



antioxidants

Special Issue Reprint

Antioxidants and Aquaculture

A Synergistic Approach for Sustainable
Aquatic Production

Edited by
Yukun Zhang, Amina Moss and Saichiro Yokoyama

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Guest Editors

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Contents

About the Editors	vii
Preface	ix
Kai Xie, Quan Li, Shuang Zheng, Huahong Wei, Tao Zhou, Yi Hu and Junzhi Zhang Dietary Soy Isoflavones Ameliorate Muscle Quality in High-Fat Diet-Fed Rice Field Eels (<i>Monopterus albus</i>) by Modulating Myogenesis, Collagen Synthesis, and Antioxidant Capacity Reprinted from: <i>Antioxidants</i> 2025 , <i>14</i> , 1195, https://doi.org/10.3390/antiox14101195	1
Shengqi Zhao, Hualiang Liang, Xiaoru Chen, Lu Zhang, Dongyu Huang, Yongli Wang, et al. Optimizing Low Fishmeal Diets with Vitamin C Supplementation: A Comprehensive Study on Growth, Immunity, and Heat Stress Resistance in Largemouth Bass (<i>Micropterus salmoides</i>) Juveniles Reprinted from: <i>Antioxidants</i> 2025 , <i>14</i> , 1175, https://doi.org/10.3390/antiox14101175	21
Nguyen Vu Linh, Luu Tang Phuc Khang, Suwanna Wisetkaso, Nguyen Dinh-Hung, Papungkorn Sangsawad, Waraphorn Sihamok, et al. Dietary Encapsulation of a Novel <i>Lysinibacillus</i> sp. PWR01 Probiotic Modulates Growth, Antioxidant, Immune Gene Expression, and Gut Health in Nile tilapia (<i>Oreochromis niloticus</i>) Against <i>Aeromonas hydrophila</i> Infection Reprinted from: <i>Antioxidants</i> 2026 , <i>15</i> , 373, https://doi.org/10.3390/antiox15030373	40
Nugroho Wiratama, Pakapon Meachasompop, Benchawan Kumwan, Yosapon Adisornprasert, Prapansak Srisapoome, Phornphan Phrompanya, et al. Dietary Probiotic <i>Bacillus subtilis</i> AAHM-BS2360 and Its Postbiotic Metabolites Enhance Growth, Immunity, and Resistance to Edwardsiellosis in <i>Pangasianodon hypophthalmus</i> Reprinted from: <i>Antioxidants</i> 2025 , <i>14</i> , 629, https://doi.org/10.3390/antiox14060629	68
Supreya Wannavijit, Punika Ninyamasiri, Wanarsa Nonkrathok, Sudaporn Tongsiri, Phisit Seesuriyachan, Yuthana Phimolsiripol, et al. Effects of Fermented Longan Peel (<i>Dimocarpus longan</i>) on Growth Performance, Digestive Enzyme Activity, Intestinal Microstructure, Immune Response, and Gene Expression of Nile Tilapia (<i>Oreochromis niloticus</i>) Raised Under Biofloc System Reprinted from: <i>Antioxidants</i> 2026 , <i>15</i> , 394, https://doi.org/10.3390/antiox15030394	98
Ioan Pet, Igori Balta, Nicolae Corcionivoschi, Tiberiu Iancu, Ducu Stef, Lavinia Stef and Iuliana Cretescu Shrimp White Spot Viral Infections Are Attenuated by Organic Acids by Regulating the Expression of HO-1 Oxygenase and β -1,3-Glucan-Binding Protein Reprinted from: <i>Antioxidants</i> 2025 , <i>14</i> , 89, https://doi.org/10.3390/antiox14010089	121
Jingwei Liu, Qian Lin, Jianchao Lu, Tianwei Jiang, Yukun Zhang and Weilong Wang Moderate Dietary Cannabidiol Enhances Growth, Restructures Gut Microbiota, and Bolsters Environmental Stress Resilience in <i>Litopenaeus vannamei</i> Reprinted from: <i>Antioxidants</i> 2026 , <i>15</i> , 475, https://doi.org/10.3390/antiox15040475	138
Ioannis Mittakos, Cosmas I. Nathanailides, Lambros E. Kokokiris, Alexandra Barbouti, Konstantina Bitchava, Evangelia Gouva, et al. Antioxidant Capacity, Lipid Oxidation, and Quality Traits of Slow- and Fast-Growing Meagre (<i>Argyrosomus regius</i>) Fillets During Cold Storage Reprinted from: <i>Antioxidants</i> 2025 , <i>14</i> , 124, https://doi.org/10.3390/antiox14020124	165

About the Editors

Yukun Zhang

Yukun Zhang is an Assistant Professor in the Research Field in Fisheries, Agriculture, Fisheries and Veterinary Medicine Area, Faculty of Fisheries, Department of Fisheries Science and Technology, Kagoshima University, Japan. His research centers on fermentation-based strategies for aquatic feed development, with particular emphasis on probiotic supplementation, gut health and immunity modulation, intestinal microbiology, and the application of machine learning techniques to feed optimization. His notable projects include the application of response surface methodology and artificial neural networks to optimize the fermentation of soybean meal and sweet potato waste for nutritional enhancement in aquafeeds, as well as investigations into the complementary effects of dietary *Bacillus subtilis* natto and *Lactobacillus plantarum* on growth, immunity, and intestinal microflora restructuring in marine finfish such as red sea bream and Japanese flounder. He has authored over 29 peer-reviewed publications in high-impact journals including *Fermentation*, *Fishes*, *Antioxidants*, and *Aquaculture Nutrition*. His key achievements are reflected in his sustained international collaborations, active contributions to advancing sustainable aquaculture through the valorization of agricultural by-products, and his integration of advanced computational models with nutritional science to promote environmentally responsible feed innovation.

Amina Moss

Amina Moss is a Lecturer in Aquaculture Nutrition at the Institute of Aquaculture, University of Stirling, United Kingdom. Her research focuses on the development of functional aquafeeds, with particular emphasis on bioactive compounds, and alternative ingredients such as halophytes. She has experience in feed trial design, nutritional biochemistry, and the evaluation of growth, health, and stress resilience in aquatic species. Her work increasingly explores micro-encapsulation strategies to enhance the stability and bioavailability of nutrients under environmental stress conditions. She has led and contributed to international projects spanning Europe, Africa, and Asia, addressing climate-resilient aquaculture and sustainable feed innovation. Her research aims to bridge fundamental nutritional science with applied solutions for industry.

Saichiro Yokoyama

Saichiro Yokoyama is an Assistant Professor in the Research Field in Fisheries, Agriculture, Fisheries and Veterinary Medicine Area, Faculty of Fisheries at Kagoshima University, Japan, where he has served since June 2006. His research centers on aquatic nutritional science, with a particular focus on the effects of nutritional components on the health of aquatic organisms, stress responses in valuable crustaceans, and the development of functional aquafeeds. His work extensively explores alternative protein sources, dietary supplements such as bovine lactoferrin, seaweed-derived fucoidan, and amino acids, and their roles in enhancing growth, immune responses, and stress tolerance in a variety of cultured marine finfish and shrimp species. He has contributed to a diverse range of international research projects, investigating topics from the interactive effects of dietary astaxanthin and cholesterol in kuruma shrimp to the efficacy of microalgae-derived lipids as fish oil replacements in larval shrimp diets. His notable projects also include research into the relationship between ascorbic acid depletion by OxyPAH exposure and malformation in fish larvae. With over 100 authored papers and approximately 3,900 indexed citations, his key achievements are reflected in his extensive publication record in high-impact journals, his active membership in the World Aquaculture Society and the Japanese

Society of Fisheries Science, and his sustained international collaborations with scholars from over a dozen countries, making significant contributions to sustainable aquaculture nutrition science.

Preface

This Reprint features eight peer-reviewed original research papers focused on the synergistic roles of antioxidants in supporting sustainable aquatic production, addressing the critical challenges faced by modern intensive aquaculture.

The motivation for this collection stems from the growing pressure on global aquaculture: as the industry expands to meet the rising demand for aquatic food, intensive farming systems have instigated multiple stressors triggering oxidative damage, immune suppression, and productivity loss, while the global restriction on antibiotic use has created an urgent need for green, nutrition-based solutions to maintain animal health and production efficiency. To respond to this demand, we organized this Special Issue to collect cutting-edge studies in this field, and we now compile these works into this Reprint to enhance their long-term accessibility and visibility.

This Reprint covers a wide range of research topics, from the physiological barrier repair effects of natural antioxidants and vitamins in finfish, the modulation of probiotics and fermented agricultural by-products on gut microbiota and immune homeostasis, to the molecular stress adaptation mechanisms of crustaceans, and the rules of growth phenotype and post-harvest antioxidant stability of aquatic products. All these works provide novel insights into oxidative stress management, functional feed innovation, and climate-resilient aquaculture solutions, bridging fundamental nutritional science and practical industry applications.

The core aim of this Reprint is to consolidate these high-quality studies into a single, accessible collection, in order to enhance the dissemination of these research findings to a broader audience. This Reprint is addressed to researchers, graduate students, industry professionals, and stakeholders in the fields of aquaculture nutrition, aquatic animal health, and sustainable food production, who are dedicated to advancing environmentally responsible and climate-resilient aquatic farming.

We would like to express our sincere gratitude to all the contributing authors, reviewers, and the editorial team of *Antioxidants*, whose dedicated efforts have made this Reprint possible.

Yukun Zhang, Amina Moss, and Saichiro Yokoyama

Guest Editors

Article

Dietary Soy Isoflavones Ameliorate Muscle Quality in High-Fat Diet-Fed Rice Field Eels (*Monopterus albus*) by Modulating Myogenesis, Collagen Synthesis, and Antioxidant Capacity

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Abstract: High-fat diets are increasingly used to improve feed efficiency in aquaculture but may deteriorate fillet quality and health; soy isoflavones, plant-derived polyphenols, have emerged as promising modulators of muscle growth, antioxidant defense, and lipid metabolism in fish. This study investigated the effects of dietary soy isoflavone supplementation on myogenesis, collagen synthesis, fatty-acid composition, and antioxidant capacity in muscle of *Monopterus albus* fed a high-fat diet. Fish were assigned to four diets: control (CON, 6.16% crude fat), high-fat without soy isoflavones (HSIF0, 11.98% crude fat), and high-fat with 50 mg/kg (HSIF50) or 100 mg/kg (HSIF100) soy isoflavones. HSIF0 significantly elevated whole-body/muscle lipids, reduced Σ SFA/ Σ MUFA/ Σ n-3/ Σ n-6 ratios ($p < 0.05$), increased Σ n-6 ($p < 0.05$), impaired water-holding capacity/texture (higher losses, lower hardness/cohesiveness/gumminess/chewiness/resilience) ($p < 0.05$), induced loosely arranged myofibers with enlarged inter-fiber spaces, downregulated myogenesis (upregulated mstn; downregulated myod/tcap/mrf4/mrf5) and collagen genes (ets1/sp1/p4ha1) ($p < 0.05$), decreased collagen/hydroxyproline ($p < 0.05$), and weakened antioxidants (higher MDA/ H_2O_2 ; lower T-AOC/GSH; downregulated nrf2/sod/cat/gpx1/gpx8) ($p < 0.05$). HSIF50 reversed these effects, enhancing Σ PUFA/ Σ n-3/EPA+DHA ($p < 0.05$), restoring structure/gene expression ($p < 0.05$), and boosting antioxidants ($p < 0.05$). In contrast, HSIF100 partially diminished benefits, indicating dose-dependency. Overall, 50 mg/kg soy isoflavones optimally mitigated high-fat-induced muscle quality decline via lipid remodeling, structural improvement, collagen promotion, and antioxidant enhancement.

Keywords: *Monopterus albus*; soy isoflavones; high-fat diet; fatty-acid composition; myogenesis; collagen synthesis; antioxidant capacity

1. Introduction

In aquaculture, high-fat diets have gained prominence due to their numerous advantages in enhancing fish production efficiency. These diets provide a concentrated energy source that is cost-effective, as lipids are generally cheaper than proteins, allowing for protein-sparing effects where dietary protein can be reduced without compromising growth [1]. However, high-fat diets promote ectopic lipid deposition in muscle and viscera, elevate malondialdehyde (MDA; lipid-peroxidation marker), and suppress antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx). These

changes increase reactive oxygen species (ROS), damaging cells, and triggering inflammation. As a result, flesh quality deteriorates—firmness declines, drip loss rises, and fatty-acid profiles shift toward pro-inflammatory n-6 polyunsaturated fatty acids (PUFAs) [2]. Studies indicate that hypoxia in Nile tilapia (*Oreochromis niloticus*) reduces growth rate, alters muscle proximate composition and flesh quality, increases lipid accumulation, and compromises texture parameters such as hardness and chewiness [3]. In grass carp (*Ctenopharyngodon idella*), high fat levels altered muscle growth traits, decreasing collagen synthesis and myofiber density, which correlated with softer textures and reduced water-holding capacity [4]. Similarly, high-fat diets in rainbow trout (*Oncorhynchus mykiss*) resulted in reduced myostatin-1 gene activity and modified lipid profiles in muscle tissue, which compromised muscle fiber development and oxidative defenses, consequently causing withered or less elastic muscle [5]. In yellow catfish (*Pelteobagrus fulvidraco*), high-fat diets induced oxidative stress, reducing fatty acid oxidation and collagen levels, further degrading muscle quality [6]. Collectively, these lines of evidence underscore an urgent need to rationalize high-fat feeding strategies in aquaculture—optimizing lipid level and source and leveraging functional additives—to sustain growth while safeguarding flesh quality and fish health. Yet, the underlying mechanisms by which high-fat diets impair muscle quality and the nutritional interventions that can effectively mitigate these effects remain insufficiently understood.

Soy isoflavones (SIFs), primarily genistein and daidzein, are bioactive phytoestrogens abundant in soybeans and soy-based feeds, known for their estrogen-mimicking properties and potential health-modulating effects [7]. They are recognized as potent antioxidants, capable of scavenging free radicals, inhibiting lipid peroxidation, and enhancing the activity of endogenous antioxidant enzymes [8]. In livestock such as broilers and pigs, SIFs enhance meat quality by directly improving antioxidative properties, which in turn helps stabilize protein matrices and mitigate the oxidative stress that would otherwise compromise meat quality. They also boost redness, water-holding capacity, and lean muscle growth [9,10]. In aquatic animals, SIFs offer similar benefits; for instance, in turbot (*Scophthalmus maximus*) [11] and juvenile golden pompano (*Trachinotus ovatus*) [12], SIFs supplemented in feeds enhance antioxidant defenses, immune responses, and fat metabolism, whereas research on grass carp indicates improvements in flesh physical properties like tenderness and water-holding capacity through enhanced collagen deposition and flavor quality [13]. Soy isoflavones activate nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent antioxidant defenses and improve redox status; as phytoestrogens they also interact with estrogen receptors, which can affect muscle signaling and extracellular-matrix homeostasis in a context- and dose-dependent manner [8]. However, excessive SIF addition can elicit adverse outcomes, particularly in fish, where high doses may disrupt endocrine functions, induce pro-oxidant, and compromise growth and quality [14]. For example, in rice field eels (*Monopterus albus*), levels above 2.5 g/kg depressed growth responses, feed efficiency ratios and triggered inflammation [15]. High SIF levels in Chinese mitten crabs (*Eriocheir sinensis*) also compromised oxidative defenses and innate immune functions [16], while in rainbow trout, they potentially induced stress responses like elevated cortisol, leading to diminished muscle quality and reproductive issues [17]. While SIFs are known to improve muscle quality in terrestrial animals and some fish, their role in rice field eel muscle quality under high-fat diets has not been studied. The dose-dependent effects of SIFs (beneficial or harmful) are poorly understood in aquaculture species. Therefore, the aim of this study was to investigate the effects of soy isoflavones on muscle quality, antioxidant capacity and collagen synthesis in rice field eels fed a high-fat diet and to determine the optimal inclusion levels.

2. Materials and Methods

2.1. Ethical Statement

All experimental procedures were conducted in strict accordance with the guidelines for the care and use of laboratory animals established by the Ministry of Science and Technology of the People's Republic of China (Guidelines for the Welfare of Laboratory Animals, 2006) and complied with international standards, including the ARRIVE guidelines for reporting animal research. This study was approved by the Committee of Laboratory Animal Management and Animal Welfare of Hunan Agricultural University (Changsha, China), and all experimental procedures con-formed to the guidelines of the Ethical Committee of Hunan Agricultural University (approval number: 2025121, approved on 8 April 2025).

2.2. Experimental Diets

The test feeds were prepared utilizing fish meal, soy protein concentrate and poultry by-product meal as main protein components, along with fish oil serving as the fat supplier. Every ingredient for the feeds came from Zhangjiajie Xinrui Feeds Co., Ltd. (Zhangjiajie, China). Soy isoflavones were provided as a commercial preparation ($\geq 98\%$, purchased from Chengdu Yuancheng Technology Co., Ltd., Chengdu, China). Four distinct feeds were designed: a control diet (CON) containing 6.16% crude fat, a high-fat diet (containing 11.98% crude fat) without soy isoflavones (HSIF0), plus two high-fat diets enriched with either 50 mg/kg soy isoflavones (HSIF50) or 100 mg/kg soy isoflavones (HSIF100). Prior to formulating the feeds, each raw material was milled into a fine consistency and blended progressively in suitable ratios to achieve uniform distribution. Soy isoflavones were first premixed with a small portion of the basal powder (1:10, *w/w*) to ensure uniform dispersion, and then the premix was homogenized with the remaining ingredients using a V-type mixer for 5 min before the addition of fish oil. Fish oil was incorporated directly into the blend to maintain even texture. The resulting diets were air-dried and kept in a cool, dry place prior to feeding. Detailed diet formulations are listed in Table 1.

Table 1. Feed formula and nutrition levels (dry matter).

Ingredient	CON	HSIF0	HSIF50	HSIF100
Fish meal	40.00	40.00	40.00	40.00
Soy protein concentrate	16.00	16.00	16.00	16.00
Poultry by-product meal	3.00	3.00	3.00	3.00
Beer yeast	5.00	5.00	5.00	5.00
Fish oil	2.20	8.20	8.20	8.20
Microcrystalline cellulose	10.26	4.26	4.26	4.26
Corn starch	20.00	20.00	20.00	20.00
Choline	0.50	0.50	0.50	0.50
Ca(H ₂ PO ₄) ₂	2.00	2.00	2.00	2.00
Premix ¹	1.00	1.00	1.00	1.00
Antioxidants ²	0.01	0.01	0.01	0.01
Mold inhibitor ³	0.03	0.03	0.03	0.03
Soy isoflavone ⁴ , mg/kg	0.00	0.00	50.00	100.00
Nutrition levels ⁵				
Moisture	7.45	7.52	7.56	7.49
Crude protein	41.36	41.26	40.96	41.51
Crude fat	6.16	11.98	12.11	12.08
Ash	10.23	10.35	10.17	10.31

Notes: ¹ Provided by MGO Ter Bio-Tech Co., Ltd. (Qingdao, China). Composition consistent with previous studies [2]. ² The main component of antioxidant is ethoxyquin. ³ The main component of mould inhibitor is calcium propionate. ⁴ $\geq 98\%$, purchased from Chengdu Yuancheng Technology Co., Ltd. (Chengdu, China). ⁵ The crude protein, crude lipid, and ash levels were measured values.

2.3. Feeding Experiment and Sample Collection

The *M. albus* utilized in this experiment were procured from Xihu Farm, situated in Changde, China, and belonged to a variety distinguished by deep yellow coloration with large spots. A total of 800 fish, with an average initial body weight of 26 g, were randomly allocated to 16 cages measuring 2.0 m × 1.5 m × 1.5 m. After a two-week acclimation period and 24 h of fasting, 50 eels were placed in each cage, and each treatment group had 4 replicates. In this study, experimental units (such as tanks or animals) were allocated to control and treatment groups using randomisation. The randomisation sequence was generated using the random number function in Excel. The acclimation procedure followed the methods from our previous work [18]. Throughout the 8-week trial period, the fish received manual feeding once per day from 17:00 to 18:30, with rations set at 3–5% of their body mass. Water quality was maintained throughout the experiment, with pH ranging from 7.1 to 7.5, dissolved oxygen above 6.5 mg/L, an average temperature of 29.0 °C, and ammonia levels below 0.5 mg/L. In cases where an individual eel exhibited signs of severe distress, such as continuous loss of appetite and inactivity on the aquatic plants for several consecutive days, it would be humanely euthanized to prevent further suffering.

After fasting for 24 h, seven eels were arbitrarily chosen from every enclosure and sedated using eugenol, followed by rapid euthanasia. Dorsal muscle was dissected from both sides of the spine. For three fish, approximately 1–2 cm³ of dorsal muscle was collected for texture profile analysis (stored in 2 mL tubes at 4 °C) and histological examination (fixed in 4% paraformaldehyde in 15 mL tubes). For one fish, ~6 g of dorsal muscle was collected for water loss determination. For another fish, ~2.0 g of dorsal muscle was placed in RNase-free 1.5 mL tubes, rapidly snap-frozen using liquid nitrogen, then preserved at –80 °C until further biochemical and gene expression evaluations. For the remaining two fish, ~10 g of dorsal muscle was placed in sealed polyethylene bags and stored at –20 °C for proximate composition and fatty acid profile analysis. The lab technician performing the outcome assessments was blinded to the group assignments. A schematic representation of the experimental design is provided in Figure S1.

2.4. Analytical Methods

2.4.1. Proximate Composition of Feed, Muscle and Whole Fish

Proximate composition of feed, muscle, and whole-body samples was analyzed according to AOAC official methods [19]. Moisture levels were quantified by oven-drying specimens at 105 °C to reach stable mass. Lipids were isolated via Soxhlet extraction with diethyl ether solvent. Mineral content was measured by incinerating materials in a muffle oven at 550 °C until mass remained unchanged.

2.4.2. Muscle Fatty Acid Composition

Approximately 2.0 g of dorsal muscle was freeze-dried and ground into a fine powder. A 100 mg portion of the sample was extracted with 2 mL of chloroform–methanol (2:1, v/v). Following centrifugation, the upper layer was harvested, and the extracted lipids underwent methylation using 2 mL of methanol with 1% sulfuric acid and 0.01% butylated hydroxytoluene (BHT) at 80 °C for 2 h, yielding fatty acid methyl esters (FAMES). The fatty acid profiles were examined via gas chromatography-mass spectrometry (GC-MS; model 7890B-5977A, Agilent, Santa Clara, CA, USA) fitted with an HP-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness). Helium served as the carrier gas, flowing at 1 mL/min, with a 1 µL sample injection volume. The column temperature program started at 50 °C for 2 min, ramped up to 220 °C at a rate of 10 °C/min, and maintained for 15 min. Fatty acid contents (mg/g dry weight) were quantified using FAME standards (Supelco, Bellefonte, PA, USA) as external references, and bar charts were subsequently

generated from the relative abundance of each fatty acid (percentage of total identified fatty acids).

2.4.3. Muscle Water-Holding Capacity

Cooking loss, liquid loss, and water loss of dorsal muscle were determined according to Honikel (1998) with modifications [20]. Sample weights were measured to the nearest 0.0001 g using an electronic analytical balance (FA1004B, Shanghai Precision & Scientific Instrument Co., Ltd., Shanghai, China).

Approximately 2.0 g of dorsal muscle (recorded as W1) was sealed in a polyethylene bag, immersed in a water bath maintained at 80 °C for 20 min, subsequently cooled to ambient temperature, and patted dry. The post-cooking weight (W2) was then measured. The percentage of cooking loss was determined by the equation: $[(W1 - W2)/W1] \times 100\%$.

Approximately 2.0 g of dorsal muscle tissue (designated W1) was enclosed in a plastic bag and refrigerated at 4 °C for 48 h. Accumulated fluid was discarded, and the tissue was reweighed (W2). The percentage of liquid loss was determined by the equation: $[(W1 - W2)/W1] \times 100\%$.

Approximately 2.0 g of dorsal muscle tissue was precisely measured (denoted as W1), positioned between two sheets of filter paper, and compressed with a 35 kg load for 5 min. Post-compression, the tissue was reweighed (denoted as W2). The percentage of water loss was determined using the equation: $[(W1 - W2)/W1] \times 100\%$.

2.4.4. Muscle Textural Properties

Muscle textural properties, including hardness, springiness, cohesiveness, gumminess, chewiness, and resilience, were measured using a texture profile analysis (TPA) method with a texture analyzer (TA.XT Plus, Stable Micro Systems, Godalming, Surrey, UK) equipped with a P/36R flat cylindrical probe. Raw dorsal muscle samples (1–2 cm³) were compressed twice to 50% of their original height at a constant speed of 1 mm/s, with a 5 s interval between compressions. Data were analyzed using the instrument's software (Exponent software, version 2.21.3) to calculate each texture parameter.

2.4.5. Muscle Histomorphology

Muscle tissue samples were fixed in 4% paraformaldehyde and processed following dehydration, clearing, embedding in paraffin, sectioning, and deparaffinization. After sectioning, the slides were stained with H&E staining. After section preparation, the slides were scanned and observed using an Eclipse Ci-L camera (Nikon, Tokyo, Japan) and a KF-FL-020 Digital Whole-Slide Pathology Scanner (Ningbo Jiangfeng Bioinformatics Technology Co., Ltd., Zhejiang, China).

