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Dietary Puerarin Enhances Growth, Immune Function, Antioxidant Capacity, and Disease Resistance in Farmed Largemouth Bass, *Micropterus salmoides*

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Abstract: Puerarin, a bioactive isoflavone extracted from *Pueraria lobata*, possesses well-documented pharmacological properties, including anti-inflammatory, antioxidant, and metabolic regulatory effects, which have been extensively studied in mammalian models and traditional medicine. Recently, its potential as a functional feed additive in aquaculture has garnered increasing attention. This study aimed to evaluate the effects of dietary puerarin supplementation on growth performance, immune response, antioxidant capacity, and disease resistance in largemouth bass, *Micropterus salmoides*. A total of 120 fish were randomly assigned to 4 dietary groups, receiving a basal diet supplemented with 0 (control), 200, 500, and 1000 mg/kg puerarin for 8 weeks. The results showed that dietary puerarin significantly ($p < 0.05$) improved weight gain, with the 200 mg/kg and 500 mg/kg groups exhibiting the best performance. Puerarin supplementation enhanced acid phosphatase (ACP), alkaline phosphatase (AKP), and lysozyme (LZM) activities, reduced malondialdehyde (MDA) levels, and increased superoxide dismutase (SOD) and catalase (CAT) activities, indicating improved immune function and oxidative stress resistance in groups receiving medium concentrations of puerarin supplementation. The expression of the *TNF-α*, *IL-6*, *IL-8*, and *HSP70* genes was significantly downregulated, especially in the 200 mg/kg and 500 mg/kg groups, suggesting anti-inflammatory and anti-stress effects, while *Nrf2* expression was upregulated in the 1000 mg/kg group, reinforcing its antioxidative role. Additionally, puerarin-fed fish exhibited significantly lower mortality rates following *Aeromonas hydrophila* infection, highlighting enhanced disease resistance. In summary, the dose-dependent effect of puerarin on largemouth bass aquaculture has been revealed in this study. Dietary supplementation with moderate doses of puerarin (200 and 500 mg/kg) effectively suppressed inflammation and enhanced immune function, while the highest dose (1000 mg/kg) may mildly activate the immune system. These findings suggest that puerarin is a promising phytogenic feed additive for improving fish health and aquaculture sustainability.

Keywords: puerarin; *Micropterus salmoides*; feeding; immunomodulation; antioxidation

Key Contribution: This study provides the first comprehensive evaluation of puerarin as a functional feed additive in largemouth bass, demonstrating its ability to enhance growth performance, strengthen immune responses, improve antioxidant defenses, and increase disease resistance.

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1. Introduction

Puerarin (7,4′-dihydroxy-8-C-glucosylisoflavone) is a major isoflavonoid compound extracted from the root of *Pueraria lobata*, commonly known as kudzu, a traditional medicinal plant in China. It belongs to the flavonoid family and is known for its diverse pharmacological effects, including antioxidant, anti-inflammatory, and metabolic regulatory properties [1]. Puerarin, a compound extensively used in traditional Chinese medicine for various conditions, such as cardiovascular diseases and liver diseases [2], has been shown to protect against oxidative stress [3,4], a key factor in many chronic diseases, including those affecting fish health. Another typical isoflavone—genistein—has been demonstrated to improve lipid metabolism and antioxidant activity and to modulate immune responses in fish species [5]. In addition, the soy isoflavone daidzein improved glucose homeostasis and insulin sensitivity in fish [6]. Given that hyperinsulinemia-induced insulin resistance is associated with immune suppression in zebrafish, and ptpn6 is identified as a key mediator [7], these findings support the broader idea that improving insulin sensitivity may help restore or enhance immune function. Therefore, given its ability to alleviate oxidative stress by reducing the generation of reactive oxygen species (ROS) and modulating glucose metabolism [8], puerarin is considered a promising candidate for improving metabolic balance and enhancing immune function in aquatic animals. These pharmacological effects are attributed to its ability to modulate key signaling pathways, such as the PI3K/Akt and AMPK pathways, which are involved in glucose metabolism and lipid regulation in both mammals and aquatic species [9–11]. Given its bioactive potential, puerarin is increasingly being explored as a dietary supplement in aquaculture due to its ability to enhance antioxidative response, immunity and glucolipid metabolism.

The largemouth bass (*Micropterus salmoides*), native to North America, was introduced to China in the early 1980s and has since become a significant species in the aquaculture industry. Renowned for its delectable taste, robust disease resistance, and rapid growth rate, the largemouth bass has established itself as a major freshwater cultured fish in China [12]. The industry has experienced substantial growth, with production increasing from 457,000 tons in 2017 to 802,000 tons in 2022 [13]. Despite its economic significance, the largemouth bass aquaculture industry faces challenges, such as genetic issues, feed quality, disease management, and the need for improved industry management practices [14]. Addressing these challenges is crucial for the sustainable development of the industry.

