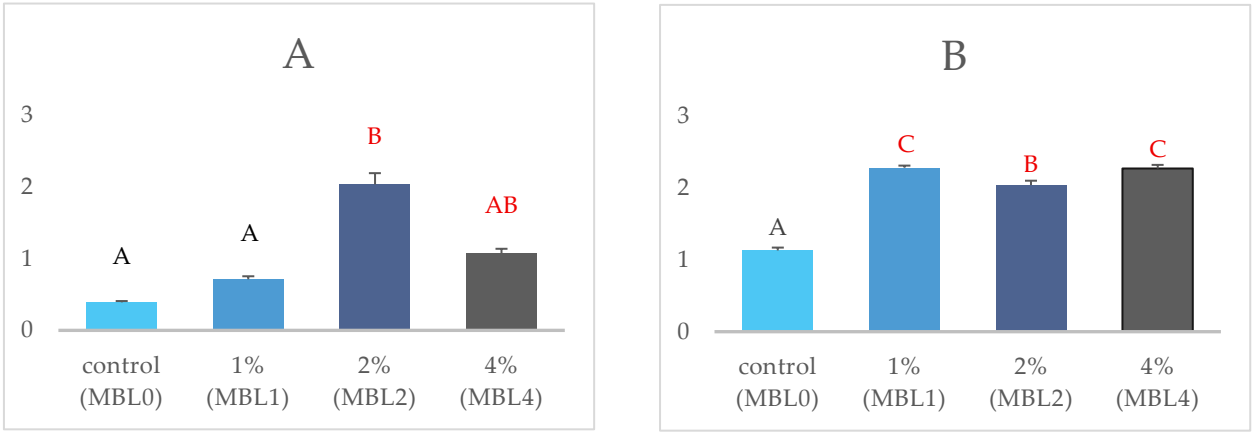

Figure 2. TNF-α (A) and IL-1 (B) of fish fed experimental diets supplemented with different levels of mulberry leaves. Different power signs indicate significant differences between groups ($P < 0.05$).

4. Conclusions

The dietary inclusion of fermented mulberry leaf meal, particularly at a 2% level, significantly enhanced innate immune responses as evidenced by increased lysozyme activity, respiratory burst activity, and upregulation of immune-related genes (TNF-α and IL-1). These results suggest that FML can improve immune competence and overall health in Nile tilapia. Moreover, FML appears to bolster the nutritional quality of the fish muscle, evidenced by an increase in protein content and a reduction in lipid content. These outcomes underscore the substantial potential of fermented mulberry leaves as a viable and sustainable feed additive in aquaculture. By fostering improved fish health and productivity through natural approaches, this study contributes significantly to the overarching objective of reducing dependence on antibiotics and promoting safer, more ecologically sound aquaculture practices. Prospective research could further explore the most effective fermentation conditions, assess the long-term impacts of FML supplementation on disease resistance under challenge conditions, and elucidate the precise molecular mechanisms through which FML modulates fish metabolism and immunity.

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References

[1] FAO. *The State of World Fisheries and Aquaculture*; Food and Agriculture Organization of the United Nations: Rome, Italy, **2014**; 223 pp.

[2] Yue, G.; Ma, K.; Xia, J. Status of Conventional and Molecular Breeding of Salinity-Tolerant Tilapia. *Rev. Aquacult.* **2024**, *16*(1), 271–286. https://doi.org/10.1111/raq.12838

[3] Arumugam, M.; Jayaraman, S.; Sridhar, A.; Venkatasamy, V.; Brown, P. B.; Abdul Kari, Z.; Tellez-Isaias, G.; Ramasamy, T. Recent Advances in Tilapia Production for Sustainable Developments in Indian Aquaculture and Its Economic Benefits. *Fishes* **2023**, *8*(4), 176. https://doi.org/10.3390/fishes8040176

[4] Ess Team. *The Global Tilapia Market in 2025: Trends, Opportunities and Challenges*; **2025**. https://essfeed.com/the-global-tilapia-market-in-2025-trends-opportunities-and-challenges-the-global-tilapia-market-in-2025-trends-opportunities-and-challenges/ (accessed 2025).