The other muscle tissue samples were embedded in OCT, snap-frozen, and stored at −80 °C. Cryosections (8–10 µm) were cut at −20 °C, air-dried, fixed in 4% PFA for 10 min, rinsed in PBS, equilibrated in 60% isopropanol for 1 min, followed by incubation in a newly prepared Oil Red O solution (made by diluting 0.5% Oil Red O isopropanol stock in a 3:2 ratio with water, followed by filtration) for 10–15 min under ambient conditions. Slides underwent a short differentiation step in 60% isopropanol (approximately 30 s), followed by a water rinse and hematoxylin counterstaining (30–60 s), and mounted with glycerol gelatin. Images were acquired with the same microscope/scanner as above.

2.4.6. Muscle Collagen Content

Muscle collagen content was determined based on hydroxyproline measurement. The hydroxyproline content was determined based on its reaction with an oxidizing agent to produce oxidative products, which subsequently react with pdimethylaminobenzaldehyde to yield a purplish-red color. The color intensity was used to calculate the hydroxyproline

content, using a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Collagen content was calculated assuming hydroxyproline accounts for 13.4% of the total collagen.

2.4.7. Muscle Antioxidant Indices

Malondialdehyde (MDA) content was measured by reacting with thiobarbituric acid to form a pink complex, which was then measured at 532 nm. Superoxide dismutase (SOD) activity was assessed by monitoring the reduction of nitrogen blue tetrazole at 450 nm. Total antioxidant capacity (T-AOC) was measured using the ferric reducing ability of plasma (FRAP) method, in which antioxidants reduce the Fe^{3+} -TPTZ complex to Fe^{2+} -TPTZ, producing a blue color with a maximum absorbance at 593 nm. The content of glutathione (GSH) was quantified by the reaction with 2-nitrobenzoic acid, producing yellow 2-nitro-5-mercaptobenzoic acid, with a characteristic absorption peak at 405 nm. Hydrogen peroxide (H_2O_2) content was quantified based on the reaction of H_2O_2 with molybdc acid to form a yellow complex, which was measured at 405 nm.

2.4.8. Real-Time Polymerase Chain Reaction

Total RNA was isolated from muscle tissues using Trizol reagent (Monad Biotech Co., Suzhou, China). RNA quality was assessed, and reverse transcription was performed with MonScript™ RTIII Super Mix (Yeasen Biotechnology, Shanghai, China). Quantitative PCR (qPCR) was conducted using SYBR® Green qPCR Mix (Monad Biotech Co., Suzhou, China), the cycling parameters were 95 °C for 3 min; 40 cycles of 95 °C for 10 s and 60 °C for 15 s; followed by a melt curve from 65 to 95 °C, 0.5 °C increments. Gene expression associated with muscle myogenesis, collagen metabolism and antioxidant were quantified, with primer sequences provided in Table 2.

Table 2. Primes of hepatic glycolipid metabolism, antioxidant and inflammatory related genes of *M. albus*.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Accession No.
<i>rpl17</i>	CGAGAACCCGACTAAATCA	GTGTAGCGACGGAAAGG	XM_020587712.1
<i>mstn</i>	TTGGCTGGGACTGGATTATTG	TTGGTGACATCTTGGTGGGG	KM103284.1
<i>myod</i>	ACATCCAGCTCCTTTCCTCC	TGCTCAATCCCAAACACACC	XM_020593504.1
<i>tcap</i>	TGGAGAAGTGTAATGGTATGGTGG	GCCTTTCCTGAGGTTGGGTT	XM_020607417.1
<i>mrf4</i>	CAAGGTGGAGATTTACGCAGC	GTTTGGGTTTCTCCTGTTTCGT	KM103283.1
<i>myf5</i>	ATGTCTTCTCATCATCCAGGTC	CCTGACGTGCTCATCTTCCTCT	KM103285.1
<i>ets1</i>	TCAGCTCAGAGGAAGTCTGAC	AGTCCTGGCCACCAAGTTTACC	XM_020600359.1
<i>sp1</i>	ACAGCCAGTGTCTTCCAACAG	AAGGATCTGCTGTGACTGCTG	XM_020600700.1
<i>p4ha3</i>	AAAGACTCTGCCAGACCCAAG	ATGTCTTCAGCCTCTGTGTCAG	XM_020588625.1
<i>nrf2</i>	TCACAGACGAGAATGATGCC	CTGCTACTGGGAAGTGAAGTGA	XM_020596408.1
<i>sod</i>	GTTGCCAAGATAGACATCACGG	TCATTGCCTCCTTTTCCAG	XM_020598412.1
<i>cat</i>	CATTGGGAAGACTACACCTATCGC	GATGAAGAAGATGGGGGTGTTG	XM_020624985.1
<i>gpx1</i>	AGATGTGAATGGGAAGGATGCC	AAACTTCGGGTCAGTCATCAGG	XM_020607739.1
<i>gpx8</i>	ATCCTGCCTTCAGATTCCTCAC	TCATTTCCTCGCACCAGCACT	XM_020593975.1

Notes: RPL17 was used as the reference gene.

2.4.9. Statistical Analysis

Data processing was carried out in Microsoft Excel 2019, while statistical evaluations were performed using R version 4.3.3. The experimental unit was the cage ($n = 4$ per treatment). For variables measured on multiple fish per cage, individual values were averaged within each cage prior to analysis. Data normality was evaluated with the Shapiro–Wilk test, and homogeneity of variance was assessed using Levene’s test. When assumptions were met, one-way ANOVA followed by Tukey’s HSD test was applied

($p < 0.05$). For non-normally distributed datasets, the Kruskal–Wallis test followed by pairwise comparisons with Bonferroni adjustment was used.

3. Results

3.1. Proximate Composition, Muscle Oil Red O-Stained Sections, and Fatty Acid Profile

In this study, no significant differences in muscle moisture content were observed among the treatment groups ($p > 0.05$). Compared with the CON group, whole-body and muscle crude lipid contents were significantly higher in the HSIF0 group ($p < 0.05$). Supplementation of the high-fat diet with 50 or 100 mg/kg soy isoflavones significantly reduced whole-body and muscle crude lipid contents compared with HSIF0 ($p < 0.05$), with the HSIF100 group showing a further decrease relative to HSIF50 ($p < 0.05$) (Figure 1A–D).

As shown in Figure 1E, the CON group exhibited almost no Oil Red O-positive staining, indicating minimal intramuscular lipid. In contrast, the HSIF0 group showed extensive red staining distributed along myosepta and between fibers, reflecting marked lipid droplet accumulation. Supplementation with 50 mg/kg and 100 mg/kg soy isoflavones markedly attenuated staining intensity, with only sparse punctate droplets.

As shown in Figure 2, the five most abundant fatty acids in *M. albus* muscle were C18:1n9c, C16:0, C18:2n6c, C22:6n3, and C16:1. Compared with the CON group, the HSIF0, HSIF50, and HSIF100 groups showed significantly lower Σ SFA, Σ MUFA, and Σ n-3/ Σ n-6 ratios, but higher Σ n-6 proportions ($p < 0.05$). In contrast, Σ PUFA and Σ n-3 proportions were significantly higher in the HSIF50 and HSIF100 groups than in the CON group ($p < 0.05$). Furthermore, compared with HSIF0, the HSIF50 group exhibited significantly lower Σ SFA and Σ n-6 proportions, but higher Σ MUFA and EPA + DHA proportions ($p < 0.05$).

3.2. Muscle Water-Holding Capacity and Textural Properties

Compared with the CON group, the HSIF0 group exhibited significantly higher cooking loss, liquid loss, and water loss ($p < 0.05$). Compared with the HSIF0 group, supplementation of the high-fat diet with 50 mg/kg soy isoflavones significantly reduced cooking loss, liquid loss, and water loss ($p < 0.05$). Furthermore, the HSIF100 group showed significantly higher liquid loss and water loss than the HSIF50 group ($p < 0.05$) (Figure 3A–C).

No significant differences in muscle springiness were observed among the dietary treatments ($p > 0.05$). Compared with the CON group, the HSIF0 group showed significantly lower hardness, cohesiveness, gumminess, chewiness, and resilience ($p < 0.05$). Supplementation of the high-fat diet with 50 mg/kg soy isoflavones (HSIF50) significantly increased these five textural parameters relative to the HSIF0 group ($p < 0.05$). However, as the level of soy isoflavone supplementation increased, cohesiveness, gumminess, and chewiness decreased significantly ($p < 0.05$) (Figure 3D–I).

3.3. Muscle Histomorphology and Expression of Myogenesis-Related Genes

As shown in Figure 4A, muscle fibers in the CON group were arranged compactly with regular morphology and minimal inter-fiber spaces. In the HSIF0 group, fibers exhibited loose arrangement, irregular shapes, and enlarged inter-fiber spaces, with partial structural disruption. Supplementation of the high-fat diet with 50 mg/kg soy isoflavones (HSIF50) resulted in tightly packed fibers, more uniform morphology, and reduced inter-fiber gaps compared with the HSIF0 group. However, in the HSIF100 group, although the fibers remained generally intact, the inter-fiber spaces were larger than in the HSIF50 group, indicating a partial decline in muscle structural integrity at higher supplementation levels.

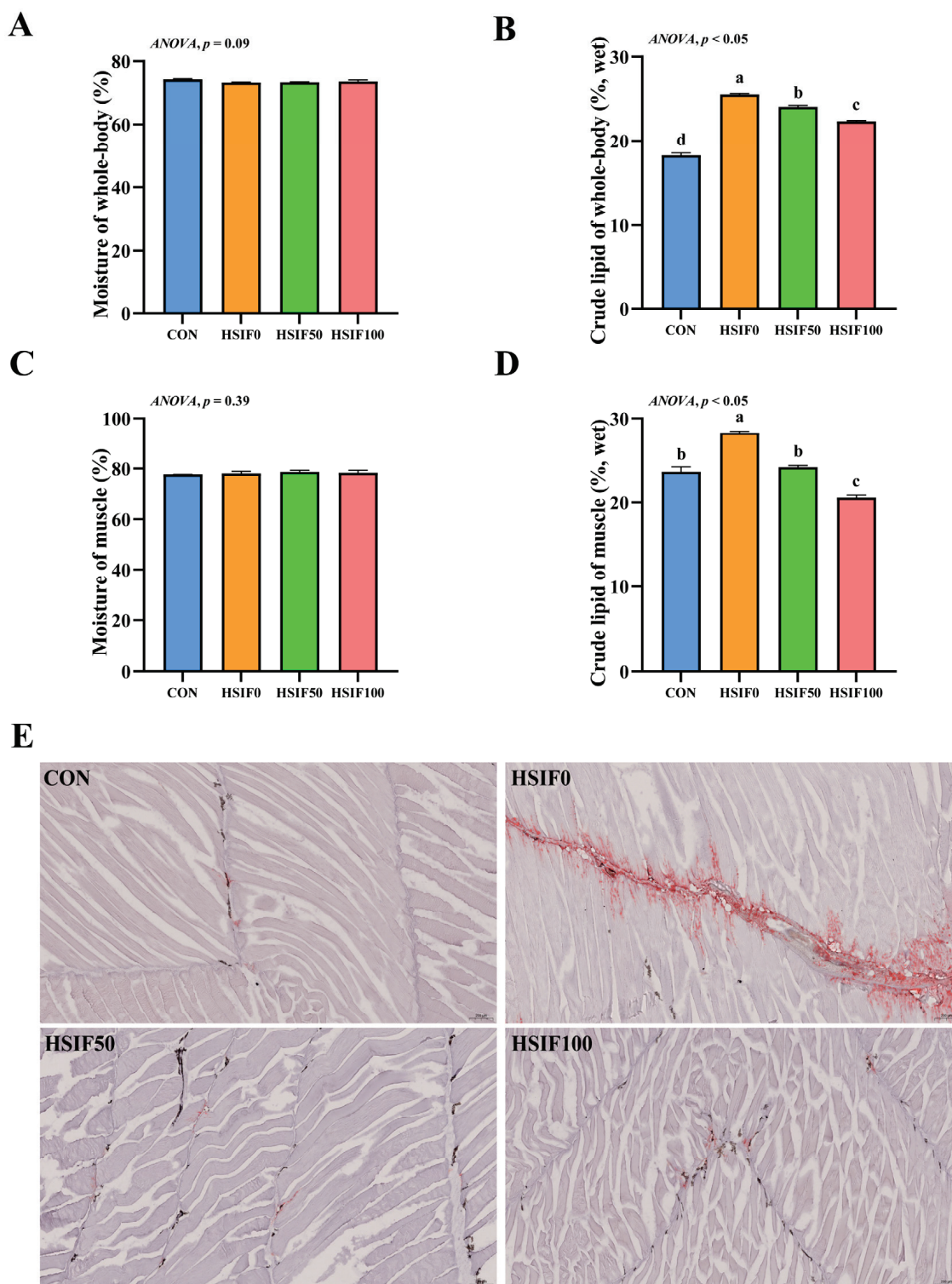


Figure 1. Effects of dietary treatments on proximate composition, muscle Oil red O-stained sections in *M. albus*. Notes: (A) Moisture of whole-body ($n = 4$); (B) crude lipid of whole-body ($n = 4$); (C) Moisture of muscle ($n = 4$); (D) crude lipid of muscle ($n = 4$); (E) The image shows Oil Red O-stained sections ($150\times$). CON: control diet (6.16% crude fat); HSIF0: high-fat diet (11.98% crude fat); HSIF50: HSIF0 supplemented with 50 mg/kg soy isoflavones; HSIF100: HSIF0 supplemented with 100 mg/kg soy isoflavones. Bars represent mean $\pm$ standard error (SE). Different superscript letters denote significant differences among groups ($p < 0.05$). CON: control diet (6.16% crude fat); HSIF0: high-fat diet (11.98% crude fat); HSIF50: HSIF0 supplemented with 50 mg/kg soy isoflavones; HSIF100: HSIF0 supplemented with 100 mg/kg soy isoflavones.

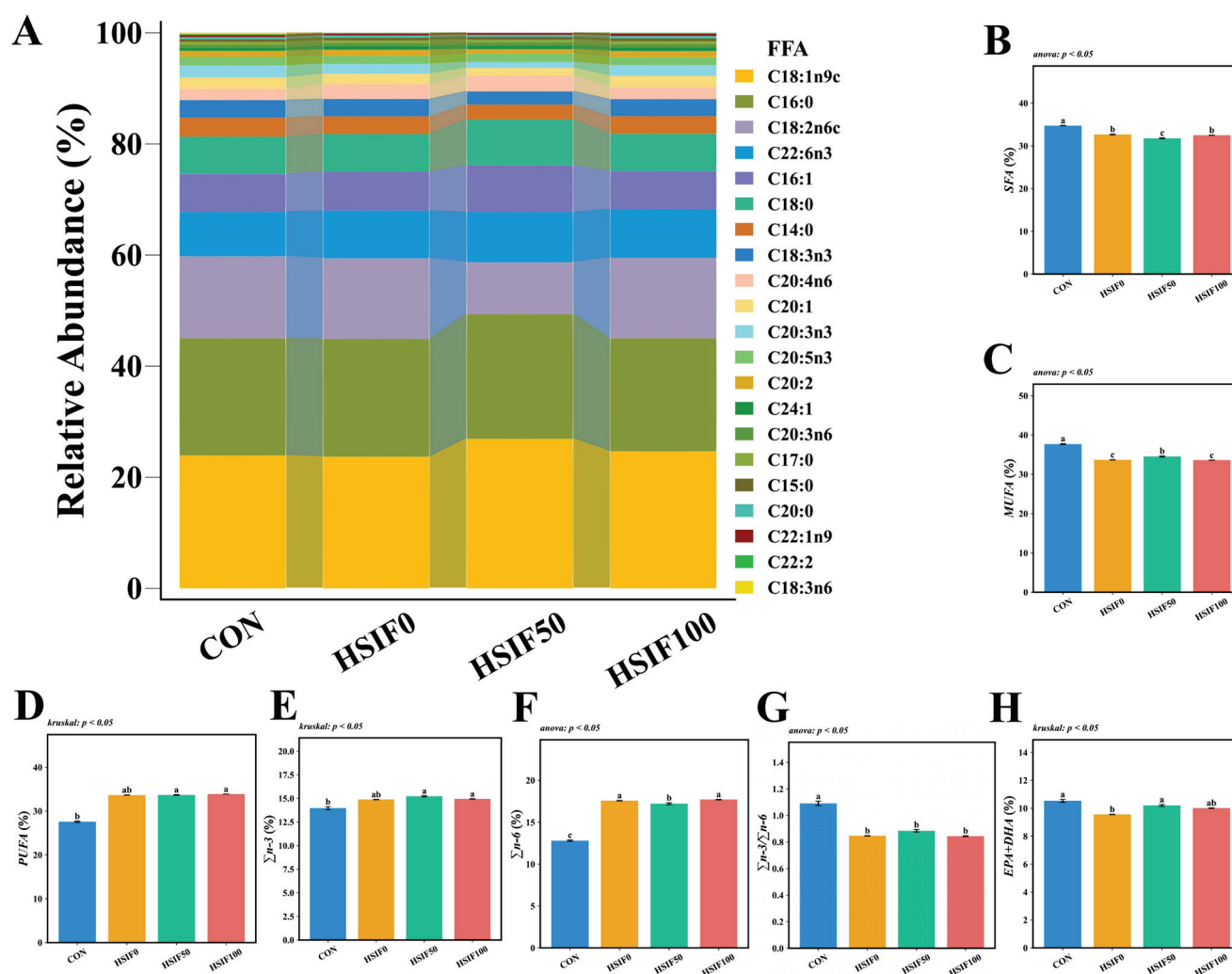


Figure 2. Effects of dietary treatments on muscle fatty acid composition in *M. albus* ($n = 4$). Notes: (A) interactive ribbon bar chart; (B) total saturated fatty acids (Σ SFA); (C) total monounsaturated fatty acids (Σ MUFA); (D) total polyunsaturated fatty acids (Σ PUFA); (E) total n-3 polyunsaturated fatty acids (Σ n-3); (F) total n-6 polyunsaturated fatty acids (Σ n-6); (G) ratio of total n-3 to total n-6 polyunsaturated fatty acids (Σ n-3/ Σ n-6). (H) eicosapentaenoic acid plus docosahexaenoic acid (EPA + DHA) Bars represent mean $\pm$ standard error (SE). Different superscript letters denote significant differences among groups ($p < 0.05$). CON: control diet (6.16% crude fat); HSIF0: high-fat diet (11.98% crude fat); HSIF50: HSIF0 supplemented with 50 mg/kg soy isoflavones; HSIF100: HSIF0 supplemented with 100 mg/kg soy isoflavones.

As shown in Figure 4B, compared with the CON group, the HSIF0 group exhibited a significant upregulation of *mstn* expression and a significant downregulation of *myod*, *tcap*, *mrf4*, and *mrf5* expression ($p < 0.05$). Compared with the HSIF0 group, supplementation of the high-fat diet with 50 mg/kg soy isoflavones significantly downregulated *mstn* expression and significantly upregulated *tcap*, *mrf4*, and *mrf5* expression ($p < 0.05$). Furthermore, in the HSIF100 group, *mstn* expression was significantly upregulated, whereas *tcap* and *mrf4* expression were significantly downregulated compared with the HSIF50 group ($p < 0.05$).

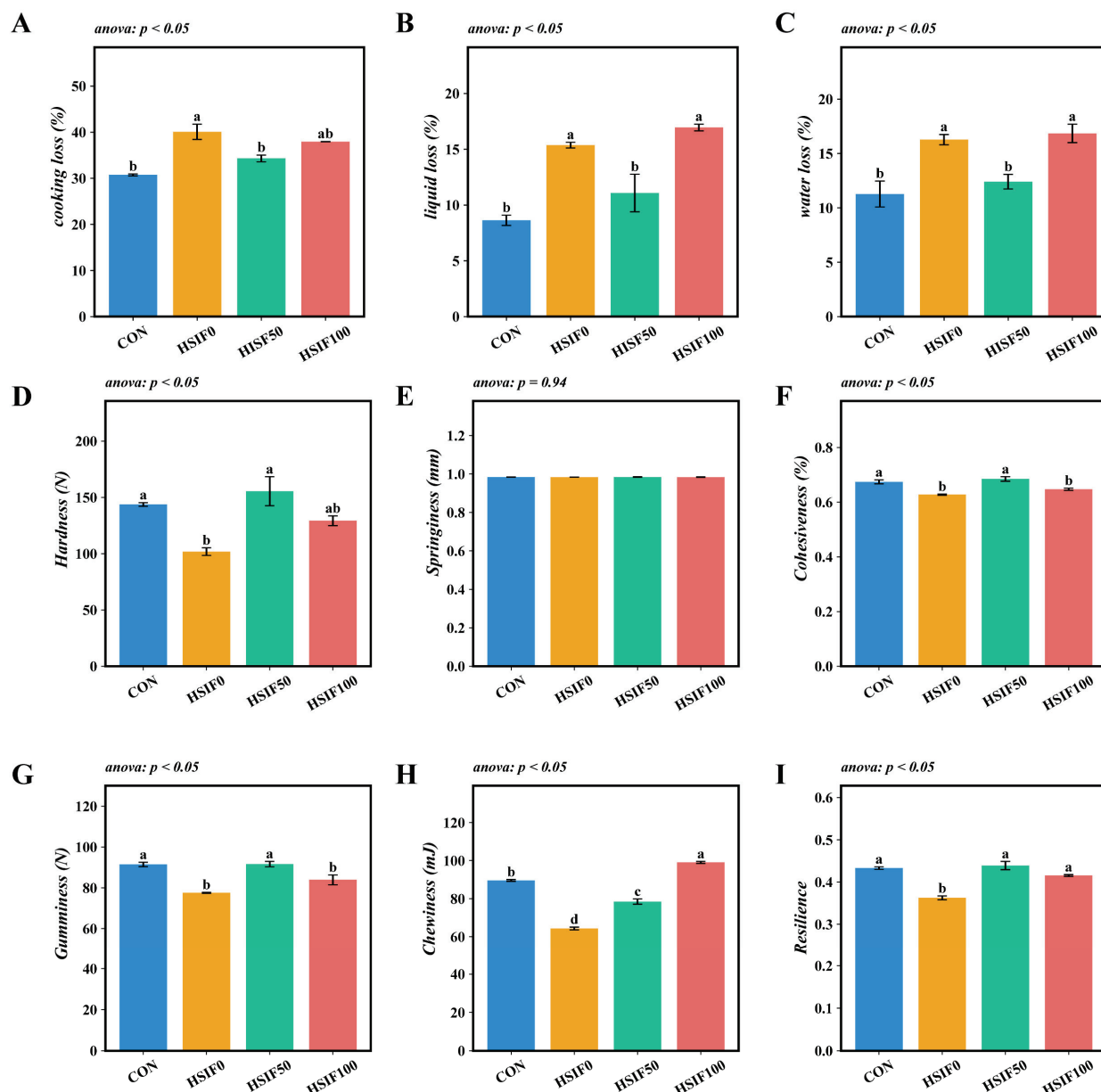


Figure 3. Effects of dietary treatments on muscle water-holding capacity and textural properties in *M. albus* ($n = 4$). Notes: (A) cooking loss of muscle; (B) liquid loss of muscle; (C) water loss of muscle; (D) hardness (E) springiness; (F) cohesiveness; (G) gumminess; (H) chewiness; (I) resilience. CON: control diet (6.16% crude fat); HSIF0: high-fat diet (11.98% crude fat); HSIF50: HSIF0 supplemented with 50 mg/kg soy isoflavones; HSIF100: HSIF0 supplemented with 100 mg/kg soy isoflavones. Bars represent mean $\pm$ standard error (SE). Different superscript letters denote significant differences among groups ($p < 0.05$). CON: control diet (6.16% crude fat); HSIF0: high-fat diet (11.98% crude fat); HSIF50: HSIF0 supplemented with 50 mg/kg soy isoflavones; HSIF100: HSIF0 supplemented with 100 mg/kg soy isoflavones.

3.4. Muscle Collagen Content and Expression of Collagen Metabolism-Related Genes

As shown in Figure 5A,B, muscle hydroxyproline and collagen contents were significantly lower in the HSIF0 group than in the CON and soy isoflavone-supplemented groups ($p < 0.05$). Furthermore, the HSIF100 group exhibited significantly lower hydroxyproline and collagen levels compared with the HSIF50 group ($p < 0.05$).