As an isoflavone, puerarin supplementation has demonstrated broad spectrum benefits in animal health, including enhanced growth performance, antioxidant defense, and immunity, particularly in aquaculture species [15,16]. Notably, puerarin enhances immune function by upregulating immune cells' proliferation and enzymatic activity [17]. These are essential for boosting the body's natural defense mechanisms against pathogens. For example, in Nile tilapia (*Oreochromis niloticus*), puerarin supplementation has been found to promote immune responses by activating key immune pathways, such as the Toll-like receptor (TLR) and NF-κB pathways, which are crucial for pathogen recognition and inflammatory responses [18]. Additionally, its strong antioxidant properties, mediated through the upregulation of enzymes, such as superoxide dismutase (SOD) and catalase (CAT) [15], help mitigate the oxidative stress commonly encountered in intensive aquaculture systems due to high stocking densities [19] and stressors, such as handling and unhealthy feedings [16,17]. Mechanistically, puerarin is proposed to activate the AMPK/Nrf2 pathway, promoting phosphorylation of AMPK and subsequent upregulation of Nrf2 expression, which drives the transcription of antioxidant genes, such as HO-1 [20]. These findings align with studies in omnivorous fish, such as Nile tilapia (*O. niloticus*) and grass carp (*Ctenopharyngodon idella*), where puerarin supplementation improved stress tolerance, immune responses, and growth performance [16,21]. However, there is limited research on

its effects in carnivorous fish, which have distinct metabolic and immune adaptations. Carnivorous species face unique challenges in aquaculture, including oxidative stress and compromised immune function due to high-carbohydrate formulated diets, which are often employed for cost-efficiency despite their physiological incompatibility [22,23]. Largemouth bass, an important aquacultural species in China with high economic value, provides a relevant model to explore this question. As carnivorous fish, they also often face challenges linked to oxidative stress, compromised immune function, and low efficiency in plant-based feed utilization. These issues underscore the critical need to evaluate whether puerarin supplementation can improve these physiological processes in largemouth bass.

Furthermore, isoflavones, including puerarin, also positively influence growth performance in aquacultural species. Puerarin has been shown to improve the feed conversion ratio (FCR) and specific growth rate (SGR), indicating enhanced nutrient utilization and overall growth. This is especially beneficial in certain species, like grass carp [21] and tilapia [18], where puerarin supplementation has led to increased weight gain and improved feed efficiency. These growth-promoting effects are likely a result of the combination of immune enhancement, reduced stress, and improved metabolic functions. In addition, isoflavones have been shown to effectively alleviate the physiological impacts of environmental stress on fish. For example, dietary soybean isoflavone supplementation enhances antioxidative responses in fish with a high stocking density and pathogen resistance [24,25]. Therefore, as an isoflavone, puerarin could be a good dietary supplement to help against environmental stress in aquaculture. However, critical knowledge gaps remain regarding the application of puerarin in aquaculture, such as puerarin's effects on carnivorous fish. Addressing these challenges is essential for maximizing its potential in sustainable fish farming.

This study aims to evaluate the effects of puerarin supplementation on the growth performance, immune function, and overall health of largemouth bass (*M. salmoides*). Specifically, we investigate survival, growth, antioxidant status, and immune-related gene expression under aquaculture conditions. By assessing its impact on disease resistance and productivity, we seek to determine whether puerarin can be effectively incorporated into largemouth bass diets to enhance aquaculture outcomes in China.

2. Materials and Methods

2.1. Chemicals and Reagents

Puerarin (purity $\geq$ 98%) was obtained from Sinochem Crop Care Co., Ltd. (Shanghai, China) and was used as the primary dietary supplement for the experimental groups. Puerarin was dissolved in 95% alcohol to prepare the stock solution, which was then incorporated into the experimental diets at final concentrations of 200, 500, and 1000 mg/kg. Since puerarin was dissolved in ethanol before mixing with the feed and the final ethanol concentration in the highest group (1000 mg/Kg) was less than 0.1%, which is far lower than the no observed effect concentration (NOEC) in fish [26], we ensured that the final ethanol concentration was negligible and unlikely to have a confounding effect on fish physiology. All other chemicals and reagents used in the study, including acid phosphatase (ACP) assay kits, alkaline phosphatase (AKP) assay kits, lysozyme (LZM) assay kits, malondialdehyde (MDA) assay kits, superoxide dismutase (SOD) assay kits, and catalase (CAT) assay kits, were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Tris–HCl, phosphate-buffered saline (PBS), EDTA, and other laboratory reagents were of analytical grade and were used as provided. The qRT-PCR reagents were purchased from Takara Co., Ltd. (Shiga, Japan), and the specific primers for immune-related genes were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). All reagents were

prepared according to the manufacturer's instructions for use in assays of antioxidant capacity, immune response, and gene expression.

2.2. Animal Acclimatization, Chronic Exposure and Sampling

A total of 150 largemouth bass (*M. salmoides*) with an average initial weight of 12 g were obtained from a commercial hatchery located in Guangzhou, China. Upon arrival, the fish were acclimatized in a recirculating aquaculture system (RAS) for a period of 14 days under controlled conditions. During the acclimatization period, water quality parameters, such as temperature (22 ± 1 °C), pH (7.5 ± 0.2), dissolved oxygen (7.5 ± 0.5 mg/L), and ammonia nitrogen (<0.5 mg/L), were monitored daily and maintained for largemouth bass culture. Fish were fed a commercial diet containing 48% crude protein and 15% crude fat (Haida, Guangzhou, China), and the feeding regime was adjusted to ensure full consumption of feed while preventing excess feed accumulation. The components of the basal diet can be found in Table 1.

Table 1. The formulation and nutritional composition of experimental diet.