[5] Aich, N.; Paul, A.; Choudhury, T. G.; Saha, H. Tilapia Lake Virus (TiLV) Disease: Current Status of Understanding. *Aquacult. Fish.* **2022**, *7*(1), 7–17. https://doi.org/10.1016/j.aaf.2021.04.007

[6] Wang, B.; Thompson, K. D.; Wangkahart, E.; Yamkasem, J.; Bondad-Reantaso, M. G.; Tattiyapong, P.; Jian, J.; Surachetpong, W. Strategies to Enhance Tilapia Immunity to Improve Their Health in Aquaculture. *Rev. Aquacult.* **2023**, *15*, 41–56. https://doi.org/10.1111/raq.12731

[7] Butt, M. S.; Nazir, A.; Sultan, M. T.; Schroën, K. *Morus alba* L.: Nature's Functional Tonic. *Trends Food Sci. Technol.* **2008**, *19* (10), 505–512. https://doi.org/10.1016/j.tifs.2008.06.002

[8] Devi, B.; Sharma, N.; Kumar, D.; Jeet, K. *Morus alba* Linn: A Phytopharmacological Review. *Int. J. Pharm. Pharm. Sci.* **2013**, *5*(2), 14–18.

[9] Lim, S. H.; Choi, C.-I. Pharmacological Properties of *Morus nigra* L. (Black Mulberry) as a Promising Nutraceutical Resource. *Nutrients* **2019**, *11*(2), 437. https://doi.org/10.3390/nu11020437

[10] Wei, Y.; Huang, J.; Sun, H.; Feng, Z.; He, Y.; Chen, Y.; Lin, S. Impact of Different Processing of Mulberry Leaf on Growth, Metabolism, and Liver Immune Function of Largemouth Bass (*Micropterus salmoides*). *Aquacult. Rep.* **2023**, *29*, 101508. https://doi.org/10.1016/j.aqrep.2023.101508

[11] Mondal, K.; Kaviraj, A.; Mukhopadhyay, P. K. Effects of Partial Replacement of Fishmeal by Mulberry Leaf Meal on Growth Performance and Digestive Enzyme Activities of *Labeo bata*. *Int. J. Aquat. Sci.* **2012**, *3*(1), 1–12.

[12] Mithilasri, M.; Parthiban, K.; Shankar, S. Nutritional and Antinutritional Profiling of Mulberry Genetic Resources Amenable for Animal Feed. *Range Manag. Agrofor.* **2023**, *44*(1), 210–215. https://doi.org/10.59515/rma.2023.v44.i1.26

[13] Novel Antioxidant Peptides from Mulberry (*Morus atropurpurea* Roxb.) Leaf Protein Hydrolysates with Hemolysis Inhibition Ability and Cellular Antioxidant Activity. *J. Agric. Food Chem.* **2019**, *67*(27), 7650–7659. https://doi.org/10.1021/acs.jafc.9b01115

[14] Hajimohammadi, A.; Mottaghitalab, M.; Hashemi, M. Influence of Microbial Fermentation of Sesame Meal and Enzyme Supplementation on Broiler Performance. *Ital. J. Anim. Sci.* **2020**, *19*(1), 712–722. https://doi.org/10.1080/1828051X.2020.1790045

[15] Kaviraj, A.; Mondal, K.; Mukhopadhyay, P. K.; Turchini, G. M. Impact of Fermented Mulberry Leaf and Fish Offal in Diet Formulation of *Labeo rohita*. *Proc. Zool. Soc.* **2013**, *66*(1), 64–73. https://doi.org/10.1007/s12595-012-0052-1

[16] AOAC. *Official Methods of Analysis*, 11th ed.; Association of Official Analytical Chemists: Washington, DC, **2000**; Vol. 11.

[17] Hasan, B.; Putra, I.; Suharman, I.; Iriani, D.; Muchlisin, Z. A. Growth Performance and Carcass Quality of River Catfish (*Hemibagrus nemurus*) Fed Salted Trash Fish Meal. *Egypt. J. Aquat. Res.* **2019**, *45*(3), 259–264. https://doi.org/10.1016/j.ejar.2019.07.005

[18] Yarahmadi, P.; Miandare, H. K.; Farahmand, H.; Mirvaghefi, A.; Hoseinifar, S. H. Dietary Fermentable Fiber Upregulated Immune Related Genes Expression, Increased Innate Immune Response and Resistance of Rainbow Trout (*Oncorhynchus mykiss*) against *Aeromonas hydrophila*. *Fish Shellfish Immunol.* **2014**, *41*(2), 326–331. https://doi.org/10.1016/j.fsi.2014.09.007