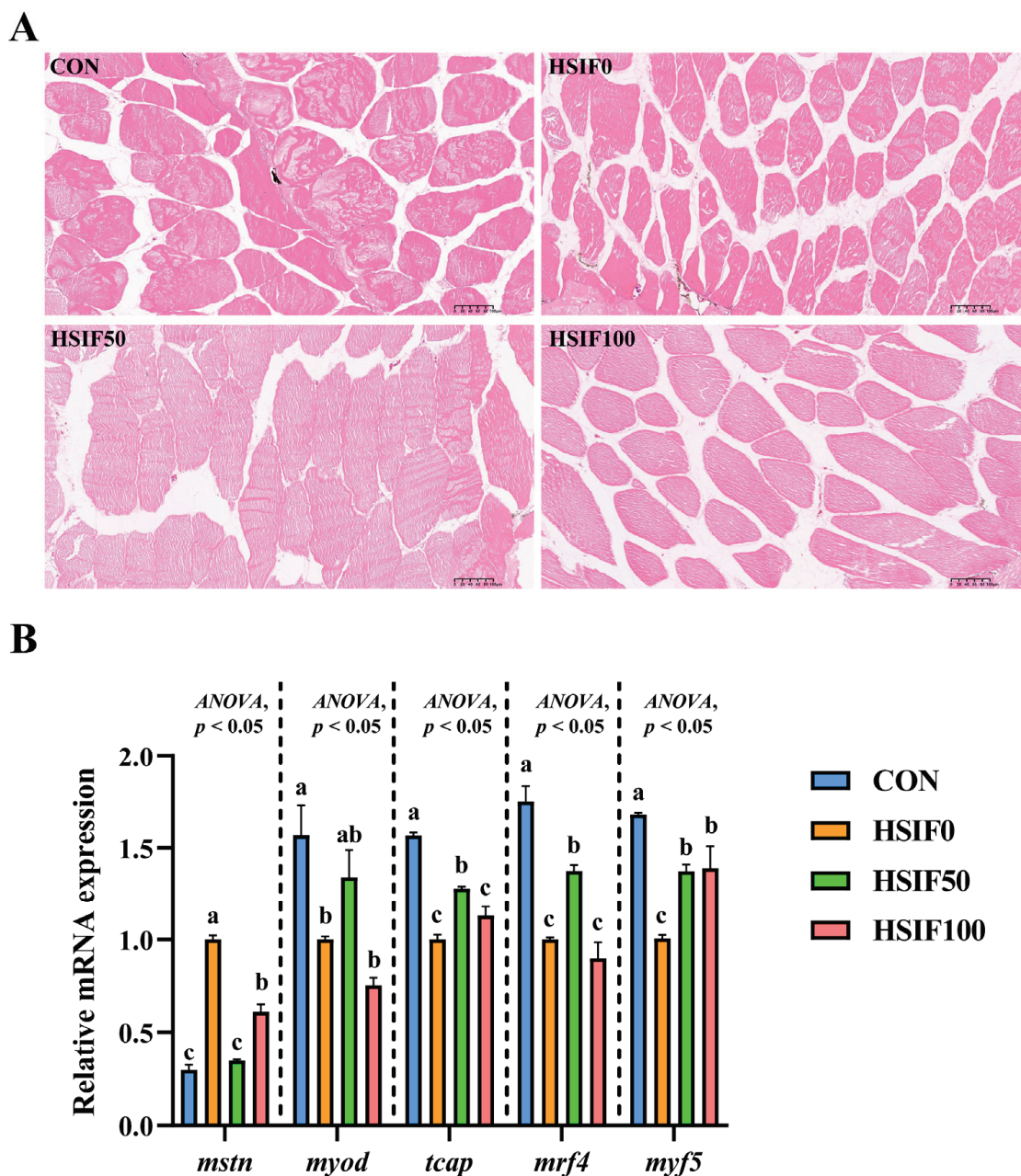


Figure 4. Effects of dietary treatments on muscle histomorphology and expression of myogenesis-related genes in *M. albus*. Notes: (A) The image shows H&E-stained sections (150×). (B) $n = 4$; *mstn*—Myostatin; *myod*—Myogenic differentiation; *tcap*—Telethonin; *mrf4*—Myogenic regulatory factor 4; *myf5*—Myogenic regulatory factor 5. *rpl17* was the internal reference gene. Bars represent mean $\pm$ standard error (SE). Different superscript letters denote significant differences among groups ($p < 0.05$). CON: control diet (6.16% crude fat); HSIF0: high-fat diet (11.98% crude fat); HSIF50: HSIF0 supplemented with 50 mg/kg soy isoflavones; HSIF100: HSIF0 supplemented with 100 mg/kg soy isoflavones.

As shown in Figure 5C, the relative expression levels of *ets1*, *sp1*, and *p4ha1* in muscle were significantly downregulated in the HSIF0 group compared with the CON group ($p < 0.05$). Supplementation of the high-fat diet with 50 mg/kg soy isoflavones significantly upregulated these genes compared with the HSIF0 group ($p < 0.05$). However, as the soy isoflavone supplementation level increased, the relative expression of *ets1*, *sp1*, and *p4ha1* declined significantly ($p < 0.05$).

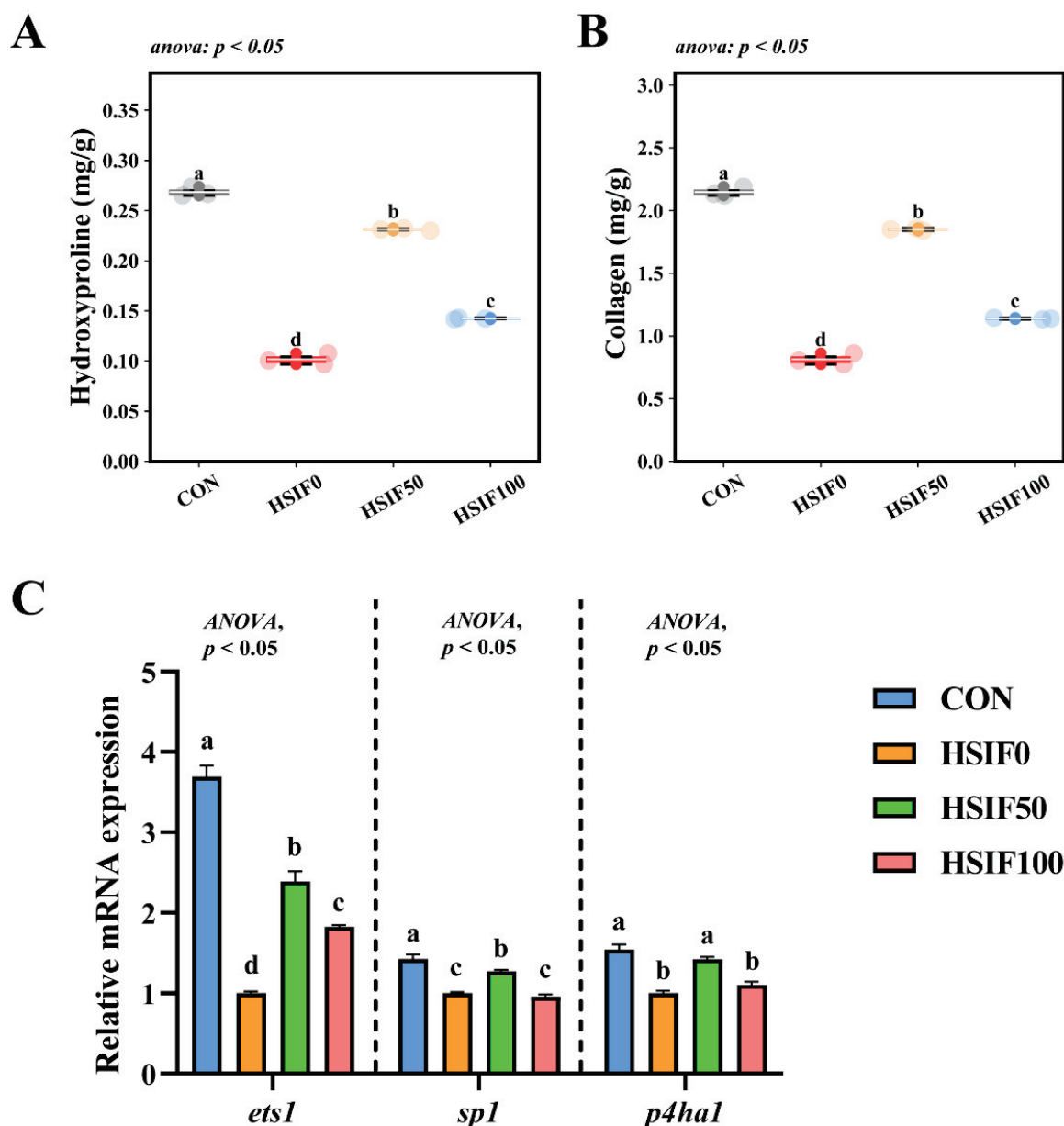


Figure 5. Effects of dietary treatments on muscle collagen content and expression of collagen metabolism-related genes in *M. albus* ($n = 4$). Notes: (A) hydroxyproline content of muscle; (B) collagen content of muscle; (C) *ets1*—ETS proto-oncogene 1; *sp1*—Sp1 transcription factor; *p4ha1*—Prolyl 4-hydroxylase subunit alpha 1. Bars represent mean $\pm$ standard error (SE). Different superscript letters denote significant differences among groups ($p < 0.05$). CON: control diet (6.16% crude fat); HSIF0: high-fat diet (11.98% crude fat); HSIF50: HSIF0 supplemented with 50 mg/kg soy isoflavones; HSIF100: HSIF0 supplemented with 100 mg/kg soy isoflavones.

3.5. Antioxidant Capacity

As shown in Figure 6A–E, muscle SOD activity did not differ significantly among treatments ($p > 0.05$). Compared with the CON group, the HSIF0 group exhibited significantly higher MDA and H_2O_2 contents, but significantly lower T-AOC activity and GSH content ($p < 0.05$). Compared with the HSIF0 group, supplementation of the high-fat diet with 50 mg/kg soy isoflavones significantly reduced MDA and H_2O_2 contents, while significantly increasing T-AOC activity and GSH content ($p < 0.05$). Furthermore, the HSIF100 group showed significantly higher MDA and H_2O_2 contents and significantly lower GSH content than the HSIF50 group ($p < 0.05$).

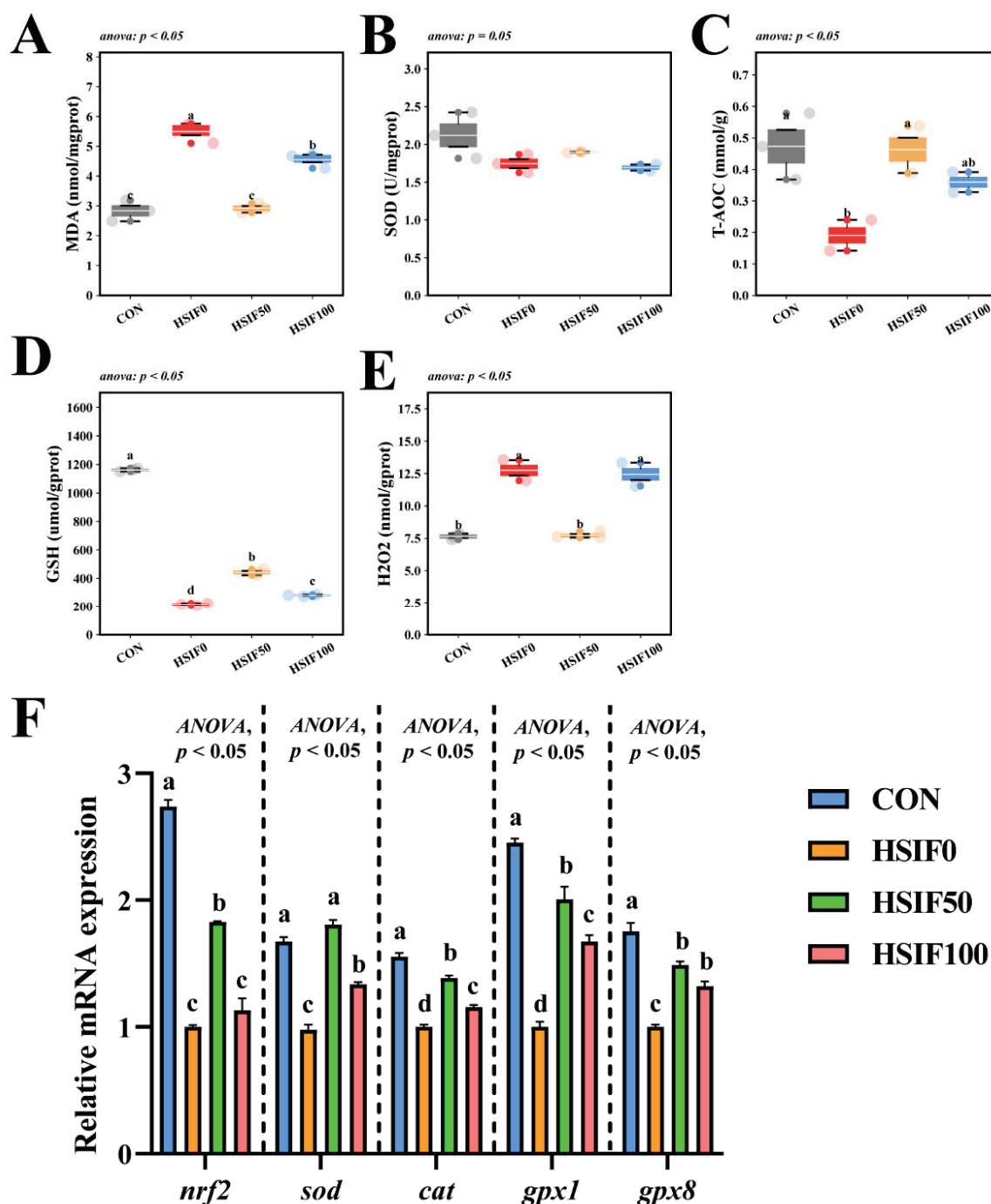


Figure 6. Effects of dietary treatments on antioxidant capacity in *M. albus* ($n = 4$). Notes: (A–E) represent reactive malondialdehyde, superoxide dismutase, total antioxidant capacity, glutathione, and hydrogen peroxide, respectively. (F) *nrf2*—Nuclear factor erythroid 2-related factor 2; *sod*—Superoxide dismutase; *cat*—Catalase; *gpx1*—Glutathione peroxidase 1; *gpx8*—Glutathione peroxidase 8. Bars represent mean $\pm$ standard error (SE). Different superscript letters denote significant differences among groups ($p < 0.05$). CON: control diet (6.16% crude fat); HSIF0: high-fat diet (11.98% crude fat); HSIF50: HSIF0 supplemented with 50 mg/kg soy isoflavones; HSIF100: HSIF0 supplemented with 100 mg/kg soy isoflavones.

As shown in Figure 6F, compared with the CON group, the HSIF0 group displayed significantly lower relative mRNA expression levels of *nrf2*, *sod*, *cat*, *gpx1*, and *gpx8* ($p < 0.05$). Compared with the HSIF0 group, dietary supplementation with 50 mg/kg soy isoflavones significantly upregulated the expression of these genes ($p < 0.05$). In addition,

as the level of soy isoflavone supplementation increased, the expression levels of *nrf2*, *sod*, *cat*, and *gpx1* were significantly downregulated ($p < 0.05$).

4. Discussion

4.1. Proximate Composition, Muscle Oil Red O-Stained Sections, and Fatty Acid Profile

In the present study, feeding rice field eels a high-fat diet (HSIF0) significantly elevated crude lipid contents in whole-body and muscle tissues compared to the control group (CON). Additionally, Oil red O-staining revealed extensive lipid droplet accumulation along myosepta and between muscle fibers in the HSIF0 group, indicative of abnormal lipid deposition under high-fat feeding conditions. This phenomenon aligns with previous findings in various fish species, such as mirror carp (*Cyprinus carpio*) [21] and yellow catfish [6], where high-fat diets promote excessive lipid accumulation in tissues, potentially impairing overall health. In contrast, supplementation with soy isoflavones effectively reduced these elevated lipid levels. This hypolipidemic effect of soy isoflavones has been well-documented in mammals and livestock, where they modulate lipid metabolism by suppressing lipogenesis, enhancing fatty acid oxidation, and improving blood lipid profiles [22]. In rodents, soy isoflavones have been shown to decrease body weight, hepatic lipid accumulation, and serum total and LDL cholesterol through mechanisms involving peroxisome proliferator-activated receptors (PPARs) and reduced intestinal cholesterol absorption [23]. Extending to aquatic species, soy isoflavones exhibit similar lipid-lowering properties in fish. In juvenile Chinese mitten crab, dietary soy isoflavones improved growth while inhibiting lipid synthesis genes and promoting decomposition, thereby reducing fat accumulation in high-fat diets [16]. Additionally, in common carp, genistein (a key soy isoflavone) modulated lipid metabolism, antioxidant activity, and immunity, alleviating high-fat-induced lipid buildup [24]. These findings suggest that soy isoflavones may mitigate high-fat-induced lipid dysregulation in rice field eels through comparable pathways, potentially involving antioxidant enhancement and gene regulation of lipid metabolism.

Regarding fatty acid composition, the high-fat diet altered muscle profiles by decreasing Σ SFA, Σ MUFA, and Σ n-3/ Σ n-6 ratios while increasing Σ n-6 across all high-fat groups compared to CON, reflecting a shift toward pro-inflammatory profiles often observed in fish under lipid overload. This is corroborated by studies in spotted seabass (*Lateolabrax maculatus*), where high-fat feeding disrupts n-3/n-6 balance, elevates n-6 PUFAs, and reduces beneficial ratios, impacting growth and tissue quality [25]. However, soy isoflavone supplementation (HSIF50 and HSIF100 groups) elevated Σ PUFA and Σ n-3 relative to CON, and in HSIF50 specifically, lowered Σ SFA and Σ n-6 while boosting Σ MUFA and EPA+DHA compared to HSIF0. This indicates that soy isoflavones can favorably modulate fatty acid profiles, potentially by enhancing desaturase activity or reducing n-6 dominance [26]. Similar effects have been reported in grass carp, where soy isoflavones combined with oils increased n-3 PUFA deposition, including EPA and DHA, in muscle tissues [27]. The observed increases in EPA+DHA likely contribute to improved muscle quality and antioxidant performance in soy isoflavone-supplemented groups; this will be proven in the indicators to be tested next. Taken together, these results indicate that soy isoflavones alleviate high-fat-induced lipid dysregulation and improve fatty-acid composition, laying the foundation for enhanced muscle quality.

4.2. Muscle Water-Holding Capacity and Textural Properties

The high-fat diet significantly impaired muscle water-holding capacity in rice field eels, as evidenced by increased cooking loss, liquid loss, and water loss compared to the control group. This deterioration is likely attributable to excessive lipid accumulation in muscle tissues, which disrupts myofibrillar protein structures, reduces the space for

water retention, and promotes drip loss during processing and storage [28]. Similarly, the HSIF0 group exhibited diminished textural properties, including lower hardness, cohesiveness, gumminess, chewiness, and resilience, reflecting a softer and less elastic muscle matrix, potentially due to fat infiltration altering collagen and protein integrity [29]. These findings are consistent with observations in other aquatic species under high-fat feeding regimes. For example, in Nile tilapia, high-fat diets compromised water-holding capacity and led to looser flesh texture by inducing lipid overload and oxidative damage [3]. Comparable effects have been reported in grass carp, where elevated dietary lipids decreased muscle pH and water-holding capacity, correlating with reduced firmness and increased tenderness [30].

Supplementation with soy isoflavones at 50 mg/kg effectively ameliorated these impairments, significantly lowering cooking loss, liquid loss, and water loss while enhancing hardness, cohesiveness, gumminess, chewiness, and resilience relative to the HSIF0 group. However, higher supplementation at 100 mg/kg resulted in elevated liquid and water losses and reduced cohesiveness, gumminess, and chewiness compared to HSIF50, possibly through over-modulation of metabolic pathways. The hypolipidemic and muscle quality-enhancing properties of soy isoflavones have been extensively documented in mammals and livestock, where they mitigate lipid peroxidation, stabilize protein matrices, and improve water retention and texture by activating antioxidant defenses and reducing fat deposition. In broilers, dietary soy isoflavones enhanced growth performance, meat quality, and water-holding capacity while decreasing oxidative stress and lipid accumulation [10]. Extending to aquatic species, soy isoflavones demonstrate analogous protective effects against high-fat-induced muscle degradation. In Chinese mitten crabs, supplementation improved lipid metabolism and flesh quality under high-fat conditions, enhancing texture and reducing losses associated with water-holding [31]. In rainbow trout, diets incorporating soy-derived isoflavones maintained muscle integrity and quality traits, countering potential anti-nutritional factors while supporting better textural properties [31]. Collectively, these findings demonstrate that moderate SIF supplementation improves water-holding capacity and textural attributes in rice field eel muscle.

4.3. Muscle Histomorphology and Expression of Myogenesis-Related Genes

To further elucidate how soy isoflavones improved water-holding capacity and texture under high-fat feeding, we assessed muscle microstructure and the expression of myogenesis-related regulators. The high-fat diet induced notable histopathological alterations in rice field eel muscle, as evidenced by HE-stained sections showing loosely arranged fibers, irregular morphology, enlarged inter-fiber spaces, and partial structural disruption. Concurrently, it upregulated the expression of *mstn* while downregulating *myod*, *tcap*, *mrf4*, and *mrf5*, indicating impaired myogenic processes under lipid overload. These changes align with observations in other fish species, like the yellow catfish [6] and grass carp [4], where high-fat diets compromise muscle histology, fiber quality, and gene regulation. These demonstrated that excessive dietary fats promote lipotoxicity, oxidative damage, and hinder muscle development [28].

Supplementation with 50 mg/kg soy isoflavones effectively ameliorated these issues, restoring compact fiber arrangement, uniform morphology, and reduced inter-fiber spaces, while downregulating *mstn* and upregulating *tcap*, *mrf4*, and *mrf5* to enhance myogenic activity. However, at the HSIF100 group, the inter-fiber gaps that widened were less pronounced, and gene expression partially reverted, suggesting a dose-dependent optimum beyond which benefits diminish. In aquatic species, soy isoflavones exhibit similar benefits in enhancing muscle quality and histology. In juvenile Chinese mitten crab, soy isoflavones improved growth, antioxidant capacity, and lipid metabolism, which could indirectly

support better muscle structure under high-fat conditions [16]. In rainbow trout, dietary soy isoflavones influenced tissue deposition and fillet quality, maintaining health without adverse effects at appropriate levels [32]. These findings imply that soy isoflavones in rice field eels likely act through antioxidant enhancement, lipid modulation, and estrogenic mimicry to optimize histological architecture and myogenic gene networks at moderate doses. The suboptimal effects at higher doses may stem from excessive isoflavone exposure, inducing stress or endocrine disruptions, as high doses have been linked to increased oxidation in tissues and potential toxicity in animals [7]. In fish, elevated soy isoflavones can alter endocrine functions and induce stress responses, such as changes in cortisol levels, which might counteract protective benefits [17]. Furthermore, these histopathological improvements and gene expression shifts in the HSIF50 group further corroborate the enhanced water-holding capacity and textural properties, while the partial histological and genetic reversals at higher doses aligned with declines in cohesiveness and chewiness. Overall, these results suggest that 50 mg/kg SIFs restore muscle fiber organization and activate myogenic pathways, thereby supporting muscle structure and function under high-fat feeding, whereas higher doses diminish these benefits.

4.4. Muscle Collagen Content and Expression of Collagen Metabolism-Related Genes

In grass carp, dietary manipulations affecting protein and lipid balance altered muscle hardness through changes in collagen metabolism, with reduced levels correlating to softer textures [33]. Similarly, in zebrafish (*Danio rerio*), high-fat feeding induced muscle mitochondrial dysfunction and impaired fiber characteristics, indirectly impacting collagen integrity via oxidative stress and metabolic shifts [34]. This aligns with findings in this study, the high-fat diet significantly reduced muscle hydroxyproline and collagen contents while downregulating the expression of collagen metabolism-related genes *ets1*, *sp1*, and *p4ha1* compared to the control group, indicating impaired collagen synthesis and structural integrity under lipid overload.

The HSIF50 group effectively reversed these deficits, elevating hydroxyproline and collagen levels while upregulating *ets1*, *sp1*, and *p4ha1* to enhance collagen metabolism. However, at the HSIF100 group, these parameters declined relative to HSIF50, suggesting an optimal dose threshold. The collagen-promoting properties of soy isoflavones are well-documented in mammals and livestock, where they support skeletal health by modulating gene expression and extracellular matrix formation. In orchidectomized rats, soy isoflavones elevated mRNA levels of type I collagen and osteocalcin, demonstrating anabolic influences on connective tissues [35]. In Chinese mitten crabs, soy isoflavones optimized gonadal and muscle parameters, including enhanced collagen content for better flavor and texture [36]. In grass carp, dietary soy isoflavones increased water-holding capacity and physical properties, largely attributed to boosted collagen deposition [27]. These results imply that soy isoflavones in rice field eels may facilitate collagen anabolism through transcriptional activation support at moderate doses. Furthermore, the elevated collagen content in the HSIF50 group further substantiates the improved textural properties and water-holding capacity, such as increased hardness and chewiness, alongside reduced cooking, liquid, and water losses, as collagen networks enhance muscle matrix stability and moisture retention. In summary, moderate SIF supplementation enhanced collagen deposition and metabolism, reinforcing muscle integrity and textural properties, whereas higher doses were less effective, pointing to a dose-dependent optimum.

4.5. Antioxidant Capacity

In aquaculture species, muscle antioxidant capacity is strongly diet-dependent, and the redox status in turn exerts significant regulatory effects on lipid metabolism and the

resulting fatty-acid composition [37]. The high-fat diet (HSIF0) significantly compromised muscle antioxidant capacity in rice field eels, as indicated by elevated MDA and H₂O₂ contents, reduced T-AOC activity and GSH levels, and downregulated expression of *nrf2*, *sod*, *cat*, *gpx1*, and *gpx8* genes, reflecting heightened oxidative stress and diminished defense mechanisms under lipid overload. This pattern accords with reports in multiple fish species: high-fat feeding drives lipid peroxidation and ROS accumulation, impairs antioxidant enzymes, and consequently aggravates inflammation and metabolic dysregulation [38–40].