Nutritional Composition	Contents (%)	Nutrient Level	Contents (%)
Fish meal	48	Crude protein	46.7
Soybean meal	8	Crude lipid	11.5
Flour	11	Crude ash	9.2
Soybean oil	6	Moisture	10.4
Chicken powder	10		
Cassava starch	8		
$Ca(H_2PO_4)_2$	1.5		
Squid ointment	4		
Gluten	2		
Premix *	1.5		
Total	100		

* Premix (per kg per content): $FeSO_4 \cdot H_2O$ 260 mg, Na_2SeO_3 (1%) 50 mg, $MnSO_4 \cdot H_2O$ 50 mg, $ZnSO_4 \cdot H_2O$ 150 mg, KI 100 mg, $CuSO_4 \cdot 5H_2O$ 20 mg, $CoCl_2$ (1%) 100 mg; inositol 100 mg, folic acid 25 mg, nicotinamide 400 mg, VB_{12} 20 mg, VB_6 800 mg, VB_2 1500 mg, VB_1 1500 mg, VK 1000 mg, VD 2,000,000 IU, biotin 8 mg, VE 5000 UI, calcium pantothenate 25 mg, and VA 8,000,000 IU.

After the acclimatization period, the fish were randomly assigned to 4 treatment groups, each consisting of 10 fish per replicate (with 3 replicates per group). The groups were as follows: (1) control group (no puerarin supplementation); (2) puerarin-supplemented groups (with 200, 500, and 1000 mg/kg puerarin in the diet, respectively). The selection of concentrations (200 mg/kg, 500 mg/kg, and 1000 mg/kg) of puerarin in the feed for this study is based on a combination of previous research in aquaculture studies. For instance, in a study on yellowfin seabream (*Acanthopagrus latus*), puerarin supplementation at 400 mg/kg improved the growth performance and antioxidant enzyme activities [16]. In another study on Chinook salmon (*Oncorhynchus tshawytscha*), puerarin supplementation (200 mg/kg) significantly improved overall health through blood flow and immune response [17]. Based on these findings, the concentrations were chosen to test a dose-dependent response and ensure the evaluation of maximum potential effects. To reduce potential bias, all sample analyses were conducted without knowledge of the treatment groups. Samples were processed and analyzed in a randomized order, and group identities were only revealed after data collection was completed. Detailed information on the fish in each sampling group were provided in Table 3.

The experiment lasted for 8 weeks, and, during this period, the fish were continuously exposed to the respective treatments via their diets. Fish were fed twice daily, and the remaining uneaten feed was removed after each feeding to minimize water quality degradation.

At the end of the experimental period, all fish were fasted for 24 h before sampling. The growth performance was evaluated by weighing each individual. The growth rate was expressed as the percentage of weight increase from each group. Then, 9 fish were randomly selected from each group and euthanized using MS-222 (Sigma-Aldrich, Saint Louis, MO, USA), and tissue samples of blood and liver were collected for subsequent analyses. Blood samples were taken from the caudal vein using a 1 mL syringe for serum separation and further immune assays. Liver tissues were carefully excised, flash-frozen in liquid nitrogen, and stored at −80 °C for later biochemical and molecular analyses. To minimize individual differences, blood or liver samples in each replicate (from three fish) were pooled together as one sample (thus, n = 3 for biological and molecular analyses).

2.3. Immune-Related Enzymes Detection

The activities of ACP, AKP, and LZM were measured to assess the immunological status of largemouth bass. Blood samples were collected from the caudal vein, and serum was separated by centrifugation at 12,000 rpm for 15 min. ACP and AKP activities were determined using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Protocol No. A060-1-1 and A059-2-2, respectively), following the manufacturer's instructions, with absorbance measured at 405 nm. The ACP activity detection was based on the 4-Aminoantipyrine method [27] and the AKP activity was based on the disodium phenyl phosphate method [28]. Lysozyme activity (Nanjing Jiancheng Bioengineering Institute, Protocol No. A050-1-1) was evaluated using a turbidimetric method, with the reaction monitored at 530 nm [29]. Each enzyme activity was expressed as units per milliliter of serum.

2.4. Oxidative Stress Detection

The oxidative stress levels in largemouth bass were assessed by measuring the activities of key antioxidant enzymes, including SOD, CAT, as well as the levels of MDA, a marker of lipid peroxidation. These parameters were measured in liver tissue samples. Firstly, liver samples were homogenized at 4 °C in 0.1 M potassium phosphate buffer with a mixer mill (MM400, Retsch, Haan, Germany) and centrifuged at 12,000 rpm for 20 min at 4 °C. Next, the supernatants were collected for biochemical analysis. SOD activities were quantified using the WST-1 method provided by a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Protocol No. A001-3-2) according to the manufacturer's protocols [30]. The enzyme activities were determined by measuring absorbance at 450 nm. CAT activity (Protocol No. A007-1-1) was evaluated using the ammonium molybdate method and measured at 405 nm [31]. The quantification of MDA (Protocol No. A003-1-2) was conducted using the thiobarbituric acid (TBA) method. MDA reacts with TBA to form a red product. The MDA levels were determined by measuring the absorbance at 532 nm [32]. The results were expressed as nmol per milligram of protein. Protein concentrations were measured using the BCA Protein Assay Kit (Nanjing Jiancheng Bioengineering Institute, Protocol No. A045-4-2) prior to enzyme activity analysis.