[19] Swain, P.; Dash, S.; Sahoo, P.; Routray, P.; Sahoo, S.; Gupta, S.; Meher, P.; Sarangi, N. Non-Specific Immune Parameters of *Labeo rohita* and Seasonal Variations. *Fish Shellfish Immunol.* **2007**, *22*(1–2), 38–43. https://doi.org/10.1016/j.fsi.2006.03.010

[20] Chandan, R.; Parry, R., Jr.; Shahani, K. Purification and Properties of Bovine Milk Lysozyme. *Biochim. Biophys. Acta* **1965**, *110* (2), 389–398. https://doi.org/10.1016/S0926-6593(65)80046-7

[21] Secombes, C. J. Isolation of Salmonid Macrophages and Analysis of Their Killing Activity. *Tech. Fish Immunol.* **1990**, *1*, 137–163.

[22] Kayansamruaj, P.; Pirarat, N.; Hirono, I.; Rodkhum, C. Increasing of Temperature Induces Pathogenicity of *Streptococcus agalactiae* and the Up-regulation of Inflammatory Related Genes in Infected Nile Tilapia (*Oreochromis niloticus*). *Vet. Microbiol.* **2014**, *172*, 265–271. https://doi.org/10.1016/j.vetmic.2014.04.013

[23] Chadzinska, M.; Savelkoul, H. F. J.; Lidy, B. M.; van Kemenade, B. M. Morphine Affects the Inflammatory Response in Carp by Impairment of Leukocyte Migration. *Dev. Comp. Immunol.* **2009**, *33*(1), 88–96. https://doi.org/10.1016/j.dci.2008.07.004

[24] Dawood, M. A.; Koshio, S. Application of Fermentation Strategy in Aquafeed for Sustainable Aquaculture. *Rev. Aquacult.* **2020**, *12*(2), 987–1002. https://doi.org/10.1111/raq.12368

[25] El-Ghafloul, M. S.; Ibrahim, M. A.; Abd El-Razek, I. M.; Abdo, S. E.; Amer, A. A.; Zaineldin, A. I.; Gewaily, M. S.; Dawood, M. A. Growth Performance and Antioxidative Status of Nile Tilapia Fed Fermented Rice Hulls. *Aquaculture* **2025**, 742586. https://doi.org/10.1016/j.aquaculture.2025.742586

[26] Siddik, M. A. B.; Julien, B. B.; Islam, S. M. M.; Francis, D. S. Fermentation in Aquafeed Processing: Achieving Sustainability in Feeds for Global Aquaculture Production. *Rev. Aquacult.* **2024**, *16*(3), 1244–1265. https://doi.org/10.1111/raq.12894

[27] Álvarez, A.; Rodríguez, A.; Chaparro, S.; Borrás, L. M.; Rache, L. Y.; Brijaldo, M. H.; Martínez, J. J. Solid-State Fermentation as a Biotechnological Tool to Reduce Antinutrients and Increase Nutritional Content in Legumes and Cereals for Animal Feed. *Fermentation* **2025**, *11*(7), 359. https://doi.org/10.3390/fermentation11070359

[28] Arbab Sakandar, H.; Chen, Y.; Peng, C.; Chen, X.; Imran, M.; Zhang, H. Impact of Fermentation on Antinutritional Factors of Legumes Seed. *Food Rev. Int.* **2023**, *39*(3), 1227–1249. https://doi.org/10.1080/87559129.2021.1931300

[29] Jiang, W.; Lin, Y.; Qian, L.; Miao, L.; Liu, B.; Ge, X.; Shen, H. Mulberry Leaf Meal as a Feed Supplement for Juvenile *Megalobrama amblycephala* "Huahai No. 1". *Fish Shellfish Immunol.* **2022**, *128*, 279–287. https://doi.org/10.1016/j.fsi.2022.07.022

[30] Ilham, I.; Sucipto, S.; Fujaya, Y. Effects of Fermented Herbal Extract as a Phytobiotic on Growth Indices, Moulting Performance, and Feed Utilization of Juvenile Tiger Shrimp (*Penaeus monodon*). *Fishes* **2024**, *9*(9), 352. https://doi.org/10.3390/fishes9090352

[31] Shi, Y.; Zhong, L.; Fan, Y.; Zhang, J.; Zhong, H.; Liu, X.; Hu, Y. Protective Effect of Mulberry Leaf Flavonoids on High-carbohydrate-induced Liver Oxidative Stress, Inflammatory Response and Intestinal Microbiota Disturbance in *Monopterus albus*. *Antioxidants* **2022**, *11*(5), 976. https://doi.org/10.3390/antiox11050976