At 50 mg/kg, soy isoflavones lowered MDA and H₂O₂, elevated T-AOC and GSH, and upregulated *nrf2*, *sod*, *cat*, *gpx1*, and *gpx8*, indicating strengthened antioxidant defenses. By contrast, 100 mg/kg partially reversed these gains, suggesting a dose-dependent optimum. Similar enhancements of antioxidant status and immune function have been reported in turbot and juvenile golden pompano under stress challenges—dietary soy isoflavones improved antioxidant status and immune responses [11,12]. Collectively, these findings suggest that moderate isoflavone dosing activates Nrf2-mediated cytoprotection and complements its hypolipidemic effects, whereas higher dosing may cross a hormetic threshold and exhibit pro-oxidant or tolerance effects. The improved redox capacity at HSIF50 also aligns with the higher muscle EPA+DHA, helping to augment antioxidant defenses and mitigate lipid peroxidation, contributing to overall muscle quality improvement. Overall, SIF at a dosage of 50 mg/kg improved muscle quality through lipid regulation, collagen synthesis, and activation of antioxidants, while 100 mg/kg resulted in decreased yields.

4.6. Economic and Regulatory Considerations

In addition to the biological benefits observed, the potential economic and regulatory aspects of soy isoflavone application in aquafeeds should also be considered. From an economic perspective, the cost of incorporating soy isoflavones must be weighed against the improvements in fish growth, flesh quality, and overall health. In practice, only about 50 g of soy isoflavones is required per metric ton of feed to achieve the optimal effect observed in this study, corresponding to an additional cost of approximately 20 RMB per ton, which is generally acceptable for commercial aquaculture operations. From a regulatory standpoint, soy isoflavones are naturally derived compounds that are generally recognized as safe and have already been approved for use in livestock and poultry feeds. However, their application in aquafeeds is less established, and determining appropriate supplementation gradients will be crucial for ensuring both safety and efficacy in aquatic species. Therefore, further studies on long-term safety, optimal inclusion levels, and regulatory compliance will be essential to facilitate the practical adoption of soy isoflavone supplementation in aquaculture.

5. Conclusions

This study demonstrated that dietary soy isoflavone supplementation can effectively mitigate the adverse effects of a high-fat diet on muscle quality, collagen deposition, and antioxidant capacity in *M. albus*. Specifically, supplementation at 50 mg/kg reduced muscle lipid accumulation, improved water-holding capacity and textural properties, promoted collagen synthesis, and enhanced antioxidant defense by modulating the expression of myogenesis-, collagen metabolism-, and antioxidant-related genes. However, excessive supplementation at 100 mg/kg partially attenuated these beneficial effects, indicating a dose-dependent response. Collectively, our findings suggest that 50 mg/kg is the optimal inclusion level of soy isoflavones in high-fat diets for *M. albus*, providing practical guidance for aquafeed formulation strategies aimed at improving flesh quality and health status in aquaculture. Beyond aquaculture, these findings may offer insights into vertebrate

muscle biology and nutrition; however, extrapolation to humans requires dedicated studies. Limitations of this study include its focus on a single species, short-term feeding duration, and the lack of protein-level or functional validation of signaling pathways. Future research should, therefore, involve cross-species validation, long-term feeding trials, and confirmatory studies of the molecular mechanisms underlying the observed effects.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antiox14101195/s1>, Figure S1: Schematic representation of the experimental design.

Author Contributions: K.X. was in charge of Conceptualization, Methodology, Writing—Original draft, Writing—Review and editing. Q.L. was responsible for Investigation, Formal analysis, Validation, Supervision, and Software. S.Z. was involved in Investigation, Validation, and Writing—Review and Editing. H.W. was Investigation, Validation. T.Z. was involved in Investigation, Validation. Y.H. was in charge of Conceptualization, Writing—Review and editing. J.Z. was responsible for Project administration, Funding acquisition, Writing—Review and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All experimental procedures were conducted in strict accordance with the guidelines for the care and use of laboratory animals established by the Ministry of Science and Technology of the People’s Republic of China (Guidelines for the Welfare of Laboratory Animals, 2006) and complied with international standards, including the ARRIVE guidelines for reporting animal research. This study was approved by the Committee of Laboratory Animal Management and Animal Welfare of Hunan Agricultural University (Changsha, China), and all experimental procedures conformed to the guidelines of the Ethical Committee of Hunan Agricultural University (approval number: 2025121, approved on 8 April 2025).

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Article

Optimizing Low Fishmeal Diets with Vitamin C Supplementation: A Comprehensive Study on Growth, Immunity, and Heat Stress Resistance in Largemouth Bass (*Micropterus salmoides*) Juveniles

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Abstract: Six dietary groups were supplemented with graded vitamin C (VC) levels: VC1 (control, 0.39 g/kg), VC2 (0.51 g/kg), VC3 (0.66 g/kg), VC4 (0.81 g/kg), VC5 (0.97 g/kg), and VC6 (1.11 g/kg). Largemouth bass (*Micropterus salmoides*) with an initial weight of 2.21 ± 0.00 g were fed these diets for 8 weeks to evaluate the effects of different VC levels on growth performance, immune response, and heat stress resistance. Heat stress was induced at a constant temperature of 33.00 ± 0.16 °C for one week. The VC3 and VC4 groups showed significantly improved growth performance (FBW, WGR, SGR) compared to VC1 ($p < 0.05$). VC4 exhibited lower ALT and AST levels before and after heat stress. Antioxidant capacity (T-AOC, GSH-Px, CAT) was significantly enhanced in VC3–VC5, with VC5 showing the highest after stress activity (except CAT). Expression of pro-inflammatory genes (*nf- κ b*, *il-8*) was downregulated in VC4 and VC5, while anti-inflammatory *il-10* was upregulated in VC4 after stress. Apoptosis-related genes (*bcl-2*, *caspase*, *bax*) and TUNEL assays indicated the strongest anti-apoptotic effects in VC3 and VC4 under heat stress ($p < 0.05$). These findings suggest that VC supplementation in low-fishmeal diets enhances growth, immune response, apoptosis resistance, and acute heat stress tolerance in fish.

Keywords: low fishmeal; vitamin C; farmed largemouth bass; immunity response; heat stress

1. Introduction

Fishmeal, as the primary protein source in aquaculture diets, is rich in protein, essential amino acids, vitamins, minerals, and other necessary nutrients [1]. Nevertheless, alongside the swift advancement of socioeconomic development, the aquaculture industry has grown significantly, leading to a mismatch between the demand for fishmeal and its availability, resulting in rising fishmeal prices and limiting the growth of the industry. To ensure the sustainable development of aquaculture, alleviate resource pressures, reduce feed costs, and promote environmentally friendly practices, low-fishmeal diets have been increasingly adopted. Despite this, research has shown that low-fishmeal diets can negatively affect the physiological metabolism of farmed species [2–5]. The supplementation of additives

such as enzymes [6], probiotics and prebiotics [7], natural plant extracts [8], and Vitamin C (VC) [9] can enhance the performance of aquatic animals fed low-fishmeal diets. Therefore, investigating the effects of specific additives in low-fishmeal diets is essential for optimizing aquaculture practices.

VC, or L-ascorbic acid, is essential for the normal growth and physiological functions of aquatic animals [10]. VC plays a critical role in promoting fish growth [11], supporting reproductive health [12], enhancing iron absorption [13], strengthening immunity [13], improving tissue hematology [14], and facilitating collagen synthesis [15]. Moreover, VC acts as a potent antioxidant by donating electrons to biomolecules, protecting cells from oxidative damage during metabolism [16], which is largely attributed to its influence on the Nrf2 signaling pathway [17]. Supplementing 148 mg/kg VC in a low-fishmeal diet for hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) enhances the Nrf2 pathway, thereby boosting antioxidant capacity [9]. Additionally, VC supplementation has been effective in reducing lipid peroxide accumulation and improving the growth performance of tuna (*Terapon jarbua*) [18,19]. However, limited research has explored the effects of VC supplementation in low-fishmeal diets within aquaculture, highlighting a significant gap that warrants further investigation.

Recent reports indicate that the total production of *Micropterus salmoides* (*M. salmoides*) in China has reached 880,000 tonnes, accounting for over 99% of global production [20,21]. This species is highly valued for its meat quality, rapid growth, and adaptability [22,23]. However, high water temperatures during summer result in elevated mortality rates in *M. salmoides* [24]. VC supplementation in the diet can effectively enhance growth rate [25], immunity [26], and antioxidant capacity in *M. salmoides* [27]. As previously noted, low-fishmeal diets may lead to nutritional deficiencies, compromised immune function, and reduced stress tolerance in fish. Given VC's ability to improve growth and immune responses, this study aims to evaluate the impact of VC supplementation in low-fishmeal diets on the growth, immunity, and stress resilience of *M. salmoides*. The study will also determine the optimal VC level in these diets. Additionally, heat stress conditions were incorporated to assess how VC supplementation affects the alleviation of acute heat stress in *M. salmoides* under low-fishmeal diet conditions.

2. Materials and Methods

2.1. Diet Preparation

Table 1 presents the primary components and dry matter content of the experimental diets. These diets primarily consist of fishmeal and chicken meal as the primary protein constituents, supplemented by fish oil and soybean oil as the principal fat inputs, among others. By reviewing relevant studies on vitamin C in aquaculture fish, based on a review of studies on VC in aquaculture fish species, optimal supplementation levels vary significantly among different animals, as reported in previous research [28–33]. Six diets with varying VC concentrations were formulated, named VC1, VC2, VC3, VC4, VC5, and VC6, corresponding to VC levels of 0.39 g/kg, 0.51 g/kg, 0.66 g/kg, 0.81 g/kg, 0.97 g/kg, and 1.11 g/kg, respectively. All ingredients were milled, passed through an 80-mesh sieve, accurately weighed, and mixed with water according to the designed formulations [34]. The mixture was then processed into puffed pellets (3 mm in diameter) using a bulking granulator (TSE65-type, Beijing, China) as described in previous studies. After pellet formation, the diets were naturally air-dried at ambient temperature. They were then placed in resealable bags, marked, and preserved at −20 °C for later use.

Table 1. Main and crude ingredients of experimental feeds.

Ingredients	VC1	VC2	VC3	VC4	VC5	VC6
Fishmeal	25	25	25	25	25	25
Chicken meal ¹	12.00	12.00	12.00	12.00	12.00	12.00
Soy concentrated protein ¹	13.41	13.41	13.41	13.41	13.41	13.41
Soybean meal ¹	15.70	15.70	15.70	15.70	15.70	15.70
Wheat gluten ¹	5.00	5.00	5.00	5.00	5.00	5.00
Blood meal ¹	2.00	2.00	2.00	2.00	2.00	2.00
Wheat flour ¹	8.00	8.00	8.00	8.00	8.00	8.00
Tapioca starch	5.00	5.00	5.00	5.00	5.00	5.00
Fish oil	2.80	2.80	2.80	2.80	2.80	2.80
Soybean oil	5.00	5.00	5.00	5.00	5.00	5.00
Calcium dihydrogen phosphate	3.00	3.00	3.00	3.00	3.00	3.00
Premix ²	2.00	2.00	2.00	2.00	2.00	2.00
Choline chloride	0.50	0.50	0.50	0.50	0.50	0.50
VC ³ (%)	0.00	0.05	0.10	0.15	0.20	0.25
Microcrystalline cellulose	0.25	0.20	0.15	0.10	0.05	0.00
L-Lysine ⁴ (98.5%)	0.23	0.23	0.23	0.23	0.23	0.23
L-Methionine ⁴	0.11	0.11	0.11	0.11	0.11	0.11
Total	100.00	100.00	100.00	100.00	100.00	100.00
Analyzed proximate composition						
Crude protein (%)	48.85	48.26	48.87	49.11	48.65	48.50
Crude lipid (%)	12.73	12.32	12.02	12.24	12.02	12.47
Actual VC content (g/kg)	0.39	0.51	0.66	0.81	0.97	1.11

¹ Fishmeal, crude protein 65.6%, crude lipid 9.5%; Chicken meal, crude protein 62.00%, crude lipid 9.00%; Soy concentrated protein, crude protein 63.00%, crude lipid 4.10%; Soybean meal, crude protein 46.00%, crude lipid 4.25%; Wheat gluten, crude protein 80.00%, crude lipid 2.00%; Blood meal, crude protein 91.45%; Wheat flour, crude protein 13.1%, crude lipid 4.0%. They are provided from Biomar Tongwei (Wuxi) Biotech Co., Ltd.

² Premixes are provided by China Wuxi Hanover Animal Health Products Co. ³ VC purchased from Biomar Tongwei (Wuxi) Biotech Co. ⁴ L-Lysine and L-Methionine were obtained from Feer Co., Ltd., Shanghai, China.

2.2. Experimental Fish

Juvenile *M. salmoides* were cultured in the recirculating aquaculture system (RAS) at the Freshwater Fisheries Research Centre of the Chinese Academy of Fisheries Sciences (CAFS), located in Yixing, China. Prior to the experiment, all juvenile fish were acclimated for two weeks in RAS breeding buckets (270 L) and fed a commercial compound diet (48% protein, 12% lipid) to adapt to the culture environment. A total of 360 healthy, uniformly sized juvenile fish (initial weight 2.21 ± 0.00 g) were randomly selected and distributed into RAS breeding buckets (20 fish per bucket), with 18 buckets set up to represent the six dietary treatments, each with triplicates. The fish received three daily feedings at 07:30, 12:30, and 17:30, with each meal provided until satiation. The culture period lasted for 8 weeks. Water quality parameters (YSI ProDSS Multiparameter Water Quality Meter, OH, USA) were recorded daily, with water temperature maintained between 26 and 30 °C, dissolved oxygen concentration above 6 mg/L, and pH between 7.0 and 7.5.

2.3. Sample Collection

When the farming trial concluded, the fish were maintained without feeding for 24 h, after which the fish in each bucket were weighed and counted for growth performance analysis. Blood was collected from the tail vein of three randomly selected fish from each bucket, placed in centrifuge tubes, and immediately centrifuged (10 min, 3000 rpm, 4 °C). The supernatant was transferred into corresponding tubes and stored at −80 °C for subsequent plasma biochemical and antioxidant capacity analyses. Intestinal samples were taken for gene expression analysis related to antioxidants, immunity, and apoptosis.

Additionally, two more fish from each bucket were randomly selected for whole-body composition analysis.

2.4. Heat Stress Experiment

At the end of the previous culture period, the remaining fish were placed back into the same RAS breeding buckets, maintaining the same groupings as before. The water temperature was incrementally raised from 28 °C to 33 °C at a rate of 0.5 °C per hour using a heating rod. The temperature was monitored every three hours with a thermometer until it stabilized at 33 ± 0.16 °C. During this period, the culture environment was maintained under the same conditions as the previous phase, with the dissolved oxygen concentration kept above 6 mg/L and the pH maintained between 7.0 and 7.5. The fish were cultured for an additional week under these conditions. Sample collection after the heat stress period was conducted following the methods outlined in Section 2.3. In addition to the previously collected samples, gills from each fish were harvested for TUNEL immunofluorescence analysis.

2.5. Measurement of Growth Indicators

The main growth indicators were calculated according to the specific formulae in Table 2.

Table 2. Formulae for calculating relevant growth properties.

Items	Full Name	Computational Formula
IBW (g)	initial body weight (g)	Total weight (g)/number of fish per bucket at the start of the culture
FBW (g)	final body weight (g)	Total weight (g)/number of fish per bucket at the end of the culture
FCR	feed conversion ratio	dry feed fed (g)/(final body weight (g) – initial body weight (g))
WGR (%)	weight gain rate (%)	$100 \times (\text{final body weight (g)} - \text{initial body weight (g)}) / \text{initial body weight (g)}$
SGR (%/day)	specific growth rate (%/day)	$100 \times [(\text{Ln (final body weight (g))} - \text{Ln(initial body weight (g))}) / \text{days}]$
SR (%)	survival rate (%)	$100 \times (\text{survival fish number} / \text{total fish number})$

2.6. Body Composition and Biochemical Analysis

The whole fish body composition and the crude composition of the feed were determined using methods referenced from previous studies within our group. Firstly whole fish moisture (%) was determined by drying in an oven at 105 °C. Whole fish and feed crude protein (%) were determined using a Kjeldahl nitrogen meter (Haineng K1100, Jinan Haineng Instrument Co., Ltd., Jinan, China). Whole fish and feed crude lipid (%) were determined on an automatic analyser (Haineng SOX606, Jinan Haineng Instrument Co., Ltd., China) using the Soxhlet extraction method. The ash (%) content of whole fish and feed was determined by burning in a muffle furnace (XL-2A, Hangzhou Zhuochi Instrument Co., Ltd., Hangzhou, China) at 560 °C. Plasma biochemical parameters ALT (alanine aminotransferase) and AST (aspartate aminotransferase) were measured using the methods recommended by the International Federation of Clinical Chemistry, employing kits purchased from Mindray Medical International Ltd. (Shenzhen, China) (ALT: 105-000442-00; AST: 15-00443-000) on the Mindray BS 400 automated biochemical analyser (Mindray Medical International Ltd., Shenzhen, China), as described in the experimental protocols of previous research [35]. Plasma antioxidant enzyme activity factors MDA (malondialdehyde), CAT (catalase), SOD (superoxide dismutase), GSH-Px (glutathione

peroxidase), GSH (glutathione) and T-AOC (total antioxidant capacity) were measured using assay kits purchased from Jiancheng Bioengineering Institute (Nanjing, China). MDA was determined using the TBA method for amino acids (A003-1-2), CAT was measured by the molybdate method (A007-1-1), SOD was assessed via the WST-1 assay (A001-3-2), GSH was quantified using the microplate method (A006-1-1), GSH-Px was determined by the colorimetric method (A005-1-2), and T-AOC was measured by the ABTS method (A015-2-1). The primary experimental methods, kits, and equipment followed those detailed in the earlier research [35], as outlined in Table 3.

Table 3. Reference table of experimental methods, kits and experimental equipment.

Items	Methods	Assay Kits/Testing Equipment
Composition of diets/whole body		
Moisture	Drying method (ID 920.36)	Electric blast drying oven (Shanghai Yiheng Scientific Instrument Co., Ltd., Shanghai, China)
Protein	Kjeldahl (ID984.13)	Auto kieldahl apparatus: Hanon K1100 (Jinan Hanon Instruments Co., Ltd., Jinan, China).
Lipid	Soxhlet (ID 991.36)	Auto fat analyzer: Hanon SOX606 (Jinan Hanon Instruments Co., Ltd., Jinan, China)
Ash	Combustion (ID 923.03)	Muffle: XL-2A (Hangzhou Zhuochi Instrument Co., Ltd., Hangzhou, China).
Plasma parameters		
ALT AST	International Federation of Clinical Chemistry recommended	Assay kits (ALT: 105-000442-00.AST: 15-00443-000 purchased from Mindray Medical International Ltd. (Shenzhen, China); Mindray BS 400 automatic biochemical analyzer (Mindray Medical International Ltd., Shenzhen, China).
Plasma parameters related antioxidant capacity		
MDA	TBA method Ammonium	Assay kits (MDA: A003-1-2. CAT: A007-1-1. SOD: A001-3-2. GSH:A006-1-1. GPx: A005-1-2.T-AOC:A015-2-1) purchased from Jian Cheng Bioengineering Institute (Nanjing, China); Spectrophotometer (Thermo Fisher Multiskan GO, Shanghai, China).
CAT	Molybdenum acid method	
SOD	WST-1 method	
GSH	Microplate method	
GSH-Px	Colorimetric method	
T-AOC	ABTS method	

Note: ALT, alanine aminotransferase; AST, aspartate aminotransferase. MDA, malondialdehyde; CAT, catalase; SOD, superoxide dismutase; GSH, glutathione; GSH-Px, glutathione peroxidase; T-AOC, total antioxidant capacity.

2.7. Methods of Analysing Apoptosis in Gills After Heat Stress

The analysis was conducted using the Aipathwell image analysis software (Alpathwell v2) from Servicebio. The procedure involved the following steps: (1) Positioning: The area to be measured was selected either automatically or manually. (2) Color Selection: The target fluorescence signal in HSI was manually selected to ensure accurate identification of the positive signal, which was then saved as a standard. (3) Calculation: The software automatically identifies DAPI-stained cell nuclei, extends the cytoplasmic boundaries, and computes the number of positive cells, area, cumulative optical density (IOD), and tissue area after reverse decolorization. (4) Analysis: The software processes high-power data of the measured area and generates a report.

2.8. Real-Time PCR Analysis

Following the method outlined by D. Huang et al. [35], RNA was extracted from perch intestines using the TRIzol method (Vazyme Biotech Co., Ltd., Nanjing, China). RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer. Real-time quantitative PCR analysis of genes related to immunity, antioxidant capacity, and apoptosis was performed on a CFX96 real-time PCR detection system (Bio-Rad) following the kit protocol (Q221-01, Vazyme, Nanjing, China). Beta-actin (*β-actin*) was selected as the internal reference gene due to its stability in prior studies within the group. Primer sequences are provided in Table 4.

Table 4. All primers and internal references used in the experiment.

Genes	Forward (5'-3')	Reverse (5'-3')	Primer Source
<i>nrf2</i>	CACAGCAGCAGCAGGAAAAG	AAGATGCTGCCGTCTGTTGA	XM_038720536.1
<i>keap1</i>	CGTACGTCCAGGCCTTACTC	TGACGGAAATAACCCCCTGC	XP_018520553.1
<i>cat</i>	CTATGGCTCTCACACCTTC	TCCTCTACTGGCAGATTCT	MK614708.1
<i>sod</i>	GGTGTITAAAGCCGTTTGTGT	CCTCTGATTTCTCCTGTCACCT	XM_038708943.1
<i>gpx</i>	ATGGCTCTCATGACTGATCCAAA	GACCAACCAGGAAGTTCTCAAA	XM_038697220.1
<i>nf-κb</i>	AGAAGACGACTCGGGGATGA	GCTTCTGCAGGTTCTGGTCT	XM_038699793.1
<i>tgf-β</i>	CACCAAGGAGATGCTGATT	CGTATGTTAGAGATGCTGAAG	XM_038693206.1
<i>il-10</i>	CGGCACAGAAATCCCAGAGC	CAGCAGGCTCACAAAATAAACATCT	XM_038696252.1
<i>tnf-α</i>	CTTCGTCTACAGCCAGGCATCG	TTTGGCACACCGACCTCACC	XM_038710731.1
<i>il-8</i>	GAGGGTACATGTCTGGGGGA	CCTTGAAGGTTTGTCTTCATCGT	XM_038713529.1
<i>bax</i>	ACTTTGGATTACCTGCGGGA	TGCCAGAAATCAGGAGCAGA	Ref. [36]
<i>bcl-2</i>	TGTGGGGCTACTTTTGGCA	TTCGACTGCCACCCCAATAC	Ref. [37]
<i>caspase3</i>	GAGGCGATGGACAAGAGTCA	CACAGACGAATGAAGCGTGG	XM_038713063.1
<i>caspase8</i>	GAGACAGACAGCAGACAACCA	TTCCATTTTCAGCAAACACATC	Ref. [38]
<i>caspase9</i>	CTGGAATGCCTTCAGGAGACGGG	GGGAGGGGCAAGACAACAGGGTG	Ref. [36]
<i>β-action</i>	GGTGTGATGGTTGGTATGG	CTCGTTGTAGAAGGTGTGAT	MH018565.1

Note: Mean values and standard error ($\pm$ SE; $n = 3 \times 3$) are presented for each parameter. *nrf2*, nuclear factor erythroid 2-related factor 2; *keap1*, recombinant kelch like ech associated protein 1; *cat*, catalase; *sod*, superoxide dismutase; *gpx*, glutathione peroxidase; *nf-κb*, nuclear factor kappa-β; *tgf-β*, transforming growth factor-β; *il-10*, interleukin-10; *tnf-α*, tumor necrosis factor-α; *il-8*, interleukin-8; *bax*, bcl-2-associated x protein; *bcl-2*, b-cell lymphoma-2; *caspase3*, *caspase8* and *caspase9* belong to cysteine aspartic acid specific proteases; *β-action*, beta-actin.

2.9. Statistical Analyses

Growth performance data and whole fish body composition are presented as Mean $\pm$ Standard Error (Mean $\pm$ S.E.) and analyzed using one-way analysis of variance (ANOVA) with Tukey's method in SPSS ver. 26 to determine statistical significance ($p < 0.05$). Independent *t*-tests were used for comparisons before and after heat stress, with $p < 0.05$ indicating statistical significance and $p < 0.01$ denoting high significance. Graphs and tables were created using GraphPad Prism software (GraphPad Prism version 9.1.0 for Windows (GraphPad Software, San Diego, CA, USA)).

3. Results

3.1. Growth Data

As shown in Table 5, the FBW, WGR, and SGR were significantly higher in the VC3 and VC4 groups compared to the VC1 group ($p < 0.05$). The FCR decreased markedly in the VC2, VC5, and VC6 groups compared to the VC1 group ($p < 0.05$). Quadratic regression analysis of SGR (Figure 1) and WGR (Figure 2) with varying VC levels indicated that the optimal VC requirement for the diet was 0.77 g/kg. According to Table 6, different VC concentrations in the diet did not significantly affect the whole-body composition of *M. salmoides* ($p > 0.05$).

Table 5. Impact of VC supplementation in low fishmeal feeds on *M. salmoides* growth performance.