2.5. Detection of Immune-Related Genes Expression

The expression levels of key immune-related genes, including tumor necrosis factor-alpha (*TNF-α*), interleukin-6 (*IL-6*), interleukin-8 (*IL-8*), nuclear factor erythroid 2-related factor 2 (*Nrf2*), and heat-shock protein 70 (*HSP70*), were analyzed using quantitative real-time PCR (qRT-PCR). The housekeeping gene *β-actin* served as an internal control. Liver tissues were collected at the end of the feeding trial, immediately frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. Total RNA was extracted by using Trizol reagent (Invitrogen, ThermoFisher, Waltham, MA, USA). RNA concentration and purity were assessed using a NanoDrop spectrophotometer (NanoDrop One, Thermo Fisher,

Waltham, MA, USA), and RNA integrity was confirmed via agarose gel electrophoresis. Complementary DNA (cDNA) synthesis was performed using the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Shiga, Japan), and the resulting cDNA was diluted 1:10 with nuclease-free water before use. The qRT-PCR was performed with an ABI 7500 Real-Time PCR System (Applied Biosystems, ThermoFisher, Waltham, MA, USA) and the cycling conditions were as follows: an initial denaturation step at 95 °C for 15 min, followed by 40 amplification cycles of 95 °C for 15 s and 60 °C for 30 s. The changes in the expression level of target genes were calculated using the comparative CT method ($2^{-\Delta\Delta Ct}$) [33] and expressed as the fold-change relative to the control. Detailed information concerning the primers is listed in Table 2.

Table 2. Oligonucleotide primers used in this study.

Primer Name	Sequence (5′ to 3′)
TNF-α Fw	CTTCGTCTACAGCCAGGCATCG
TNF-α Rv	TTTGGCACACCGACCTCACC
IL-6 Fw	GGACCGCTTTGAAACTCT
IL-6 Rv	GCTCCCTGTAACGCTTGT
IL-8 Fw	CGTTGAACAGACTGGGAGAGATG
IL-8 Rv	AGTGGGATGGCTTCATTATCTTGT
Nrf2 Fw	CAGACAGTTCCTTTGCAGGC
Nrf2 Rv	AGGGACAAAAGCTCCATCCA
HSP70 Fw	GCAGACGCAGACCTTCACCA
HSP70 Rv	TGCGCTTCCAGACCTCCAAC
β-actin Fw	AAAGGGAAATCGTGCGTGAC
β-actin Rv	AAGGAAGGCTGGAAGAGGG

Abbreviations: forward (Fw), reverse (Rv), 5 prime (5′), and 3 prime (3′).

2.6. Effects of Puerarin on Resistance to Aeromonas hydrophila Infection

To evaluate the protective effects of puerarin against bacterial infection, *M. salmoides* were challenged with *A. hydrophila* (the bacteria strain was a gift from Prof. Xiaozhen Yang, Shanghai Ocean University) after the feeding trial. At the end of the feeding experiment, after sampling for biochemical and molecular tests, 20 fish per group were randomly selected and intraperitoneally injected with 200 μL of *A. hydrophila* suspension at 1×10^6 CFU/mL, prepared from a fresh bacterial culture in tryptic soy broth (TSB). Control fish were injected with an equal volume of sterile phosphate-buffered saline (PBS). In total, 5 fish received PBS and 15 received bacteria per group. After the injection, fish were transferred to separate tanks and monitored for 24 h. Mortality was recorded, and dead fish were removed for bacterial reisolation to confirm *A. hydrophila* infection. The cumulative mortality rate was calculated at the end of the challenge period. The survival data were used to assess the protective effects of puerarin supplementation, and comparisons between groups were analyzed statistically. The number of fish used in each test is presented in Table 3.

Table 3. Fish allocation in each treatment group for experimental assessments.

Treatment Group (mg/kg Puerarin)	Initial Fish (n)	Growth Assessment (n)	Biochemical/ Molecular Analyses (n)	Bacterial Challenge (n)	PBS-Injected Controls (n)	*A. hydrophila*-Injected (n)
0 (Control)	30	30	9	20	15 *	5 *
200	30	30	9	20	15 *	5 *
500	30	30	9	20	15 *	5 *
1000	30	30	9	20	15 *	5 *

* Note: there were 15 *A. hydrophila*-injected and 5 PBS-injected fish per group. The remaining fish were unused and kept for growing in our aquaculture system.

2.7. Statistical Analysis

Statistical evaluation of the data was carried out using SPSS software version 22.0. For growth performance, 10 individuals were used in each replicate and in total 3 replicates were employed (n = 30). For biological and molecular analyses, n = 3. The normality of the data distribution was assessed using the Shapiro–Wilk test, while Levene's test was employed to evaluate the homogeneity of variances among treatment groups. Our results indicated that all data were naturally normally distributed, and homogeneity of variance was confirmed, allowing for parametric statistical analysis. Therefore, we performed a one-way analysis of variance (ANOVA) to determine whether significant differences existed among the treatment groups. Duncan's multiple range test (DMRT) was selected as the post hoc test due to its ability to maintain high statistical power while controlling type I error rates. DMRT is particularly useful in situations where multiple comparisons are required, and differences between means need to be ranked effectively, making it suitable for our study's dataset.

3. Results

3.1. Effects of Puerarin on Growth Performance of M. salmoides

The effects of dietary puerarin supplementation on weight gain (%) and survival rate (%) in *M. salmoides* are presented in Figure 1. Weight gain was significantly higher in fish fed with 200 mg/kg, 500 mg/kg, and 1000 mg/kg puerarin compared to the control group ($p < 0.05$), with no significant differences among the supplemented groups (Figure 1A). These results indicate that puerarin supplementation effectively promotes growth in largemouth bass. On the other hand, survival rate showed no significant differences among all groups ($p > 0.05$), suggesting that puerarin supplementation did not negatively impact fish survival (Figure 1B). The consistently high survival rates across treatments indicate that puerarin is well tolerated in juvenile largemouth bass diets.