[32] Zhang, M.; Pan, L.; Fan, D.; He, J.; Su, C.; Gao, S.; Zhang, M. Effects of Fermented Feed by Mixed Strains and Their Effects on the Survival, Growth, Digestive Enzyme Activity and Intestinal Flora of *Penaeus vannamei*. *Aquaculture* **2021**, *530*, 735703. https://doi.org/10.1016/j.aquaculture.2020.735703

[33] Neves, N. O. D. S.; De Dea Lindner, J.; Stockhausen, L.; Delziovo, F. R.; Bender, M.; Serzedello, L.; Perez Fabregat, T. E. H. Fermented Plant-Based Feeds with *Lactobacillus acidophilus* Improves the Survival and Intestinal Health of Juvenile Nile Tilapia (*Oreochromis niloticus*) Reared in a Biofloc System. *Animals* **2024**, *14*(2), 332. https://doi.org/10.3390/ani14020332

[34] Dumas, A.; France, J.; Bureau, D. P. Modelling Growth and Body Composition in Fish Nutrition: Where Have We Been and Where Are We Going?. *Aquacult. Res.* **2010**, *41*(2), 161–181. https://doi.org/10.1111/j.1365-2109.2009.02323.x

[35] Jobling, M. Fish Nutrition Research: Past, Present and Future. *Aquacult. Int.* **2016**, *24*(3), 767–786. https://doi.org/10.1007/s10499-014-9875-2

[36] Miao, L.; Charles, O.; Lin, Y.; Gong, Y.; Zhu, W.; Wang, L.; Fu, J.; Zhang, Z.; Dong, Z. Interactive Effects of Mulberry Leaf Meal and Bamboo Charcoal Additive on Growth Performance, Anti-oxidant Capacity, and Disease Resistance of Genetically Improved Farmed Tilapia (GIFT) Juvenile (*Oreochromis niloticus*). *Aquacult. Rep.* **2020**, *18*, 100483. https://doi.org/10.1016/j.aqrep.2020.100483

[37] Rombout, J.; Huttenhuis, H.; Picchietti, S.; Scapigliati, G. Phylogeny and Ontogeny of Fish Leucocytes. *Fish Shellfish Immunol.* **2005**, *19* (5), 441–455. https://doi.org/10.1016/j.fsi.2005.03.007

[38] Uribe, C.; Folch, H.; Enríquez, R.; Moran, G. Innate and Adaptive Immunity in Teleost Fish. *Vet. Med.* **2011**, *56* (10), 486–503. https://doi.org/10.17221/3294-VETMED

[39] Magnadottir, B. Immunological Control of Fish Diseases. *Mar. Biotechnol.* **2010**, *12*(4), 361–379. https://doi.org/10.1007/s10126-010-9279-x

[40] Balcázar, J. L.; De Blas, I.; Ruiz-Zarzuela, I.; Cunningham, D.; Vendrell, D.; Múzquiz, J. L. The Role of Probiotics in Aquaculture. *Vet. Microbiol.* **2006**, *114*(3–4), 173–186. https://doi.org/10.1016/j.vetmic.2006.01.009

[41] Vine, N. G.; Leukes, W. D.; Kaiser, H. Probiotics in Marine Larviculture. *FEMS Microbiol. Rev.* **2006**, *30*(3), 404–427. https://doi.org/10.1111/j.1574-6976.2006.00017.x

[42] Lim, K. C.; Yusoff, F. M.; Karim, M.; Natrah, F. M. Carotenoids Modulate Stress Tolerance and Immune Responses in Aquatic Animals. *Rev. Aquacult.* **2023**, *15* (2), 872–894. https://doi.org/10.1111/raq.12767

[43] Ahmadifar, E.; Pourmohammadi Fallah, H.; Yousefi, M.; Dawood, M. A.; Hoseinifar, S. H.; Adineh, H.; Doan, H. V. The Gene Regulatory Roles of Herbal Extracts on the Growth, Immune System, and Reproduction of Fish. *Animals* **2021**, *11*(8), 2167. https://doi.org/10.3390/ani11082167

[44] Adebo, J. A.; Njobeh, P. B.; Gbashi, S.; Oyedeji, A. B.; Ogundele, O. M.; Oyeyinka, S. A.; Adebo, O. A. Fermentation of Cereals and Legumes: Impact on Nutritional Constituents and Nutrient Bioavailability. *Fermentation* **2022**, *8*(2), 63. https://doi.org/10.3390/fermentation8020063