Diets (VC mg/kg)	IBW (g)	FBW (g)	FCR	WGR(%)	SGR (%/Day)	SR (%)
VC1	2.22 ± 0.00	41.16 ± 0.20 a	0.72 ± 0.01 ^c	1758.10 ± 8.87 a	4.87 ± 0.01 a	96.70 ± 1.67
VC2	2.22 ± 0.01	44.35 ± 0.55 b	0.69 ± 0.01 ^a	1897.50 ± 22.25 b	4.99 ± 0.02 b	100.00 ± 0.00
VC3	2.22 ± 0.01	47.22 ± 1.14 c	0.71 ± 0.01 ^c	2025.20 ± 46.73 c	5.09 ± 0.04 c	96.70 ± 1.67
VC4	2.22 ± 0.00	47.24 ± 0.19 c	0.72 ± 0.00 ^c	2032.80 ± 5.97 c	5.10 ± 0.00 c	98.30 ± 1.67
VC5	2.21 ± 0.01	43.97 ± 0.48 b	0.68 ± 0.00 ^{ab}	1889.40 ± 21.64 b	4.98 ± 0.02 b	100.00 ± 0.00
VC6	2.21 ± 0.00	42.90 ± 0.36 ab	0.68 ± 0.00 ^{ab}	1844.10 ± 15.41 b	4.95 ± 0.01 b	98.30 ± 1.67

Note: Mean values and standard error (±SE) are presented for each parameter. The results of this data analysis are presented as mean ± standard error (Mean ± S.E.). Values with distinct alphabetical superscripts differ significantly ($p < 0.05$).

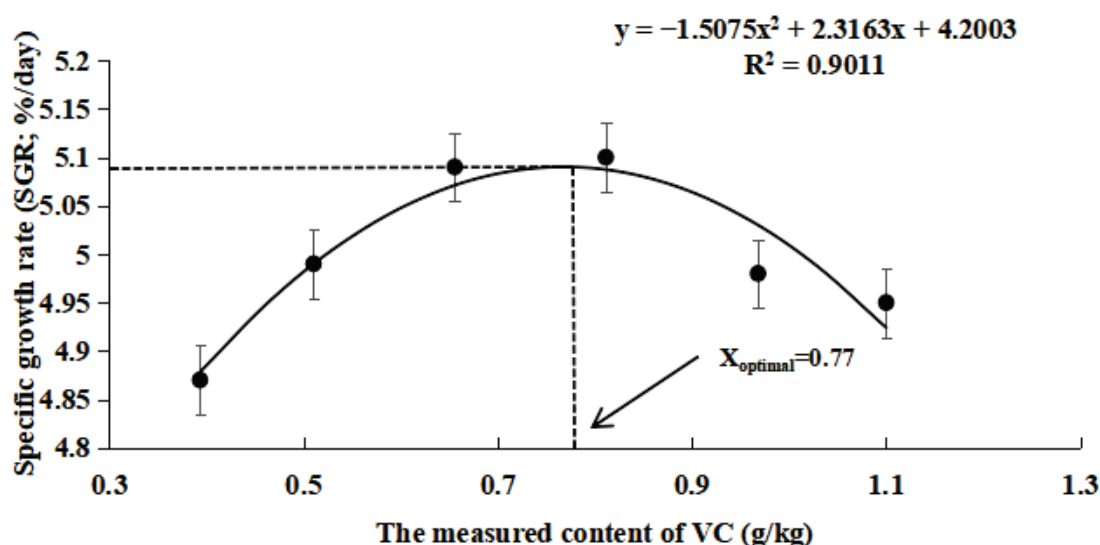


Figure 1. Quadratic regression relationship between specific growth rate (SGR, %/day) and VC supplementation levels in low-fishmeal diets.

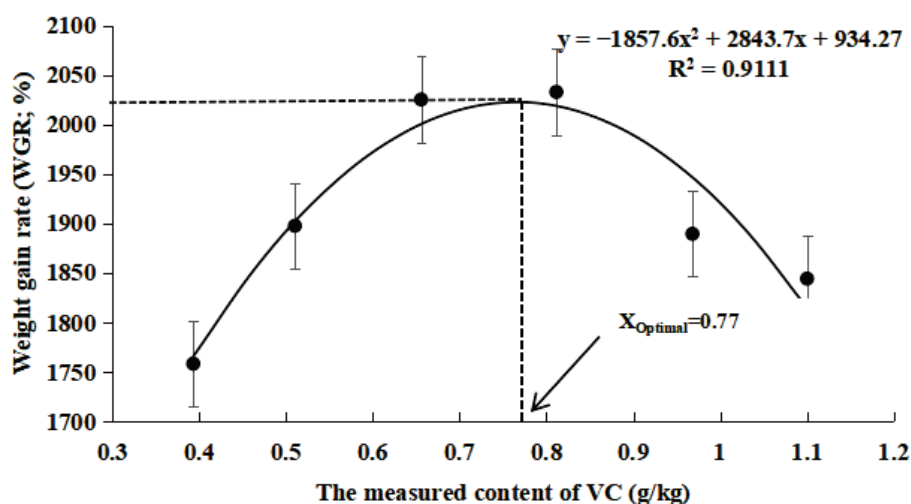


Figure 2. Quadratic regression relationship between weight gain rate (WGR, %) and VC supplementation levels in low-fishmeal diets.

Table 6. Impact of different VC addition levels on whole fish body composition of *M. salmoides* (dry weight; %).

Diets (VC mg/kg)	Moisture (%)	Lipid (%)	Ash (%)	Protein (%)
VC1	69.58 ± 0.19	9.77 ± 0.23	3.04 ± 0.18	16.79 ± 0.19
VC2	69.48 ± 0.49	9.47 ± 0.68	3.12 ± 0.18	16.84 ± 0.19
VC3	69.35 ± 0.46	9.56 ± 0.37	3.03 ± 0.12	16.56 ± 0.09
VC4	69.17 ± 0.62	9.82 ± 0.75	3.13 ± 0.15	17.39 ± 0.35
VC5	69.35 ± 0.39	9.45 ± 0.41	3.26 ± 0.03	17.29 ± 0.48
VC6	69.49 ± 0.20	10.59 ± 0.37	3.11 ± 0.06	16.46 ± 0.25

Note: Mean values and standard error ($\pm$ SE; $n = 3 \times 3$) are presented for each parameter.

3.2. Plasma Biochemical Analysis Before and After Heat Stress

Figure 3 illustrates the effect of dietary VC levels on plasma biochemical indices of *M. salmoides* before and after heat stress. No significant impact on ALT and AST concentrations was observed in all groups before heat stress ($p > 0.05$). After heat stress, both ALT and AST levels in the VC4 group were markedly lower than those in the VC1 group ($p < 0.05$). *t*-test analysis revealed significant increases in ALT and AST levels after heat stress across all groups ($p < 0.01$).

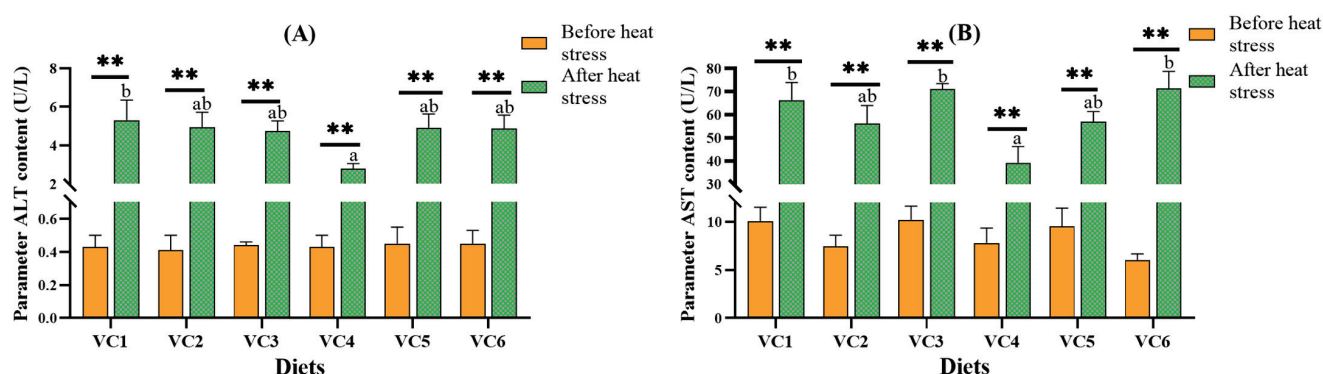


Figure 3. Effect of different levels of VC added to low fishmeal diets on plasma biochemical parameters of *M. salmoides* before and after heat stress. Data after heat stress are marked with lowercase letters 'a–b'. After *t*-test, '**' for before and after heat stress data shows a extremely significant contrast ($p < 0.01$). Columns of identical color marked with different letters show significant variations between data points ($p < 0.05$). (A), ALT; (B), AST.

3.3. Analysis of Plasma Antioxidant Capacity

Figure 4 presents the effects of different VC levels in the diet on the plasma antioxidant capacity of *M. salmoides* before and after heat stress. Before stress, the activity of T-AOC (Figure 4A) was significantly higher in the VC3 and VC4 groups compared to the VC1 group ($p < 0.05$). SOD activity (Figure 4B) was significantly increased in the VC6 group ($p < 0.05$). MDA content (Figure 4C) was lowest in the VC3 group ($p < 0.05$). CAT activity (Figure 4E) was markedly higher in the VC3 and VC6 groups than in the VC1 group ($p < 0.05$). GSH-Px activity (Figure 4E) was significantly higher in the VC4 group than in the VC1 group ($p < 0.05$). After heat stress, the activities of T-AOC (Figure 4A), SOD (Figure 4B), and GSH-Px (Figure 4E) were considerably higher in the VC5 group compared to the VC1 group ($p < 0.05$). MDA content (Figure 4C) remained lowest in the VC3 group ($p < 0.05$), while GSH levels (Figure 4D) were significantly higher in the VC4 group ($p < 0.05$). The highest CAT activity (Figure 4E) was observed in the VC3 and VC6 groups ($p < 0.05$). *t*-test analysis revealed that the activities of T-AOC and SOD were significantly higher after heat

stress compared to before heat stress ($p < 0.05$), with GSH levels significantly elevated in the VC2-VC5 groups.

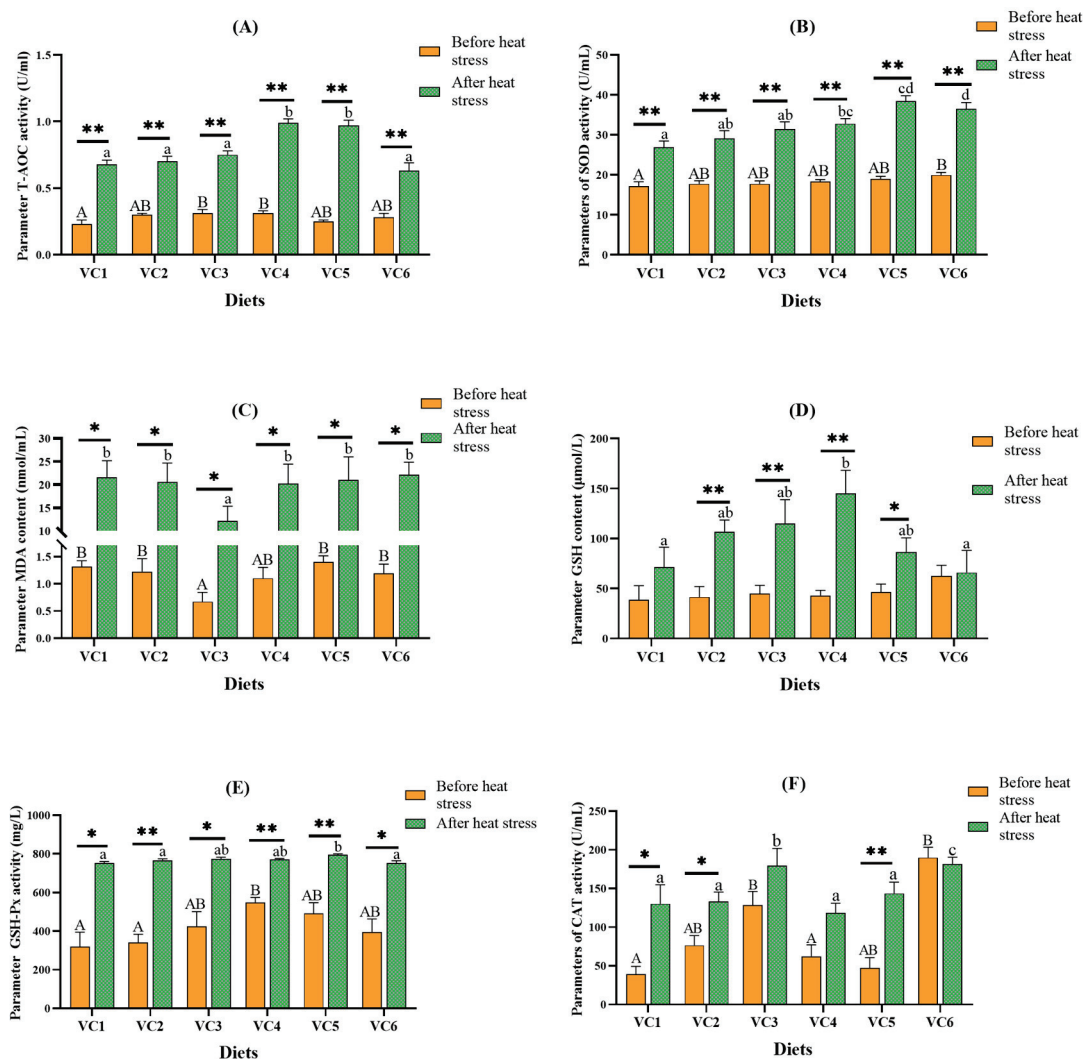


Figure 4. Effect of different levels of VC added to low fishmeal diets on plasma antioxidant capacity of *M. salmoides* before and after heat stress. Data before heat stress are marked with uppercase letters 'A–B' and data after heat stress are marked with lowercase letters 'a–d'. After *t*-test, '*' for before and after heat stress data shows a statistically significant contrast ($p < 0.05$) and '**' shows extremely significant ($p < 0.01$). Columns of identical color marked with different letters show significant variations between data points ($p < 0.05$). (A), T-AOC; (B), SOD; (C), MDA; (D), GSH; (E), GSH-Px; (F), CAT.

3.4. Analysis of Antioxidant-Related Genes in the Intestine

Figure 5 illustrates the impact of varying VC levels in the diet on the antioxidant *nrf2* signaling pathway in the intestines of *M. salmoides* before and after heat stress. Before heat stress, different VC levels did not substantially affect the expression of *nrf2* (Figure 5A), *keap1* (Figure 5B), *sod* (Figure 5C), or *gpx* (Figure 5D) ($p > 0.05$). However, the expression of *cat* (Figure 5E) in the VC3 group was substantially higher than in the VC1 group ($p < 0.05$). After heat stress, the expression levels of *nrf2* (Figure 5A), *sod* (Figure 5C), *gpx* (Figure 5D), and *cat* (Figure 5E) showed no significant differences between the groups ($p > 0.05$). The expression of *keap1* (Figure 5B) was notably higher in the VC3 group compared to the VC1 group ($p < 0.05$). *t*-test analysis revealed that after heat stress, *gpx* expression (Figure 5D)

was significantly higher between the groups than before heat stress ($p < 0.05$), with *keap1* (Figure 5B) showing similar trends in the VC1-VC4 groups.

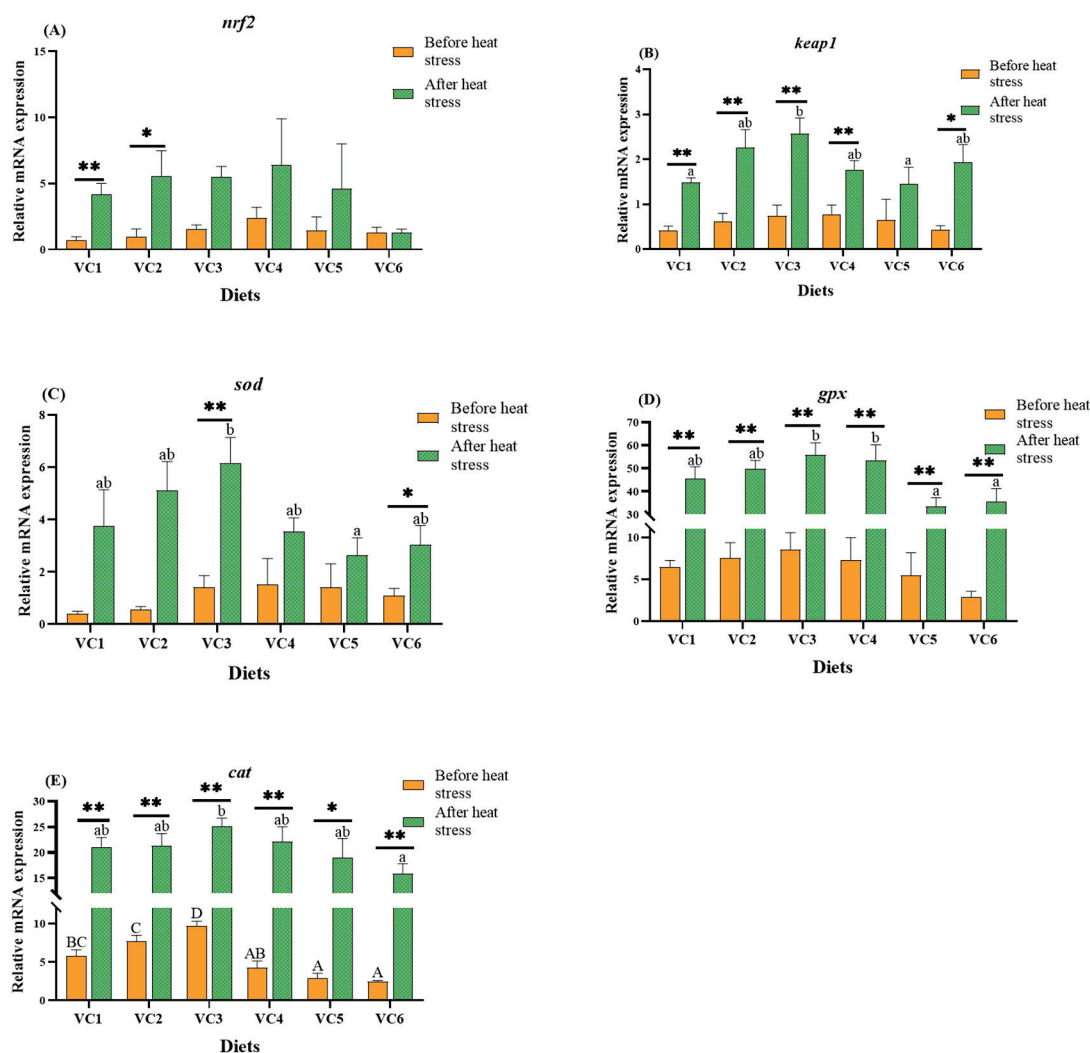


Figure 5. Effects of different levels of VC added to low fishmeal diets on antioxidant-related genes in the intestine of *M. salmoides* before and after heat stress. Data before heat stress are marked with uppercase letters 'A–D' and data after heat stress are marked with lowercase letters 'a–b'. After *t*-test, '*' for before and after heat stress data shows a statistically significant contrast ($p < 0.05$) and '**' shows extremely significant ($p < 0.01$). Columns of identical color marked with different letters show significant variations between data points ($p < 0.05$). (A), *nrf2*; (B), *keap1*; (C), *sod*; (D), *gpx*; (E), *cat*.

3.5. Analysis of Immune-Related Genes in the Intestine

Figure 6 demonstrates the effects of different VC levels on antioxidant-related genes in the intestines of *M. salmoides*. Before heat stress, expressions of *nf-κb* (Figure 6A), *il-8* (Figure 6D), and *tnf-α* (Figure 6E) showed a gradual decrease, being substantially lower in the VC4 and VC5 groups compared to the VC1 group ($p < 0.05$). The expression of *tgf-β* (Figure 6B) exhibited a gradual increase, reaching the highest level in the VC6 group ($p < 0.05$). The expression of *il-10* (Figure 6C) was markedly higher in the VC3 group than in the VC1 group ($p < 0.05$). After heat stress, expressions of *nf-κb* (Figure 6A), *il-8* (Figure 6D), and *tnf-α* (Figure 6E) followed a trend of initial decrease followed by an increase, with the lowest levels of *nf-κb* (Figure 6A) and *il-8* (Figure 6D) observed in the VC4 group ($p < 0.05$), and the lowest *tnf-α* (Figure 6E) levels in the VC5 group ($p < 0.05$). The expression of *tgf-β*

(Figure 6B) was significantly higher in the VC3 group ($p < 0.05$), while the expression of *il-10* showed an initial increase followed by a decrease. *t*-test analysis revealed that expressions of *il-10* (Figure 6C) and *tnf- α* (Figure 6E) were highly significant after heat stress compared to before heat stress ($p < 0.01$), while *il-8* (Figure 6D) expressions also showed significant changes in the VC1-VC3 groups.

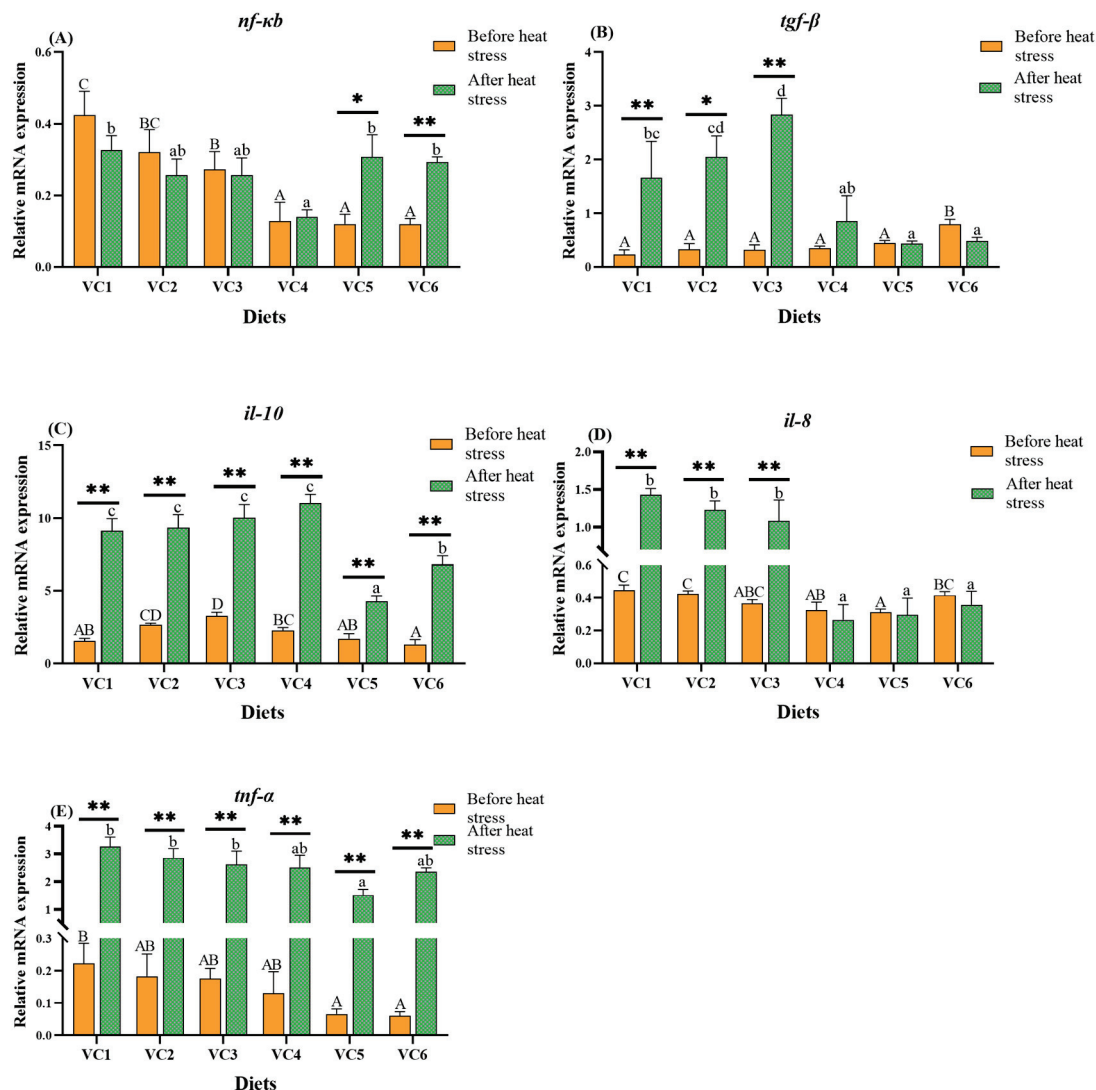


Figure 6. Effects of different levels of VC added to low fishmeal diets on intestinal immune-related genes in *M. salmoides* before and after heat stress. Data before heat stress are marked with uppercase letters 'A–D' and data after heat stress are marked with lowercase letters 'a–d'. After *t*-test, '*' for before and after heat stress data shows a statistically significant contrast ($p < 0.05$) and '**' shows extremely significant ($p < 0.01$). Columns of identical color marked with different letters show significant variations between data points ($p < 0.05$). (A), *nf-κb*; (B), *tgf-β*; (C), *il-10*; (D), *il-8*; (E), *tnf-α*.