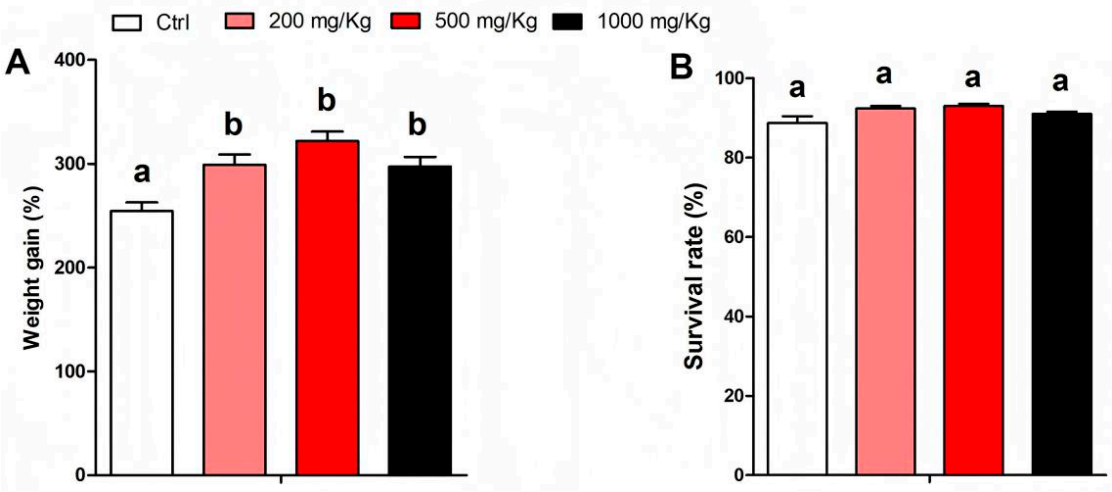


Figure 1. Effects of dietary puerarin supplementation on the weight gain and survival rate of *M. salmoides*. (**A**) Weight gain (%) of largemouth bass after feeding with different levels of puerarin (200 mg/kg, 500 mg/kg, and 1000 mg/kg) compared to the control group. (**B**) Survival rate (%) of largemouth bass. Values are expressed as mean ± SD (n = 30). Bars with different letters (a, b) indicate significant differences ($p < 0.05$) among groups.

3.2. Effects of Puerarin on Immunological and Antioxidative Status of M. salmoides

Dietary puerarin supplementation significantly influenced the immune response and antioxidant capacity of *M. salmoides* (Figure 2). ACP and AKP activities increased significantly ($p < 0.05$) in fish fed with 200 mg/kg and 1000 mg/kg puerarin, suggesting enhanced

innate immunity, while LZM activity remained unchanged ($p > 0.05$) (Figure 2A–C). Puerarin also improved oxidative stress resistance, as evidenced by significantly decreased MDA levels ($p < 0.05$) in the 200 and 500 mg/kg groups, as the 1000 mg/kg group showed no additional decrease compared to 500 mg/kg (Figure 2D). Additionally, SOD activity significantly increased at 500 mg/kg and CAT activity significantly increased at 1000 mg/kg, reflecting enhanced antioxidant defenses (Figure 2E,F).