3.6. Expression of Apoptosis-Related Genes

Figure 7 illustrates the effects of different VC levels in the diet on apoptosis-related gene expression in the intestines of *M. salmoides* before and after heat stress. Before heat stress, the expression of *bcl-2* (Figure 7B) was significantly higher in the VC3 group compared to the other groups ($p < 0.05$). The expression levels of caspase 8 (Figure 7D) and caspase 9 (Figure 7E) were significantly lower in the VC4 group compared to the VC1 group ($p < 0.05$). No significant differences were observed in the expression of *bax* (Figure 7A) and caspase 3 (Figure 7C) ($p > 0.05$). After heat stress, the expressions of *bax* (Figure 7A),

caspase 8 (Figure 7D), and caspase 3 (Figure 7C) were significantly lower in the VC4 group compared to the VC1 group ($p < 0.05$). The expression of bcl-2 (Figure 7B) initially increased and then decreased, with the highest expression observed in the VC3 group ($p < 0.05$). The expressions of caspase 3 (Figure 7C) and caspase 9 (Figure 7E) were not markedly affected by VC addition ($p > 0.05$). *t*-test analysis revealed that the expression of bax (Figure 7A), caspase 9 (Figure 7E), and bcl-2 (Figure 7B) showed highly significant differences after heat stress compared to before heat stress ($p < 0.01$).

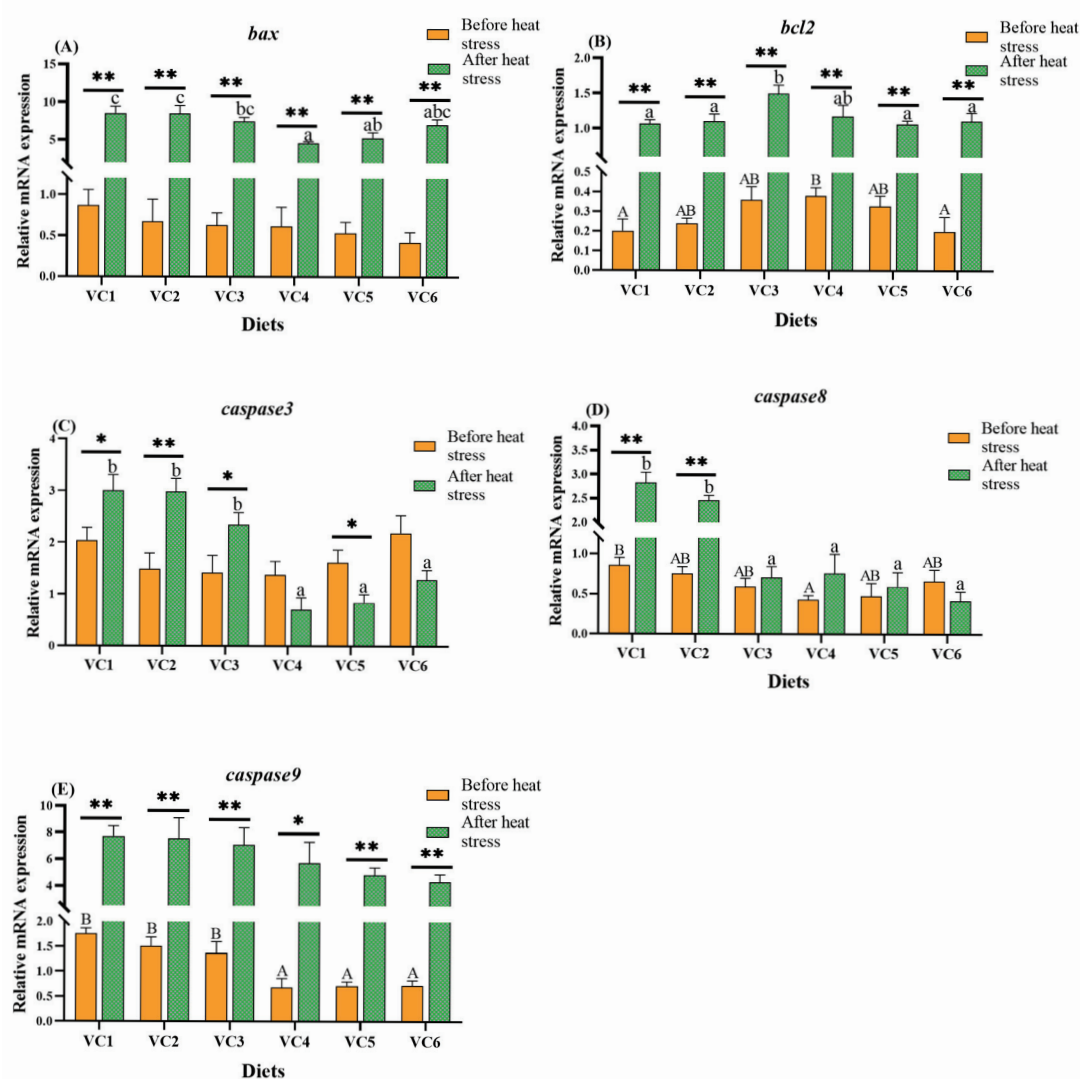


Figure 7. Effects of different levels of VC added to low fishmeal diets on intestinal apoptosis-related genes in *M. salmoides* before and after heat stress. Data before heat stress are marked with uppercase letters 'A–B' and data after heat stress are marked with lowercase letters 'a–c'. After *t*-test, '*' for before and after heat stress data shows a statistically significant contrast ($p < 0.05$) and '**' shows extremely significant ($p < 0.01$). Columns of identical color marked with different letters show significant variations between data points ($p < 0.05$). (A), bax; (B), bcl-2; (C), caspase 3; (D), caspase 8; (E), caspase 9.

3.7. TUNEL (Red) Fluorescence Analysis

Table 7 presents the results of TUNEL (red) fluorescence analysis of apoptosis in the gills of *M. salmoides* after heat stress, showing the effects of different VC levels. The density of red-positive cells was significantly lower in the VC3–VC5 groups compared to the VC1 group ($p < 0.05$), with the lowest density observed in the VC4 group ($p < 0.05$). The red mean density was also markedly lower in the VC4 and VC6 groups than in the VC1 group

($p < 0.05$). As shown in Figure 8, the VC3–VC5 groups exhibited the lowest number of apoptotic cells, with increased VC supplementation.

Table 7. Effects of different VC addition levels on apoptosis in *M. salmoides* gills after heat stress.

Diets	VC1	VC2	VC3	VC4	VC5	VC6
Red Positive Cells%	0.45 ± 0.01^c	0.43 ± 0.02^c	0.19 ± 0.02^a	0.17 ± 0.04^a	0.28 ± 0.02^b	0.36 ± 0.05^{bc}
Red Positive Cells Density, number/ 10^4 pixels	0.25 ± 0.02^b	0.20 ± 0.03^{ab}	0.21 ± 0.01^{ab}	0.17 ± 0.04^a	0.19 ± 0.01^{ab}	0.22 ± 0.00^{ab}
Red Mean Density	0.55 ± 0.06^b	0.50 ± 0.04^{ab}	0.49 ± 0.06^{ab}	0.37 ± 0.03^a	0.44 ± 0.01^{ab}	0.39 ± 0.03^a

Note: Mean values and standard error ($\pm$ SE; $n = 3 \times 3$) are presented for each parameter. Red Positive Cells% = Red Cells Amount/Total Cells Amount; Red Positive Cells Density (number/ 10^4 pixels) = Red Cells Amount/issue Area (pixel); Red Mean Density = Red Positive Density/Red Positive Area (pixel). Values with distinct alphabetical superscripts differ significantly ($p < 0.05$).

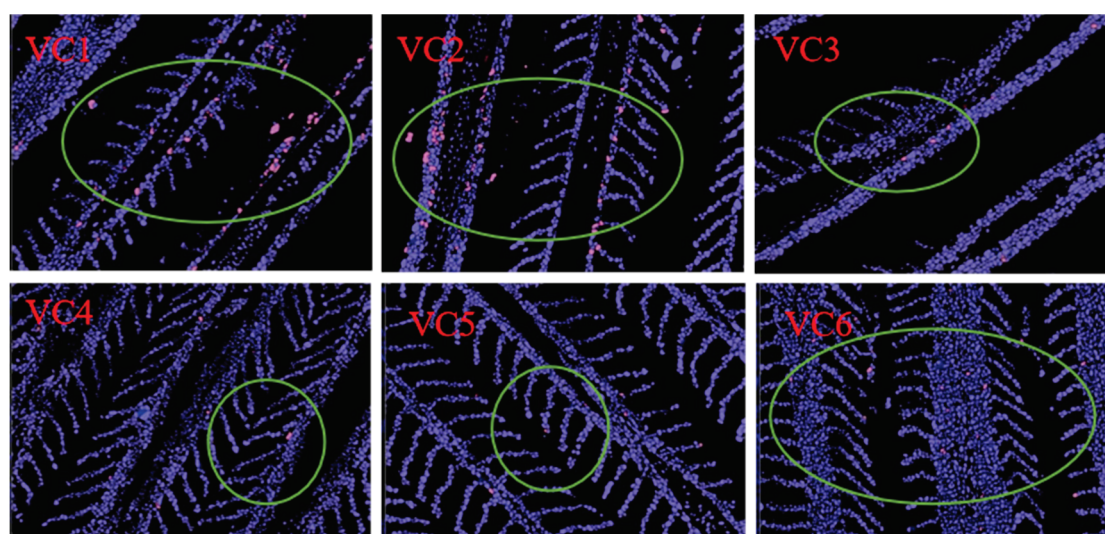


Figure 8. The immunofluorescence of gills of *M. salmoides* after heat stress stained with TUNEL (red). The red dots in the picture represent apoptotic cells. In order to show them more easily, we labelled them using green circles.

4. Discussion

4.1. Effects of VC Supplementation in Low Fishmeal Diets on the Growth Performance of *M. salmoides*

VC is an essential nutrient for fish growth [39]. Dietary VC supplementation has been shown to significantly enhance growth performance in species such as gibel carp (*Carassius gibelio*) [40] and Amur carp (*Cyprinus carpio haematopterus*) [41]. In this experiment, the addition of VC to the diet resulted in an initial increase in FBW, WGR, and SGR, followed by a decline. The optimal growth results were observed in the VC3 and VC4 groups. VC supplementation can enhance iron absorption [12], improve immune function [14], and contribute to collagen synthesis [42], all of which support fish growth, health, and nutrient absorption. Consistent with these findings, the addition of VC to a low-fishmeal diet improved the growth performance of juvenile *M. salmoides* in this study. Additionally, based on quadratic regression analysis of SGR and WGR, the optimal dietary VC requirement for *M. salmoides* fed low-fishmeal diets was estimated to be 0.77 g/kg. However, when VC supplementation exceeded 0.81 g/kg, a decline in growth performance was observed. This aligns with previous research on juvenile cobia (*Rachycentron canadum* L.), which showed growth inhibition when VC levels in the diet exceeded 150 mg/kg [29]. Similarly, a study on hybrid groupers (*Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂) found that excessive

VC levels led to growth suppression similar to the control group [43]. High doses of VC may disrupt physiological processes, thereby slowing growth. This finding is supported by earlier studies, which suggested that VC levels above a certain threshold do not further improve growth performance [44]. Additionally, in our study, the VC supplementation did not significantly affect the whole-body composition of *M. salmoides*, which aligns with findings from research on walleye pollock (*Gadus chalcogrammus*) [45].

4.2. Effects of VC Supplementation in Low Fishmeal Diets on Plasma ALT and AST Levels in *M. salmoides*

Plasma ALT and AST levels serve as indicators of physiological changes and stress in fish, with elevated concentrations reflecting liver and tissue damage [46]. In this experiment, under low fishmeal conditions, no significant changes in ALT and AST levels were observed prior to heat stress across the different VC supplementation groups. However, after heat stress, the VC4 group showed significantly reduced ALT and AST levels compared to the VC1 group. These results align with findings from studies on coho salmon (*Oncorhynchus kisutch*) [47], juvenile Chinese sucker (*Myxocyprinus asiaticus*) [48], and juvenile striped catfish (*Pangasianodon hypophthalmus*) [49], where appropriate VC supplementation effectively reduced plasma ALT and AST levels. These observations suggest that VC supplementation in low fishmeal diets can reduce ALT and AST levels, indicating its potential to protect hepatocytes from damage. Additionally, independent sample *t*-tests revealed that ALT and AST levels were significantly higher after stress compared to before stress within the same VC supplementation groups. This indicates that the temperature conditions in the experiment likely induced oxidative stress in fish, leading to excessive ALT and AST in the blood [50]. In summary, under heat stress conditions, VC supplementation in low fishmeal diets for *M. salmoides* can reduce ALT and AST levels, thereby improving fish health.

4.3. Effects of VC Supplementation in Low Fishmeal Diets on Plasma and Intestinal Antioxidant Capacity of *M. salmoides*

Antioxidant enzyme activities and related gene expression are closely linked to tissue health [51]. *Nrf2* plays a pivotal role in antioxidant functions, regulating factors such as *sod*, *cat*, and *gpx* [52,53]. These enzymes counteract the toxicity of reactive oxygen species (ROS), while the activity of T-AOC and the levels of GSH effectively reflect the antioxidant capacity of fish [54]. In our experiment, prior to heat stress, the VC3 group exhibited higher expression levels of *cat* and greater CAT enzyme activity than the VC1 group. The downstream factors of *nrf2* and enzyme activity indicators, including T-AOC and GSH-Px, peaked in the VC3 and VC4 groups. VC supplementation in the diets of juvenile yellow catfish (*Pelteobagrus fulvidraco*) [33] and black carp (*Mylopharyngodon piceus*) [55] enhances the activities of SOD, CAT, and GSH-Px. VC also promotes *nrf2/keap1* signaling and gene expression, further boosting antioxidant capacity [56]. After heat stress, the VC3 group demonstrated the highest expression levels of *keap1* and *nrf2*. T-AOC and GSH levels were highest in the VC4 group, while SOD and GSH-Px levels peaked in the VC5 group. Furthermore, studies have shown that *Nrf2* expression increases during stress, indicating its protective role, and it is positively correlated with the expression of antioxidant factors [57]. However, sustained elevation of *Nrf2* may lead to free radical damage, apoptosis, and tumorigenesis [58,59]. In contrast, increased expression of *Keap1* enhances the regulation of *Nrf2* abundance, reduces its gene expression, and thereby contributes to more effective antioxidant responses [58]. Heat stress experiments on golden shiners (*Notemigonus crysoleucas*) [60] and rats [61] have shown that dietary VC enhances heat stress resistance and cellular responses. In gibel carp (*Carassius gibelio*), VC activates the *nrf2/keap1* pathway following acute stress, mitigating oxidative damage [40]. Before and after heat stress, MDA levels in the VC3 group were significantly lower compared

to the VC1 group, indirectly indicating that adding 0.66 g/kg of VC benefits fish health. Heat stress increases mitochondrial respiration and ROS production [62]. The upregulation of genes (*keap1*, *sod*, *cat*) and the enhanced activities of antioxidant enzymes (CAT, SOD, GSH-Px) suggest that adding VC at this level improves antioxidant defenses and reduces oxidative damage induced by heat stress. Therefore, incorporating VC into low fishmeal diets enhances the antioxidant capacity of *M. salmoides* under heat stress, helping to alleviate the impacts of heat stress.

4.4. Effects of VC Supplementation in Low Fishmeal Diets on Intestinal Immune Function in *M. salmoides*

The NF- κ B pathway plays a pivotal role in physiological processes such as inflammation and immune responses [63], regulating downstream immune factors [64]. VC has been demonstrated to modulate the innate immune system in fish [65]. In this experiment, the NF- κ B pathway was activated before heat stress, with the expression of the anti-inflammatory factor *il-10* being highest in the VC3 group. Additionally, the expression of *tgf- β* positively correlated with VC levels, while the pro-inflammatory factors *il-8* and *tnf- α* were lowest in the VC5 group. Similar studies on juvenile grass carp [66] and yellow catfish [33] showed that VC supplementation down-regulated pro-inflammatory factors and up-regulated anti-inflammatory factors, enhancing immune capacity. Earlier research also indicated that VC enhanced immune-related gene expression in heat-stressed gibel carp, boosting lysozyme and complement C3 levels [40]. After heat stress in the present study, the NF- κ B pathway remained activated, with the highest *tgf- β* expression observed in the VC3 group. The VC4 group exhibited the most reduced *il-8* levels, and the VC5 group showed the lowest *tnf- α* expression. These results suggest that VC supplementation in a low fishmeal diet activates the NF- κ B pathway, promotes anti-inflammatory factor expression, inhibits pro-inflammatory factors, and protects gut health. *t*-tests revealed that after heat stress, *il-10* and *tnf- α* expressions significantly increased compared to pre-stress levels, while *tgf- β* and *il-8* expressions were most evident in the VC1-VC3 groups. Heat stress activates both innate and adaptive immune responses in fish, including the upregulation of pro-inflammatory cytokines like *il-1 β* , *il-6*, and *tnf- α* , indicating an inflammatory response [67]. Thus, VC supplementation in low fishmeal diets after heat stress promotes the expression of anti-inflammatory factors, reduces pro-inflammatory responses, and enhances immune function.

4.5. Effects of VC Supplementation in Low Fishmeal Diets on Intestinal Apoptosis in *M. salmoides*

Apoptosis is regulated by the *bcl-2* family, where *bax* promotes apoptosis and *bcl-2* inhibits it [68]. *Caspase 8* and *caspase 9* require activation by *caspase 3*, and elevated mRNA levels of these *caspsases* are indicative of increased apoptosis [69,70]. In this experiment, prior to heat stress, the expression of *bcl-2* was highest in the VC4 group, while *caspase 3* expression was lowest. Expression levels of *bax*, *caspase 8*, and *caspase 9* decreased with increasing VC supplementation. These results suggest that VC supplementation in a low-fishmeal diet enhances anti-apoptotic capacity, a conclusion also supported by studies on grass carp, where VC supplementation reduced pro-apoptotic genes (*bax* and *caspase 3*) and increased *bcl-2* expression [68,71]. After heat stress, the VC4 group exhibited the lowest expressions of *bax* and *caspase 8*, with *bcl-2* peaking in the VC3 group. Although changes in *caspase 3* and *caspase 9* expression were not significant, *caspase 3* expression initially decreased before increasing, while *caspase 9* expression declined with higher VC levels. Studies on gibel carp [40] and abalone [56] have shown that VC supplementation during stress reduces apoptosis. In a study on pikeperch (*Sander lucioperca*), acute heat stress significantly increased the expression of pro-apoptotic genes *bax*, *caspase 3*, and *caspase 9*. In this experiment, adding 0.81 g/kg VC to the diet effectively reduced apoptosis

in fish [72]. Furthermore, *bax*, *bcl-2*, and *caspase 3* expressions were significantly higher after heat stress compared to before, likely due to cell damage or apoptosis induced by high temperatures [73]. Based on our findings, VC-enriched diets likely promote anti-apoptotic gene expression while reducing pro-apoptotic gene expression. These results were further confirmed by immunofluorescence experiments on gills. After TUNEL (red) staining, it was observed that VC supplementation at levels of 0.66–0.97 g/kg (VC3–VC5 groups) in low fishmeal diets effectively reduced apoptosis under heat stress conditions.

5. Conclusions

In conclusion, the inclusion of VC in low fishmeal diets mitigates the growth inhibition, antioxidant capacity reduction, and immune suppression associated with low fishmeal while effectively reducing apoptosis in fish. Additionally, based on quadratic regression analysis of SGR and WGR, the optimal dietary VC requirement for *M. salmoides* fed low-fishmeal diets was estimated to be 0.77 g/kg. Under heat stress conditions, VC supplementation significantly enhances the antioxidant, immune, and anti-apoptotic capacities of fish, thereby improving their resistance to acute heat stress.

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Dietary Encapsulation of a Novel *Lysinibacillus* sp. PWR01 Probiotic Modulates Growth, Antioxidant, Immune Gene Expression, and Gut Health in Nile tilapia (*Oreochromis niloticus*) Against *Aeromonas hydrophila* Infection

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Abstract

Encapsulated probiotics, which are promising approaches for improving aquaculture species' performance and health, have incompletely characterized dose-dependent physiological and immunological effects. This study represents the first report evaluating the probiotic efficacy of a novel encapsulated *Lysinibacillus* sp. PWR01, originally isolated from rubber latex nodules, in Nile tilapia (*Oreochromis niloticus*). A total of 300 Nile tilapia (10.80 ± 0.07 g) was allocated to four experimental groups receiving 0, 10^4 , 10^5 , and 10^6 CFU/g of encapsulated *Lysinibacillus* sp. PWR01 in their diet. At 8 weeks of the feeding trial, growth performance, feed utilization, serum antioxidant status, intestinal bacterial counts and immune-related gene expression were analyzed. Results demonstrated that fish fed 10^6 CFU/g achieved a final weight of 51.48 g, representing a 9.88% increase compared with the control (46.85 g), while weight gain (WG) improved by 12.82% and specific growth rate (SGR) by 6.34%. Feed conversion ratio (FCR) was reduced by up to 18.42% at 10^5 CFU/g relative to the control. Encapsulated groups enhanced serum superoxide dismutase activity without altering malondialdehyde levels, increased total intestinal bacterial and lactic acid bacteria counts, and selectively upregulated *TLR2* and *MHC II* mRNA transcript levels. Histological analysis further revealed increased intestinal villus height and width in encapsulated-fed groups, while liver architecture remained normal across treatments. Multivariate analyses demonstrated strong positive associations among growth performance, gut microbiota enrichment, and immune gene expression. Resistance to *Aeromonas hydrophila* infection was significantly improved at higher dietary doses, with post-challenge survival reaching 61.67–75% in supplemented groups versus 45.00% in controls. These findings demonstrate that latex-derived *Lysinibacillus* sp. PWR01 acts as a strain-specific immunomodulatory probiotic that enhances growth, antioxidant capacity,

microbial community balance, and disease resistance to Nile tilapia. Future studies should investigate the role of the Nrf2 antioxidant pathway, clarify TLR2-mediated immune signaling, and assess gut microbiota–immune system interactions within commercial-scale production systems.

Keywords: antioxidant activity; disease resistance; gut bacteria; probiotic supplementation; sustainable aquaculture

1. Introduction

Aquaculture is the world’s fastest-growing food production sector, now providing over half of all fish for human consumption [1]. Nile tilapia (*Oreochromis niloticus*) is one of the most widely farmed freshwater fish globally, valued for its rapid growth and hardiness, and its production has reached record highs in recent years [2–4]. Intensive farming practices needed to meet rising demand often lead to high stocking densities and degraded water quality, which increase fish stress and susceptibility to diseases [5,6]. Bacterial outbreaks in tilapia culture cause severe economic losses; for example, *Aeromonas hydrophila* (*A. hydrophila*), the causative agent of motile *Aeromonas* septicemia, is a prevalent pathogen that can induce high mortality in tilapia populations [7,8]. Therefore, ensuring fish health and disease resistance in such intensive systems has become a paramount challenge in aquaculture.

Historically, disease control in aquaculture has relied on antibiotics. However, the overuse of antibiotics has led to the emergence of antibiotic-resistant bacteria and residual drug accumulation in the environment [9,10]. These issues pose risks to animal and human health, prompting many countries to restrict antibiotic use in farmed fish. In response, there is growing interest in sustainable, eco-friendly disease management strategies. Probiotics have emerged as a viable alternative to antibiotics in aquaculture, offering a means to prevent infections through modulation of the host microbiota and immunity rather than chemical therapeutics [11–13]. The use of probiotics in fish farming has expanded significantly in the past decade [14], reflecting their promise in improving fish health and reducing dependence on antimicrobial drugs.

Probiotics are defined as viable beneficial microorganisms that, when delivered to the host in sufficient quantities, confer measurable health benefits [15,16]. In aquaculture, dietary probiotic supplementation has been extensively reported to enhance growth performance, feed utilization efficiency, and immunological responses of fish [16–18]. Previous studies have shown that probiotic supplementation can increase weight gain (WG), improve feed conversion ratio (FCR), and stimulate the fish immune system, thereby improving survival rate against bacterial infections [19–22]. Among the diverse available candidates for aquaculture application, *Bacillus* species are especially popular in aquaculture due to their spore-forming ability, which confers high stability in feed and the gastrointestinal tract [23]. Numerous studies on Nile tilapia have demonstrated the benefits of *Bacillus*-based probiotics. Dietary inclusion of *Bacillus* (e.g., *B. subtilis*, *B. licheniformis* or their combinations) has been shown to significantly promote growth performance, enhance innate immune parameters, and elevate resistance to bacterial pathogens [24–26]. These beneficial outcomes are mechanistically attributed to improved nutrient digestibility, favorable modulation of gut microbiota composition toward health-promoting communities, and production of bioactive compounds that inhibit pathogens [27,28]. As a consequence, *Bacillus*-based probiotics have firmly become an effective and widely adopted feed additive in tilapia culture to sustain productivity and host health. Parallel to this well-established literature, con-

temporary research is increasingly directing attention toward novel bacterial genera with underexplored probiotic potential. *Lysinibacillus*, a spore-forming genus phylogenetically closed to *Bacillus* has emerged as a candidate of particular interest. Recent investigations have demonstrated that select *Lysinibacillus* strains exhibit broad-spectrum antibacterial activity against major aquatic pathogens, including *A. hydrophila*, *Vibrio* spp., and *Streptococcus* spp. [29–31], suggesting functional diversity same as typically documented for conventional *Bacillus* probiotics. In addition, *Lysinibacillus* species have shown promising probiotic properties in preliminary studies. Yao, et al. [31] isolated a *Lysinibacillus* strain from the gut of yellow croaker (*Larimichthys crocea*) that exhibited strong antimicrobial activity against several fish pathogens, including *A. hydrophila*. These findings indicate that *Lysinibacillus* could serve as a beneficial probiotic in aquaculture. Notably, *Lysinibacillus* shares the advantages of spore formation and environmental resilience with *Bacillus* [32], but its use in fish diets has not been as extensively studied. To date, most evidence for *Lysinibacillus* efficacy comes from in vitro assays or trials in species like zebrafish (*Danio rerio*) [30], whiteleg shrimp (*Litopenaeus vannamei*) [29] or common carp (*Cyprinus carpio*) [33] rather than in major culture species like tilapia. Those results underscore the probiotic potential of *Lysinibacillus*; however, there remains a clear knowledge gap regarding its in vivo effects in economically important fish like tilapia, especially under practical farming conditions.

Another major determinant of probiotic efficacy in aquafeeds is delivery performance and viability at the intestinal target site. To exert beneficial effects, probiotic cells must successfully withstand feed processing, storage, immersion/leaching in water, and the acidic and bile-rich conditions of the gastrointestinal tract [34,35]. Even in spore-forming bacteria, viability losses and suboptimal intestinal colonization can occur, which may reduce functional dosing and compromise consistency of outcomes, particularly in intensive systems where stressors are frequent [36]. Although *Lysinibacillus* species possess endospores, there is limited empirical evidence regarding their survival efficiency in tilapia gastrointestinal conditions when administered in non-encapsulated form. Potential reductions in viable cell delivery to the intestine may therefore constrain probiotic efficacy and justify the exploration of protective delivery systems [36]. To address these limitations, encapsulation technologies (e.g., alginate-based microcapsules and related biopolymer matrices) have increasingly been adopted to protect probiotics from processing stress and gastrointestinal degradation, reduce premature leaching in water, and enable controlled release within the intestine [37,38]. Recent aquaculture studies have demonstrated that encapsulation can significantly enhance probiotic survival during feed processing and improve intestinal recovery rates compared with free-cell formulations [39–41], leading to more consistent physiological and immunological outcomes.

Critically, no peer-reviewed investigation has thus far examined the efficacy of an encapsulated *Lysinibacillus* strain in Nile tilapia subjected to *A. hydrophila* challenge, leaving a critical translational gap in both delivery strategy and host–pathogen context. Although preliminary trials in model species suggest probiotic potential, direct evidence in Nile tilapia remains lacking. This gap limits assessment of whether *Lysinibacillus* can provide comparable or complementary benefits to established *Bacillus*-based probiotics in tilapia aquaculture. Therefore, the current study aims to investigate dietary encapsulated *Lysinibacillus* sp. PWR01 in Nile tilapia, with emphasis on growth performance, antioxidant status, intestinal bacterial counts, immune-related gene expression, and resistance to *A. hydrophila* infection. By integrating an emerging probiotic with a protective delivery technology and evaluating its efficacy under pathogen challenge in a major aquaculture species, this study addresses a clearly defined research gap and contributes to the translational development of *Lysinibacillus*-based probiotics for tilapia culture.

2. Materials and Methods

2.1. Bacterial Culture and Experimental Fish

Lysinibacillus sp. PWR01 was isolated, characterized, and identified from our previous study [37]. The 16S rRNA sequencing data has been uploaded to the National Center for Biotechnology Information (NCBI) database under accession number PX415241.1. The strain was originally isolated from rubber latex nodules collected from a rubber plantation in Surat Thani Province, Thailand. The isolate was cultured on nutrient agar (NA; HiMedia, Thane, India).

The bacterial pathogen *A. hydrophila* used for the challenge experiment was isolated from diseased fish during an outbreak at a local aquaculture farm and was obtained from the Centex Shrimp Center, Faculty of Science, Mahidol University, Thailand.

A total of 300 one-month-old Nile tilapia were obtained from a local fish farm in Chiang Mai Province, Thailand. At the start of the experiment, fish had a mean body weight of 10.80 ± 0.07 g. Fish were acclimated under laboratory conditions prior to the feeding trial and challenge test. Before the experiment commenced, 10 randomly selected fish were screened to confirm the absence of bacterial and viral infections.

2.2. Diet Preparation

The experimental diets followed a protocol from a previous study [38]. Briefly, polysaccharides were extracted from dried *Gracilaria fisheri* seaweed collected from Thung Sai Chai, Chaiya District, Surat Thani Province, Thailand. Dried seaweed (20 g) was soaked in a 6% (*w/v*) sodium hydroxide (NaOH) solution at 28 °C for 5 h. Rinsed with distilled water, the seaweed was cut into 1.0–1.5 cm segments and hot-water extracted. Distilled water was added to the mixture, and the pH was adjusted to 6.2–6.8. The mixture was boiled for 1.5 h, stirred occasionally, and then filtered through two layers of fine cloth. The filtrate was frozen at −20 °C for 24 h, dried in a hot-air oven at 60 °C until constant weight, ground into a fine powder, and stored at room temperature for feed formulation.

Probiotic diets were formulated by incorporating *Lysinibacillus* sp. PWR01 spores. Spore production was accomplished through nutrient depletion in Difco Sporulation Medium. Subsequently, the purified spores were enumerated and purified according to the methodology outlined by Goncalves, et al. [42]. The purified spores were incorporated into the experimental diets at concentrations of 0 (control), 10^4 , 10^5 , and 1×10^6 CFU/g of feed (Figure 1).

For encapsulation, purified spores were suspended in a sterile sodium alginate at a final concentration of 2% (*w/v*). Encapsulation was performed by the dropwise addition of this mixture into a 0.45 M CaCl₂ solution under continuous gentle stirring, allowing bead solidification to proceed for 45 min at room temperature. The resultant gel beads, with a mean particle diameter of approximate [4–5 mm], were collected through filtration using filter paper, rinsed three times with sterile distilled water to remove residual calcium ions, and subsequently dried using hot air drying at 50–70 °C for 4–5 h. The encapsulation spore preparations were stored at 4 °C for further experiments.

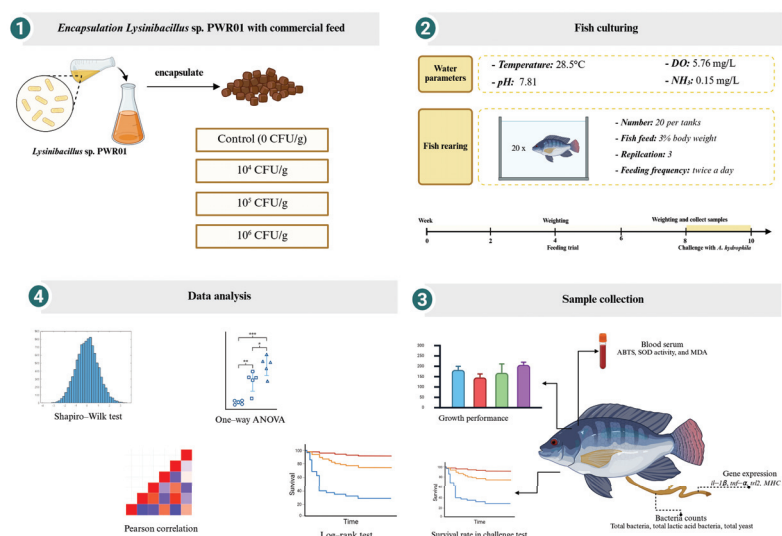


Figure 1. Schematic overview of the experimental design. Asterisks denote the level of statistical significance, where * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

2.3. Rearing Conditions

Three replicate tanks were assigned to each dietary treatment, with 20 fish stocked per tank. Experimental diets were administered twice daily at 09:00 and 16:00 during a eight-week feeding trial (from October to December 2025). Fish were observed for 30 min after each feeding to ensure the absence of uneaten feed. Any remaining feed was collected and weighed at the end of each day to estimate feed requirements for the subsequent day. The average feed intake for juvenile Nile tilapia was approximately 4% of body weight throughout the experimental period.

Water quality parameters were monitored and maintained within optimal ranges for Nile tilapia throughout the experiment [43]. These parameters included temperature (28.5 °C), pH (7.8), dissolved oxygen (5.76 mg/L), and NH_3 (0.15 mg/L).

2.4. Sample Collection

Fish were weighed at two-week intervals to assess growth performance. Mortality was recorded daily, and dead fish were promptly removed from the tanks. Survival rate was calculated at weeks 2 and 4 to determine cumulative survival.

At the end of the 8-week feeding trial, three fish per replicate tank were randomly selected and anesthetized with clove oil [44]. Briefly, the stock solution was diluted in tank water to achieve a working concentration of 100 mg/L. Individual fish were immersed in the anesthetic solution until complete sedation was conformed. Blood and tissue samples were then collected for subsequent analyses. Blood was drawn from the caudal vein using a 1 mL syringe, allowed to clot at 4 °C for 4 h, and centrifuged at $1000\times g$ for 10 min at 4 °C to obtain serum for antioxidant analysis [45].

Mid-intestinal tissue samples (approximately 50 mg) were collected, placed into sterile tubes containing 200 μL of TRIzol™ reagent (Invitrogen™, Life Technologies, Carlsbad, CA, USA), and stored at -80°C for gene expression analysis. In addition, midgut and liver tissues were fixed in 10% neutral buffered formalin for histological examination. The distal intestine was collected for total bacterial count analysis and stored in sterile 0.85% (w/v) NaCl solution until processing.

2.5. Growth Performance Indices

Fish growth performance was assessed using the following indices according to previously described methods [45,46]:

- Weight gain (WG, %) = [(Final body weight – Initial weight)/Initial weight] × 100.
- Specific growth rate (SGR, %) = [ln (Final weight) – ln (Initial weight)]/days × 100.
- Feed conversion ratio (FCR) = [Feed consumed (g)/Weight gain (g)].
- Daily weight gain (DWG, g/day) = (Final body weight – Initial body weight)/days.
- Periodic growth rate (PWG, g/day) = [(Final weight of period – Initial weight of the period)/period day].

2.6. Blood Biochemical Analysis

2.6.1. ABTS Radical Scavenging Activity

Antioxidant activity was determined using the ABTS radical scavenging assay following the method described by Boonkong, et al. [47] with minor modification. Briefly, 20 µL of serum sample was mixed with 200 µL of 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) solution. The mixture was gently vortexed and incubated in the dark at room temperature for 10 min. Absorbance was measured at 734 nm using a microplate reader. Antioxidant activity was quantified by comparison with a Trolox standard curve ranging from 0 to 2.5 mg/mL.

2.6.2. Superoxide Dismutase Activity

Superoxide dismutase (SOD) activity was measured using a commercial assay kit (CheKine™ Micro Superoxide Dismutase Activity Assay Kit, KTB1030; Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Serum samples were incubated with the provided reagents, and absorbance was recorded at 450 nm using a Varioskan LUX microplate reader (Thermo Scientific, Vantaa, Finland) [48].

2.6.3. Malondialdehyde Assay

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) levels following the method of Karatas and EM [49]. Briefly, 50 µL of serum was mixed with 100 µL of thiobarbituric acid (TBA) reagent consisting of 0.375% TBA and 0.25 N HCl in 15% trichloroacetic acid (TCA). The mixture was incubated at room temperature for 5 min and then heated at 95 °C for 5 min. After cooling on ice, absorbance was measured at 532 nm. MDA concentration was calculated using a standard curve ranging from 0 to 300 µmol/L.

2.7. Histological Analysis

For each treatment group, anterior intestinal and liver tissues ($n = 5$) were collected and subjected to histological processing. The samples were first fixed and then dehydrated through a sequential ethanol gradient consisting of 10%, 20%, 30%, 50%, 70%, and 100% ethanol. Following dehydration, the tissues were clarified by two immersions in xylene, each lasting 60 min.

Subsequently, the specimens were infiltrated with a transitional mixture of xylene and paraffin (1:2, v/v) prior to paraffin processing. This was followed by two successive paraffin infiltration steps, after which the tissues were maintained in molten paraffin overnight before being embedded into paraffin blocks.

Paraffin-embedded samples were sectioned at a thickness of 5 µm using a rotary microtome (Leica 2025, Wetzlar, Germany). The sections were stained with hematoxylin and eosin (H&E; Solarbio, G1120, Beijing, China) to evaluate tissue architecture and cellular characteristics. Microscopic examination was carried out using a CX43 light microscope (Olympus, Hachioji-shi, Tokyo, Japan) fitted with an E620 digital imaging system. Morphometric measurements were performed according to the methodological framework described by previous studies [45,50].

2.8. Intestinal Bacterial and Yeast Counts

The intestine was thoroughly washed with sterile saline solution (0.85% NaCl) and homogenized for the determination of total bacterial count, total lactic acid bacteria (LAB), and total yeast count using the tenfold serial dilution method. Approximately 1 g of intestinal tissue was homogenized in 9 mL of sterile saline to obtain an initial 10^{-1} dilution. Tenfold serial dilutions were subsequently prepared up to 10^{-6} . For each appropriate dilution, 0.1 mL aliquots were spread-plated in triplicate onto selective media. Plate count agar (PCA; HiMedia Laboratories, Mumbai, India) was used for enumeration of total bacteria, De Man, Rogosa and Sharpe (MRS) agar (HiMedia Laboratories, India) for total LAB, and potato dextrose agar (PDA; HiMedia Laboratories, India) for total yeast. PCA and MRS plates were incubated at 37 °C for 24–48 h under aerobic conditions, whereas PDA plates were incubated at 28 °C for 72 h. Plates containing 30–300 colonies were selected for counting. Colony-forming units were calculated by multiplying the colony number by the corresponding dilution factor and expressed as $\log_{10}$ CFU/g of intestinal tissue.

2.9. Gene Expression by Quantitative PCR (qPCR)

Total RNA was extracted from mid-intestinal tissue samples using TRIzol™ reagent following homogenization with a Bullet Blender® homogenizer (Next Advance, New York, NY, USA) based on previous studies [51]. Homogenized samples were incubated at room temperature for 2–3 min, after which 100 µL of chloroform was added. The mixtures were vortexed briefly, incubated for 2 min, and centrifuged at 12,000 rpm for 15 min at 4 °C. The aqueous phase containing RNA was collected and further purified using a Total RNA extraction kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop™ One/OneC spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) [52].

Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using a reverse transcription kit (BIO-RAD, Hercules, CA, USA). Quantitative real-time PCR (qPCR) was conducted in a 20 µL reaction volume containing 100 ng of cDNA, 0.4 µL of each primer (10 µM), and 10 µL of 2× iTaq Universal SYBR Green Supermix (BIO-RAD, USA). Amplification was performed using a CFX Connect™ Real-Time PCR Detection System (BIO-RAD, USA) under the following conditions: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s [45]. A melt curve analysis was included to confirm amplification specificity. Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method [53], with β -actin used as the reference gene. All the primers were adopted from Sheng, et al. [54] study. Primer sequences are provided in Table 1.

Table 1. Primer sequences used for quantitative PCR analysis in this study.

Gene	Primer Direction	Sequence (5'→3')	Accession Number
β -actin	Forward	GGCATCACACCTTCTACAACGA	KJ126772.1
	Reverse	ACGCTCTGTCAGGATCTTCA	
TNF- α	Forward	GAACACTGGCGACAAAACAGA	AY428948
	Reverse	TTGAGTCGCTGCCTTCTAGA	
IL-1 β	Forward	AGCTCCATGCAGTGATGCTG	XM_019365841.2
	Reverse	TGTTTTTATCCGTCACCTCCTCC	
IFN- β	Forward	CCATGAGCAGGAGAACTTCT	—
	Reverse	GTGAGCTCGCCTCTTCGTACA	
TLR2	Forward	AAAAGCATAGATGAGTTCCACATCC	JQ809459.1
	Reverse	GTAAGACAAGGCATCACAAACACC	
MHC II	Forward	ATCACTGACTGGGATCCCTCCTC	NM_001279562.1
	Reverse	CTCGAGCCTTCCTCCTGTAGTAGAT	

2.10. *Aeromonas hydrophila* Challenge Test

Aeromonas hydrophila used in the present study was cultured in tryptic soy broth (HiMedia, India) at 28 °C for 16 h. Bacterial cells were harvested by centrifugation at $12,000 \times g$ for 15 min at 4 °C, washed three times with sterile phosphate-buffered saline (PBS), and resuspended in sterile PBS. Bacterial concentration was determined by tenfold serial dilution and plate counting on tryptic soy agar plates.

Fish in the negative control group were injected intraperitoneally with sterile PBS, whereas fish in the remaining treatments were injected with *A. hydrophila*. All challenged fish were intraperitoneally injected with *A. hydrophila* at a dose of 2.5×10^6 CFU/g body weight, following a previously described protocol [55]. After challenge, fish were monitored daily, and cumulative mortality was recorded over a 14-day period.

After the challenge trial, surviving and moribund Nile tilapia were managed under strict biosecurity and hygienic disposal procedures to prevent environmental release of the pathogen. All remaining fish were humanely euthanized using an overdose of tricaine methanesulfonate (MS-222; 300 mg/L) for the euthanasia of experimental fish [56]. Death was confirmed by cessation of opercular movement and absence of reflex responses before further handling. All dead and euthanized fish were collected in sealed, leakproof biosecure containers and transported to a licensed facility for high-temperature incineration, consistent with aquatic animal health and biosafety recommendations [57]. In addition, tanks, equipment, and effluent water were disinfected with sodium hypochlorite before disposal, in accordance with established aquaculture biosecurity protocols.

2.11. Statistical Analysis

Prior to statistical analysis, microbial count data were log-transformed to satisfy assumptions of normality and homoscedasticity. Data normality was assessed using the Shapiro–Wilk test [58]. Differences among treatments were analyzed by one-way analysis of variance (ANOVA), followed by Duncan’s multiple range test using SPSS software (version 29.0.2.0; IBM Corp., Armonk, NY, USA). In addition, fish survival during the 14-day post-challenge period following *A. hydrophila* injection was analyzed using Kaplan–Meier survival curves, and differences among treatments were evaluated with the log-rank test. Pearson correlation analysis and principal component analysis (PCA) were conducted using standardized (z-score transformed) datasets including all quantitative indicators measured in this study (growth performance indices, antioxidant parameters, intestinal microbial counts, histological parameters and immune-related gene expression levels). Variables included in PCA were screened to ensure complete datasets (no missing values) and adequate variance (variance > 1 after scaling). PCA suitability was verified using the Kaiser–Meyer–Olkin (KMO) measure of sampling adequacy and Bartlett’s test of sphericity prior to dimensionality reduction. Principal components with eigenvalues > 1 were retained for interpretation. All statistical analyses and graphical visualizations were conducted using R software (version 4.3.1; R Foundation for Statistical Computing, Vienna, Austria). The following R packages were used as *stats* for correlation analysis, *FactoMineR* and *factoextra* for PCA, *survival* and *survminer* for survival analysis, and *ggplot2* for data visualization. Results are expressed as mean $\pm$ standard deviation. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Growth Performance Analysis

Lysinibacillus sp. PWR01 encapsulation concentration affected growth performance from week 4 onward, while survival remained unchanged throughout the experiment (Table 2). At week 4, FW ranged from 26.00 g in the control to 27.63 g at 10^5 CFU/g. WG

(15.18–16.87 g), PWG (137.83–156.66%), SGR (2.92–3.14%/day), and DWG (0.54–0.60 g/day) showed higher mean values at 10^5 – 10^6 CFU/g than in the control. FCR at week 4 was similar among treatments (0.83–0.84). However, these parameters have no different significant among treatments.

Table 2. Growth performance parameters of Nile tilapia (*Oreochromis niloticus*) fed diets containing different *Lysinibacillus* sp. PWR01 encapsulation concentrations (0, 10^4 , 10^5 , and 10^6 CFU/g) measured at weeks 4 and 8.

Parameter	Encapsulation Concentration (CFU/g)			
	0 (Control)	10^4	10^5	10^6
Initial weight (g)	10.82 ± 0.08	10.80 ± 0.09	10.77 ± 0.03	10.83 ± 0.10
Week 4				
Survival rate (%)	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Final weight (g)	26.00 ± 1.09	26.25 ± 0.65	27.63 ± 0.54	27.33 ± 0.73
Weight gain (g)	15.18 ± 1.08	15.45 ± 0.73	16.87 ± 0.55	16.50 ± 0.83
Percent weight gain (%)	137.83 ± 9.81	143.09 ± 7.74	156.66 ± 5.31	152.36 ± 9.04
Specific growth rate (%/day)	2.92 ± 0.11	2.96 ± 0.11	3.14 ± 0.07	3.08 ± 0.12
Daily weight gain (g/day)	0.54 ± 0.07	0.55 ± 0.03	0.60 ± 0.03	0.59 ± 0.03
FCR	0.84 ± 0.05	0.83 ± 0.03	0.84 ± 0.02	0.83 ± 0.01
Week 8				
Survival rate (%)	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Final weight (g)	46.85 ± 1.08 ^b	47.03 ± 2.42 ^b	49.45 ± 1.08 ^{ab}	51.48 ± 0.54 ^a
Weight gain (g)	36.03 ± 1.13 ^b	36.23 ± 2.37 ^b	38.68 ± 1.08 ^{ab}	40.65 ± 0.44 ^a
Percent weight gain (%)	333.17 ± 12.20 ^c	335.45 ± 20.68 ^{bc}	359.29 ± 10.00 ^{ab}	375.23 ± 1.86 ^a
Percentage specific growth rate (%/day)	4.89 ± 0.09 ^b	4.90 ± 0.16 ^b	5.08 ± 0.07 ^{ab}	5.20 ± 0.01 ^a
Daily weight gain (g/day)	0.64 ± 0.02 ^b	0.65 ± 0.04 ^b	0.69 ± 0.02 ^{ab}	0.73 ± 0.01 ^a
FCR	1.52 ± 0.17 ^b	1.37 ± 0.07 ^b	1.24 ± 0.02 ^a	1.31 ± 0.04 ^a

Data are presented as the mean ± standard deviation (SD) ($n = 3$ per treatment). Within the same row and sampling time (week 4 or week 8), mean values followed by distinct superscript lowercase letters (a, b, c) indicate statistically significant differences among encapsulation concentrations. These differences were determined using one-way ANOVA followed by Duncan's multiple range test ($p < 0.05$). Means sharing at least one common superscript letter are not significantly different ($p > 0.05$).

At week 8, FW increased significantly with encapsulation concentration ($r = 0.75$, $p < 0.01$) (Figure 2), reaching 51.48 g at 10^6 CFU/g compared with 46.85 g in the control, representing a 9.88% increase relative to the control. WG was also positively correlated with concentration ($r = 0.75$, $p < 0.01$), increasing from 36.03 g in the control to 40.65 g at 10^6 CFU/g, corresponding to a 12.82% improvement. PWG showed a strong positive correlation ($r = 0.73$, $p < 0.01$), ranging from 333.17% to 375.23% across the same treatments (+12.62% compared with control). SGR increased with encapsulation concentration ($r = 0.72$, $p < 0.01$), with the highest value observed at 10^6 CFU/g (5.20%/day), 6.34% higher than the control (4.89%/day). DWG exhibited a positive but non-significant association with concentration ($r = 0.39$, $p = 0.21$), reaching 0.73 g/day at 10^6 CFU/g, which was 14.06% greater than the control (0.64 g/day). FCR showed a negative, non-significant correlation with encapsulation concentration ($r = -0.25$, $p = 0.42$), with lower values at 10^5 – 10^6 CFU/g (1.24–1.31) than in the control (1.52), indicating an improvement in feed efficiency of up to 18.42% at 10^5 CFU/g.

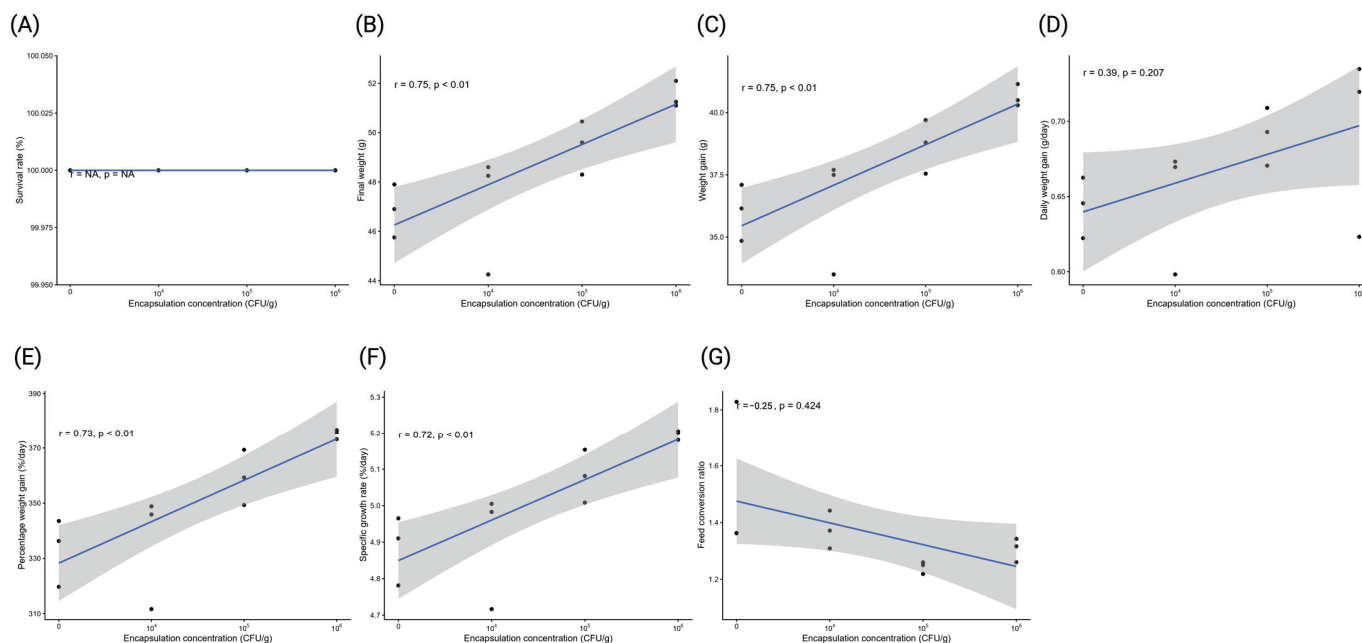


Figure 2. Correlations between encapsulation concentration (CFU/g) and growth-related parameters measured at week 8: (A) survival rate, (B) final weight, (C) weight gain, (D) daily weight gain, (E) percent weight gain, (F) specific growth rate, and (G) feed conversion ratio. Solid lines indicate good linear regression fit and shaded areas represent 95% confidence intervals. Pearson correlation coefficients (r) and p -values are shown in each panel.