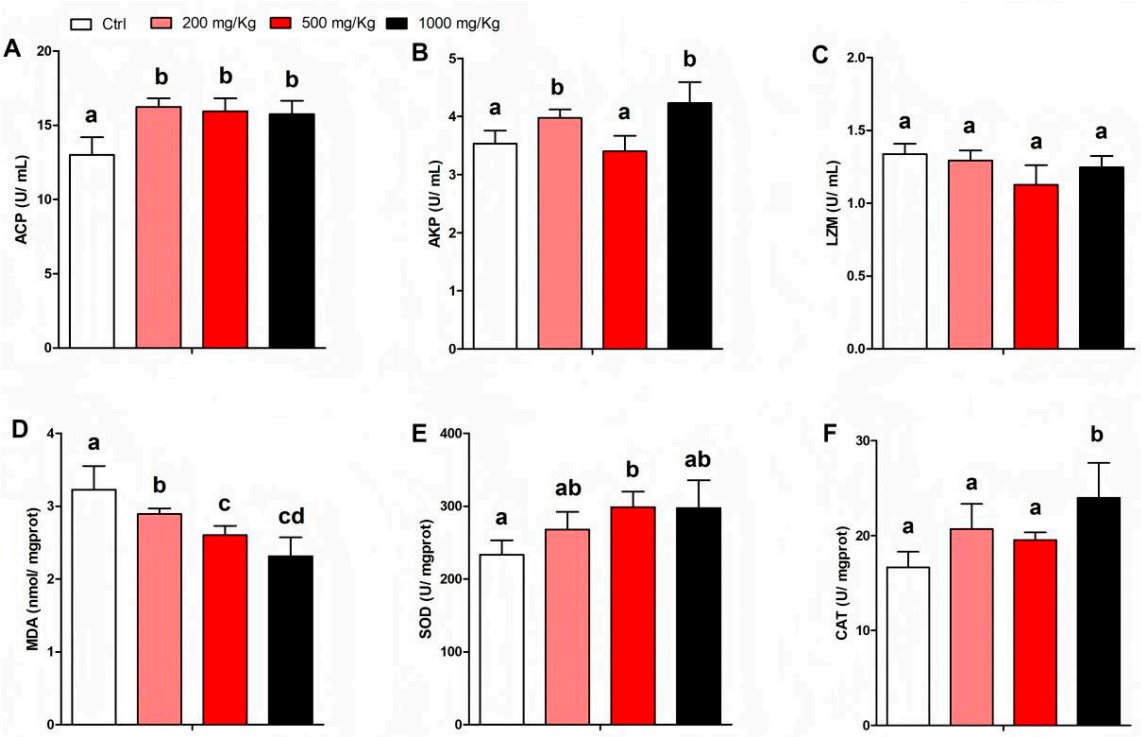


Figure 2. Effects of dietary puerarin supplementation on immune-related and antioxidative parameters in *M. salmoides*. (**A**) ACP (acid phosphatase) activity, (**B**) AKP (alkaline phosphatase) activity, and (**C**) LZM (lysozyme) activity in serum. (**D**) MDA (malondialdehyde) levels, (**E**) SOD (superoxide dismutase) activity, and (**F**) CAT (catalase) activity in liver tissue. Values are expressed as mean ± SD (n = 3). Bars with different letters indicate significant differences ($p < 0.05$) among groups.

3.3. Expression of Immune-Related Genes

The effects of dietary puerarin on the expression of immune-related genes in largemouth bass (*M. salmoides*) are shown in Figure 3. The expression levels of *TNF-α*, *IL-6*, and *IL-8*, which are key inflammatory cytokines, were significantly downregulated ($p < 0.05$) in the 200 and 500 mg/kg groups, except for IL-6 expression at 200 mg/Kg, which showed a trend toward downregulation that was not statistically significant, suggesting an anti-inflammatory effect. However, *TNF-α* and *IL-6* expression increased in the 1000 mg/kg group, indicating a possible immune-stimulatory response at higher dosages. *Nrf2* expression, which plays a crucial role in antioxidant regulation, was significantly decreased ($p < 0.05$) in the 200 and 500 mg/kg group, whereas it was significantly increased in 1000 mg/kg group. In addition, *HSP70* expression, associated with cellular stress responses, was downregulated in a dose-dependent manner and was significantly lower in the 1000 mg/kg group compared to the control ($p < 0.05$).

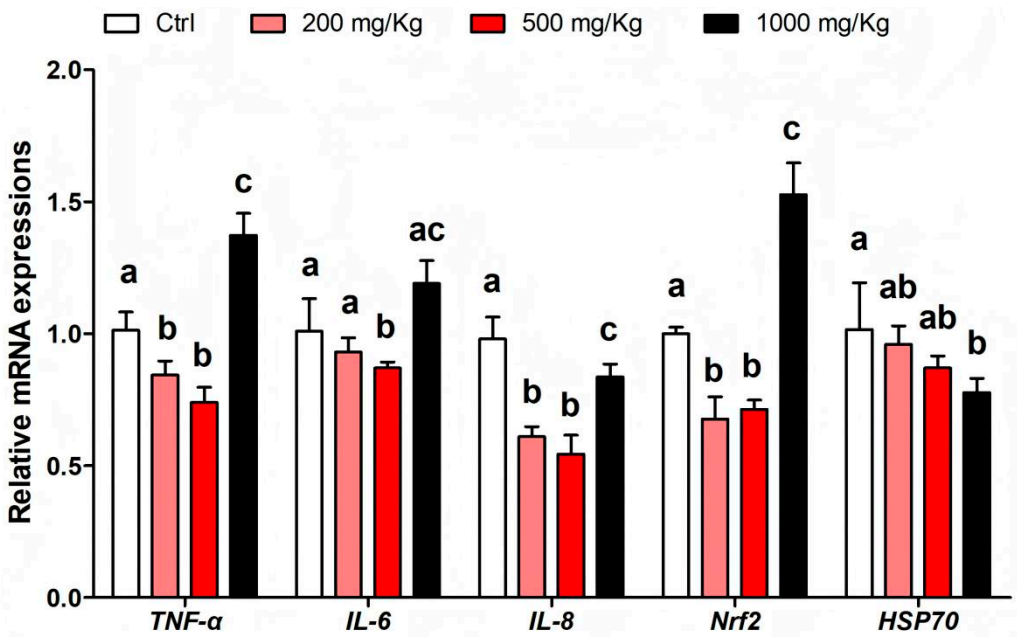


Figure 3. Effects of puerarin on immune-related gene expression in *M. salmoides*. The relative mRNA expression levels of tumor necrosis factor-alpha (*TNF-α*), interleukin-6 (*IL-6*), interleukin-8 (*IL-8*), buclear factor erythroid 2-related factor 2 (*Nrf2*), and heat-shock protein 70 (*HSP70*) were quantified by quantitative real-time PCR (qRT-PCR). Values are expressed as mean ± SD (n = 3). Bars with different letters indicate significant differences ($p < 0.05$) among groups.

3.4. Effects of Puerarin on Resistance to A. hydrophila Infection

Dietary puerarin supplementation significantly enhanced disease resistance in *M. salmoides*, as indicated by the reduced mortality rate after *A. hydrophila* infection (Figure 4). No mortality of fish was observed under PBS injection. Fish in the puerarin-fed groups (200, 500, and 1000 mg/kg) had significantly lower mortality ($p < 0.05$) than the control feeding group, with no differences among supplemented groups ($p > 0.05$).

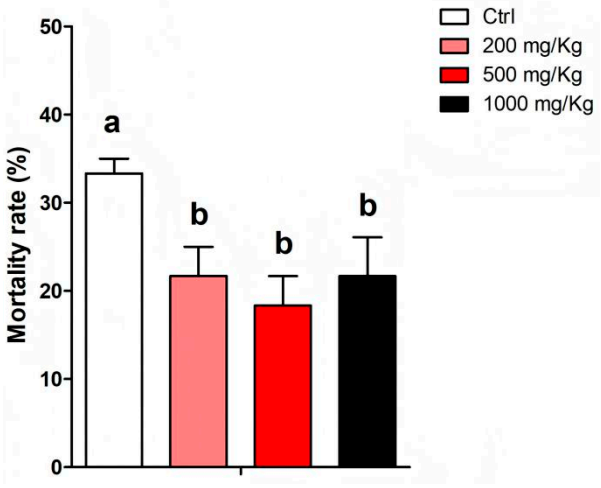


Figure 4. Effects of dietary puerarin supplementation on mortality rate after *A. hydrophila* challenge in *M. salmoides*. Mortality was recorded over 24 h. Values are expressed as mean ± SD (n = 20). Bars with different letters indicate significant differences ($p < 0.05$) among groups.

4. Discussion

The results of this study demonstrate that dietary puerarin supplementation significantly enhances growth performance, immune function, antioxidative capacity, and disease resistance in largemouth bass (*M. salmoides*). These findings align with previous research

in aquatic species, such as yellowfin seabream (*A. latus*) [16], and terrestrial models, like mice [34], underscoring the conserved physiological benefits of puerarin across taxa. The improvements in growth performance observed in this study suggest that puerarin plays a key role in optimizing nutrient utilization and metabolic regulation, particularly in carnivorous fish that have limited capacity to metabolize high-carbohydrate diets [35]. The increased weight gain and specific growth rate recorded in puerarin-fed fish are consistent with findings in yellowfin seabream, where puerarin supplementation enhanced digestive enzyme activity, feed conversion efficiency, and hepatic function [16]. Similar benefits have been reported in mice, where puerarin improved protein lipid metabolism [34]. These effects may be attributed to puerarin's activation of AMP-activated protein kinase (AMPK), a master regulator of cellular energy homeostasis. In mice, puerarin improved protein–lipid metabolism by stimulating AMPK-mediated glucose uptake and lipid oxidation, a pathway potentially conserved in fish [36]. Although direct evidence of AMPK activation in aquatic species remains limited, our results suggest that puerarin may similarly enhance energy metabolism in largemouth bass, thereby supporting growth under dietary challenges. However, despite the significant improvements in growth and disease resistance observed at the 200 mg/kg and 500 mg/kg puerarin supplementation levels, no significant additional benefits were observed at the highest dose (1000 mg/kg) in either parameter. One possible explanation for the lack of further improvements at the 1000 mg/kg dose is metabolic saturation. It is possible that at the moderate doses (200 and 500 mg/kg), the fish's physiological capacity to utilize puerarin was maximized, and that further increases in dosage do not lead to enhanced growth or immune responses. This could reflect a biological threshold where the additional dose does not provide extra benefits. Furthermore, the cost-effectiveness of using higher doses must also be considered. In aquaculture systems, where efficiency and cost are crucial, the lack of additional benefits at the higher dose indicates that moderate doses may offer the best balance of efficacy and economic feasibility.

In addition to its role in growth, puerarin significantly enhanced immune function and antioxidative status, as indicated by increased ACP, AKP, and LZM activities, along with elevated SOD and CAT levels. The reduction in malondialdehyde (MDA) levels suggests that puerarin effectively mitigates lipid peroxidation, which is crucial for maintaining cellular integrity and preventing oxidative stress-induced damage [37]. These results align with previous studies in farmed chinook salmon (*O. tshawytscha*) fingerlings, where puerarin improved immune responses and circulatory health, contributing to better disease resistance [17]. The qRT-PCR analysis further supports puerarin's immunomodulatory role, as fish fed puerarin-supplemented diets showed downregulation of pro-inflammatory cytokines (*TNF-α*, *IL-6*, *IL-8*) and upregulation of *Nrf2*, a key regulator of antioxidant defenses. This suggests that puerarin helps maintain immune homeostasis by suppressing excessive inflammation while enhancing antioxidative defense mechanisms. The Nrf2–Keap1 pathway, which plays a central role in regulating oxidative stress responses, appears to be a key target of puerarin [38,39]. By promoting Nrf2 activation, puerarin may facilitate the transcription of antioxidant enzymes, such as SOD and CAT, enhancing the fish's ability to counteract oxidative stress [10]. In contrast, the suppression of NF-κB activation, as evidenced by lower *TNF-α*, *IL-6*, and *IL-8* gene expression, suggests that puerarin helps regulate immune responses, preventing excessive inflammation that could otherwise impair growth and metabolic function [40,41]. However, the downregulation of *Nrf2* expression at 200 and 500 mg/kg, in contrast with its upregulation at 1000 mg/kg in this study, may reflect a dose-dependent activation of the Nrf2 pathway. At lower doses, the activation of Nrf2 could be balanced by negative feedback mechanisms or compensatory pathways, leading to downregulation. In contrast, at higher doses (1000 mg/kg), the Nrf2 pathway

may become fully activated, resulting in upregulation as part of an adaptive response to increased oxidative stress.

The modulation of immune-related gene expression provides deeper insight into puerarin's regulatory effects. *TNF-α*, *IL-6*, and *IL-8* are key pro-inflammatory cytokines that mediate the fish's response to infections and environmental stress [41]. Elevated levels of these cytokines can be beneficial in acute immune responses; however, chronic overexpression may lead to immune dysregulation and energy diversion from growth-related processes [42]. In this study, the downregulation of *TNF-α*, *IL-6*, and *IL-8* in the 200 mg/kg and 500 mg/kg groups suggests that puerarin prevents excessive inflammation, thereby conserving energy for growth and metabolic efficiency. These findings align with research in zebrafish, where puerarin reduced pro-inflammatory gene expression and oxidative damage, leading to improved liver health [43]. Interestingly, the upregulation of *TNF-α* and *IL-6* at 1000 mg/kg puerarin suggests that excessive supplementation may trigger a mild immune activation, which could indicate a dose-dependent effect on immune modulation. One possible explanation is that moderate puerarin supplementation helps maintain immune homeostasis, reducing excessive inflammation while still supporting immune defense. However, at higher concentrations, immune overactivation or loss of regulatory balance might occur, leading to an upregulation of inflammatory markers. Alternatively, this could represent a form of immune priming, where the immune system is mildly activated in preparation for potential pathogenic challenges. To further investigate this, histological examination of key immune organs, such as the spleen, gut-associated lymphoid tissue (GALT), and anterior kidney, would be essential in future studies. This approach would help clarify the dose-dependent immune effects of puerarin and guide safe puerarin supplementation in aquaculture.

In addition to regulating inflammatory cytokines, puerarin also influenced heat-shock protein 70 (*HSP70*) expression, which plays a crucial role in cellular stress responses and protein homeostasis. *HSP70* functions as a molecular chaperone, protecting cells from oxidative damage and protein misfolding under stressful conditions [44]. In this study, *HSP70* expression remained stable at low and moderate puerarin doses but decreased in the 1000 mg/kg group, suggesting homeostasis or reduced cellular stress upon treatment. Similar effects have been observed in *Drosophila melanogaster*, where puerarin supplementation improved stress resilience by downregulating *HSP70* expression and antioxidant enzyme activity [45].

In addition to immune enhancement, puerarin significantly improved disease resistance in largemouth bass, as indicated by reduced mortality following *A. hydrophila* challenge. Similarly, a previous study showed that dietary supplementation of Chinese herbs, such as astragalus and cypress, significantly enhanced resistance to *A. hydrophila* in juvenile largemouth bass by modulating gut immunity and antioxidant pathways [46]. *A. hydrophila* is a ubiquitous, opportunistic bacterial pathogen that causes motile aeromonad septicemia (MAS) in freshwater fish, leading to hemorrhagic lesions, fin rot, organ damage, and high mortality [47]. This pathogen thrives in aquatic environments, particularly under conditions of poor water quality, high organic loads, or environmental stressors [47]. This protective effect may stem from the combined impact of immune modulation, oxidative stress reduction, and gut health improvements. Similar findings have been reported in piglets [48] and chicks [49,50], where puerarin supplementation enhanced resistance to bacterial infections by regulating immune gene expression and mucosal immunity. Notably, phytogenic compounds, like puerarin, can reshape gut microbial communities, favoring commensals (e.g., *Bacillus* spp.) that competitively exclude pathogens and secrete antimicrobial metabolites [51]. However, direct evidence of puerarin's impact on fish gut microbiota

remains scarce, warranting metagenomic and metabolomic analyses to clarify its role in aquatic species.

Additionally, our study did not specifically address potential sex differences in the immune responses or growth performance of largemouth bass. Given that the fish were juveniles at the time of the study, it is likely that sexual dimorphism in immune function and growth was not yet fully apparent. However, sex can influence immune responses, hormone levels, and growth rates. The lack of sex-specific data is a limitation of this study and investigations of effects of puerarin on adult fish with different sex should be conducted in the future research. Furthermore, while this study provides valuable insights into the acute effects of puerarin on juvenile fish, long-term studies are needed to evaluate the chronic effects of puerarin supplementation over the lifespan of largemouth bass. Furthermore, there are some other limitations in our study that require further exploration. For example, as a dietary supplement, the stability of puerarin in aquafeeds should be taken into consideration in future research. Another limitation is the small sample size (n = 3) used for biochemical and molecular analyses due to pooling. While pooling was necessary to reduce variability and conserve resources, this small sample size may have reduced the statistical power of the analyses. We recommend that future studies use larger sample sizes to improve the robustness of the data and increase the reliability of the conclusions.

5. Conclusions

Overall, this study provides strong evidence that puerarin supplementation is an effective strategy for enhancing growth performance, immune response, and antioxidative capacity in juvenile largemouth bass. The observed improvements in inflammation control and disease resistance highlight the potential of puerarin as a functional feed additive in aquaculture. Under the highest dosage of puerarin used in this study, the observed effects were not significantly greater than those seen at moderate doses (200 and 500 mg/kg), suggesting that moderate puerarin supplementation may be more beneficial for enhancing largemouth bass immunity and growth. However, an optimization study is essential to determine the most effective puerarin dosage for maximizing its benefits in aquaculture. While this study provides preliminary data, further research is needed to refine the optimal concentration, evaluate interactions with other feed components, and assess long-term effects on fish health. Additionally, future research should focus on exploring its effects on metabolic adaptations at different life stages and also in different fish species to further optimize its application in commercial aquafeeds. This will provide valuable insights into its broader implications for sustainable aquaculture development.

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