3.2. Antioxidant and Lipid Peroxidation Analysis

Encapsulation concentration significantly altered antioxidant enzyme activity and radical scavenging capacity, while lipid peroxidation remained unchanged (Figure 3).

Relative to the control, serum ABTS activity declined at the highest encapsulation level. ABTS activity was comparable among the control, 10^4 CFU/g, and 10^5 CFU/g groups (2.61–2.85 $\mu\text{mol TE/L}$), but was significantly lower at 10^6 CFU/g (1.31 $\mu\text{mol TE/L}$; $p < 0.05$). Specifically, ABTS activity at 10^6 CFU/g was approximately 2.0-fold lower than the control. A strong negative correlation was observed between encapsulation concentration and ABTS activity ($r = -0.80$, $p < 0.001$).

Serum SOD activity increased with encapsulation concentration. Mean SOD activity ranged from 13.34 U/mL at 10^4 CFU/g to 38.99 U/mL at 10^6 CFU/g. At 10^6 CFU/g, SOD activity was approximately 2.9-fold higher than the lowest-dose group (10^4 CFU/g) and markedly elevated relative to the control ($p < 0.05$). SOD activity showed a significant positive correlation with encapsulation concentration ($r = 0.48$, $p < 0.01$).

MDA levels did not differ significantly among treatments, ranging from 1.84 to 2.54 $\mu\text{mol/L}$. No significant correlation was detected between encapsulation concentration and MDA level ($r = -0.07$, $p = 0.68$).

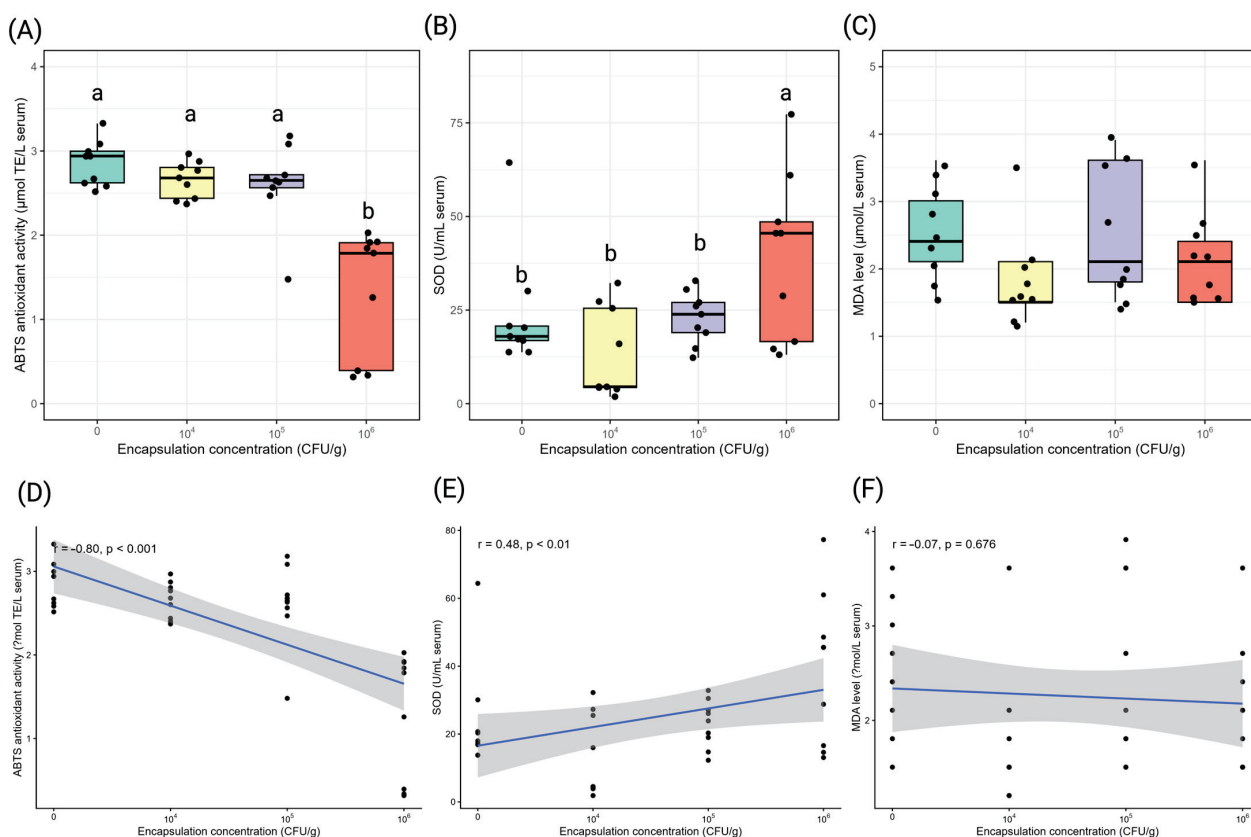


Figure 3. Effects of *Lysinibacillus* sp. PWR01 encapsulation concentration (CFU/g) on blood serum oxidative stress and antioxidant parameters of Nile tilapia (*Oreochromis niloticus*). (A) ABTS radical scavenging activity (μmol TE/L), (B) SOD activity (U/mL), and (C) MDA level (μmol/L). Different lowercase letters indicate significant differences among treatments ($p < 0.05$). Pearson correlation analyses between encapsulation concentration and (D) ABTS activity, (E) SOD activity, and (F) MDA level.

3.3. Microbial Counts Analysis

Encapsulation concentration significantly influenced total intestinal bacterial and LAB counts, whereas yeast counts were unaffected (Figure 4).

Total intestinal bacterial count increased progressively with encapsulation concentration. Mean bacterial counts rose from 7.47 log₁₀ CFU/g intestine in the control group to 7.70 log₁₀ CFU/g at 10⁴ CFU/g, 8.34 log₁₀ CFU/g at 10⁵ CFU/g, and 8.96 log₁₀ CFU/g at 10⁶ CFU/g ($p < 0.05$). Compared with the control, total bacterial abundance increased by 0.87, and 1.49 log units at 10⁵, and 10⁶ CFU/g, respectively. Encapsulation concentration showed a strong positive correlation with total bacterial count ($r = 0.72$, $p < 0.001$).

Total LAB count also differed significantly among treatments. LAB counts increased from 2.12 log₁₀ CFU/g intestine in the control to 2.29 log₁₀ CFU/g at 10⁴ CFU/g and peaked at 2.66 log₁₀ CFU/g at 10⁵ CFU/g, with a similar value at 10⁶ CFU/g (2.64 log₁₀ CFU/g; $p < 0.05$). Relative to the control, LAB abundance increased by 0.54, and 0.52 log units at 10⁵, and 10⁶ CFU/g, respectively. A positive correlation was observed between encapsulation concentration and LAB count ($r = 0.34$, $p < 0.05$).

Total intestinal yeast count remained stable across treatments, ranging from 2.64 to 2.75 log₁₀ CFU/g intestine, with no significant differences among encapsulation concentrations. In addition, no significant correlation was detected between encapsulation concentration and yeast count.

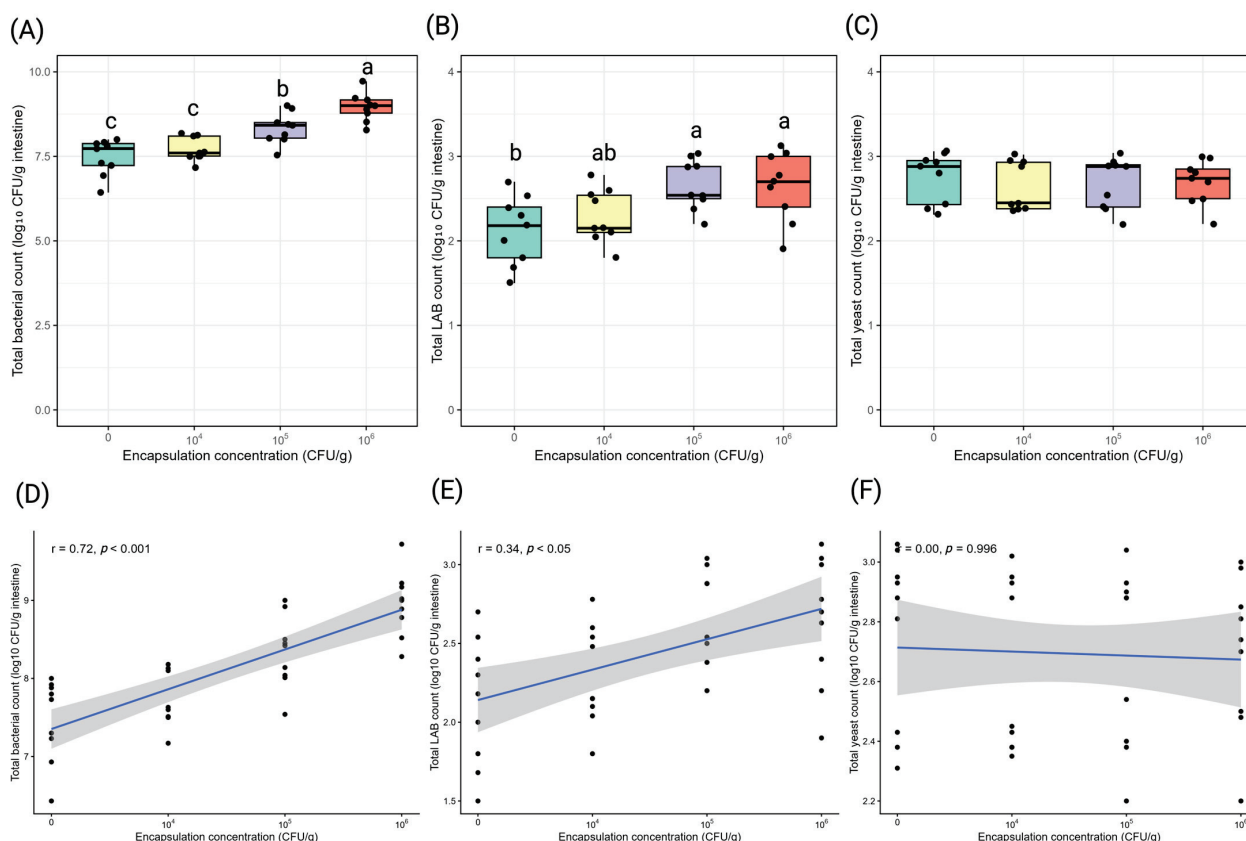


Figure 4. Effects of *Lysinibacillus* sp. PWR01 encapsulation concentration (CFU/g) on Nile tilapia (*Oreochromis niloticus*) intestinal microbial populations. (A) Total bacterial count, (B) total LAB count, and (C) total yeast count. Different lowercase letters indicate significant differences among treatments ($p < 0.05$). Pearson correlation analyses between encapsulation concentration and (D) total bacterial count, (E) LAB count, and (F) yeast count. Dots represent individual data points.

3.4. Histological Characteristics

Encapsulation supplementation significantly increased villus height and villus width, while muscular layer thickness remained unchanged and liver histology exhibited normal structural organization across all treatments (Figure 5).

Mean villus height increased progressively with encapsulation concentration, ranging from 71.10 μm in the control group to 91.90 μm in the 10⁶ CFU/g treatment (Figure 5B). The highest supplementation level produced significantly greater villus height than the control ($p < 0.05$), whereas the 10⁴ and 10⁵ CFU/g treatments showed intermediate values without significant differences from adjacent groups (Figure 5B). Correlation analysis indicated a strong positive association between encapsulation concentration and villus height ($r = 0.79$, $p < 0.001$) (Figure 5C).

Villus width showed a similar trend. Mean values increased from 52.22 μm in the control group to 67.74 μm at 10⁶ CFU/g (Figure 5B). The 10⁶ CFU/g treatment exhibited significantly greater villus width compared with the control ($p < 0.05$), while the 10⁴ and 10⁵ CFU/g treatments displayed intermediate measurements without significant differences (Figure 5B). A significant positive correlation was detected between encapsulation concentration and villus width ($r = 0.65$, $p < 0.001$) (Figure 5C).

Muscular layer thickness varied within a narrow range among treatments, from 19.96 μm to 22.44 μm (Figure 5B). No statistically significant differences were detected among groups ($p > 0.05$). The correlation between encapsulation concentration and muscular thickness was not significant ($r = -0.13$, $p = 0.465$) (Figure 5C).

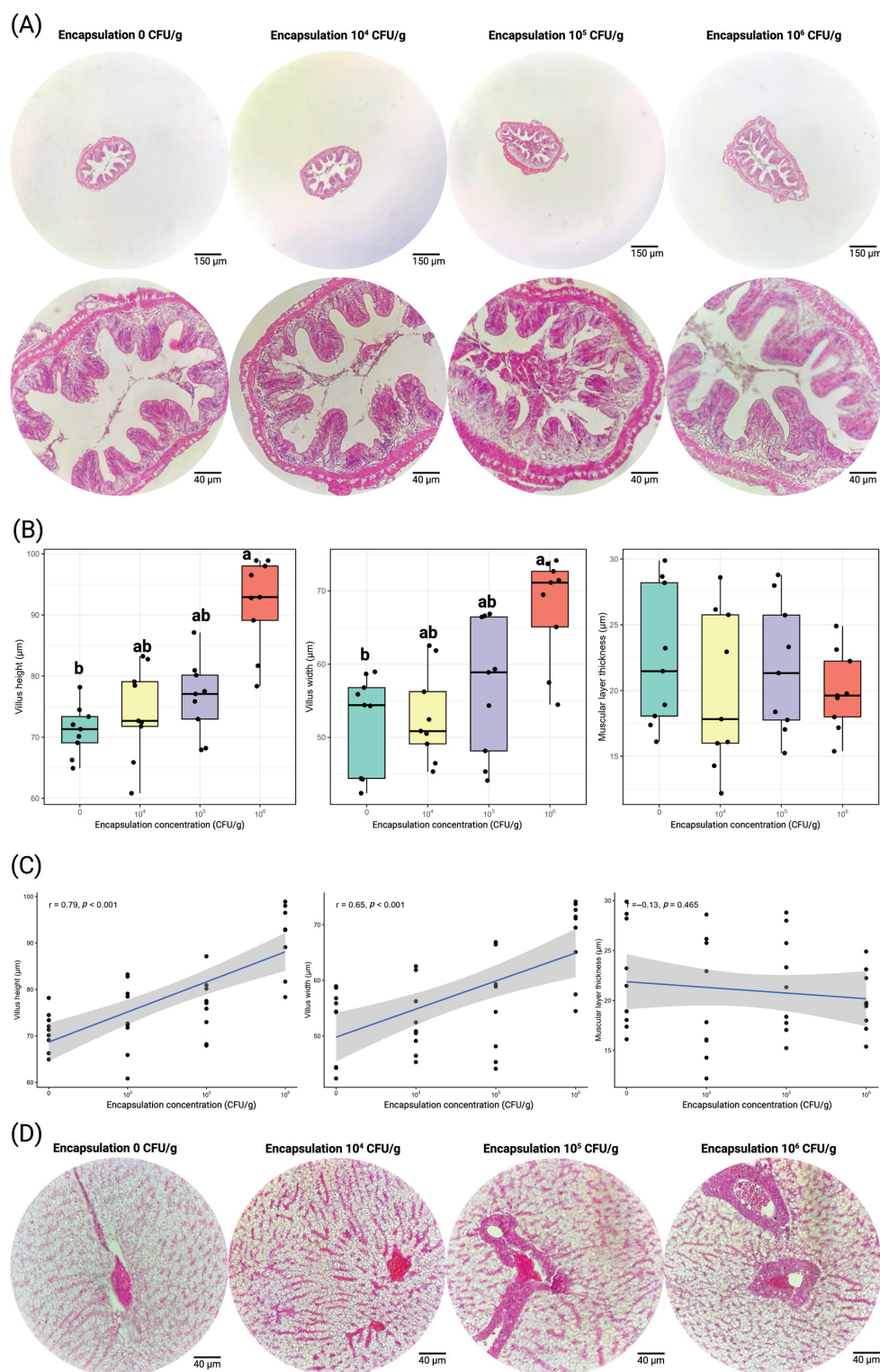


Figure 5. Histological structure and morphometric characteristics of Nile tilapia (*Oreochromis niloticus*) intestinal and hepatic tissues under different *Lysinibacillus* sp. PWR01 encapsulation concentrations (CFU/g). **(A)** Representative H&E-stained cross-sections of the anterior intestine at encapsulation levels of 0, 10^4 , 10^5 , and 10^6 CFU/g. **(B)** Boxplots showing villus height, villus width, and muscular layer thickness across treatments; different letters indicate significant differences ($p < 0.05$). **(C)** Correlation between encapsulation concentration and intestinal morphometric parameters. **(D)** Representative H&E-stained liver sections illustrating hepatocyte organization, sinusoidal spaces, and central vein structure across treatments. Scale bars: 150 μ m (upper intestinal overview), 40 μ m (intestinal and liver details). Dots represent individual data points.

Histological examination of the liver revealed well-preserved hepatic architecture in all treatments (Figure 5D). Hepatocytes were arranged in typical cords radiating from the central vein and separated by clearly visible sinusoidal spaces. Hepatocyte cytoplasm appeared homogeneous with centrally located nuclei, and no cellular degeneration, vacuolation, or structural disruption was observed across treatments. Sinusoidal structures remained regular and continuous, and the central vein maintained normal morphology without dilation or congestion. No necrotic areas, inflammatory cell infiltration, or tissue disorganization were detected in any group (Figure 5D). The structural integrity of hepatocytes and sinusoidal networks was comparable among all encapsulation concentrations.

3.5. Gene Expression

Encapsulation increased the transcript levels of *TLR2* and *MHC II*, while *IL-1 β* , *TNF- α* , and *IFN- β* showed no consistent upregulation across doses (Figure 6). At 10^4 – 10^6 CFU/g, *TLR2* transcripts significantly increased from the control (1.00) to 1.61–2.53, respectively, corresponding to $\log_2$ fold changes of 0.58–1.25. *MHC II* transcripts significantly rose from 1.00 in the control to 2.67–3.71 at 10^4 – 10^6 CFU/g, with $\log_2$ fold changes of 1.39–1.87, respectively. In contrast, *IL-1 β* transcript levels were 1.26–0.94 at 10^4 – 10^6 CFU/g, yielding $\log_2$ fold changes of 0.28–−0.14. *TNF- α* transcripts decreased relative to the control to 0.67, 0.56 and 0.62 at 10^4 – 10^6 CFU/g, corresponding to $\log_2$ fold changes of −0.86, −1.10, and −0.79. *IFN- β* transcript levels were 1.17, 0.98, and 0.89 at 10^4 – 10^6 CFU/g, with $\log_2$ fold changes of 0.17, −0.06, and −0.19.

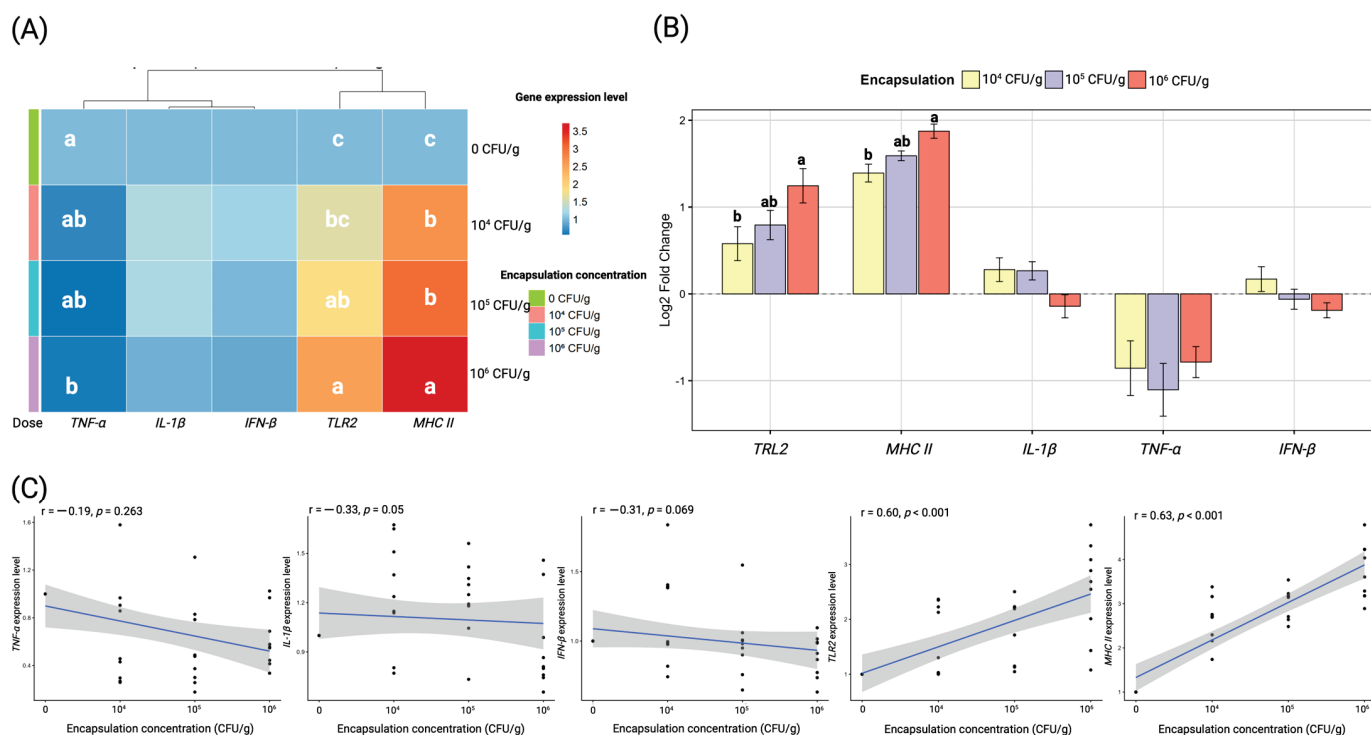


Figure 6. Dose-dependent effects of encapsulated *Lysinibacillus* sp. PWR01 on immune-related gene expression. (A) Heatmap of relative transcript levels (*TNF- α* , *IL-1 β* , *IFN- β* , *TLR2*, and *MHC II*) across treatments (0, 10^4 , 10^5 , and 10^6 CFU/g). (B) $\log_2$ fold changes (mean $\pm$ SE) relative to the control. (C) Pearson correlations between encapsulation concentration and gene expression levels. Different lowercase letters indicate significant differences among treatments ($p < 0.05$). Dots represent individual data points.

Pearson correlation analysis showed positive associations between encapsulation concentration and *TLR2* ($r = 0.60$, $p < 0.001$) and *MHC II* expression ($r = 0.63$, $p < 0.001$), whereas correlations with *IL-1 β* , *TNF- α* , and *IFN- β* were not significant ($p \geq 0.05$).

3.6. Pearson Correlation and PCA

Multivariate analysis showed significant associations among growth, antioxidant, microbiota, and immune variables and a clear separation of samples by encapsulation level (Figure 7). The Pearson correlation heatmap identified significant positive and negative correlations ($p < 0.05$) among multiple parameters, while self-correlations were excluded and non-significant relationships were masked. Positive correlations ($r > 0.50$, $p < 0.05$) were observed between growth-related indices (FW, WG, DWG, and PWG) and immune markers TLR2 and MHC II, whereas negative correlations ($r < -0.50$, $p < 0.05$) were detected between FCR and these growth indices. Microbiota variables (total bacteria, total LAB, and total yeast) showed significant positive correlations with TLR2 and MHC II ($r = 0.48$ – 0.67 , $p < 0.05$), while TNF- α was negatively correlated with several growth and microbiota parameters ($r = -0.41$ to -0.60 , $p < 0.05$). Principal component analysis explained 56.7% of the total variance on the first two axes, with PC1 accounting for 45.6% and PC2 for 11.1%. Samples were separated along PC1 according to encapsulation levels, with minimal overlap between 0 CFU/g and 10^6 CFU/g groups, whereas intermediate overlap was observed between 10^4 and 10^5 CFU/g groups.

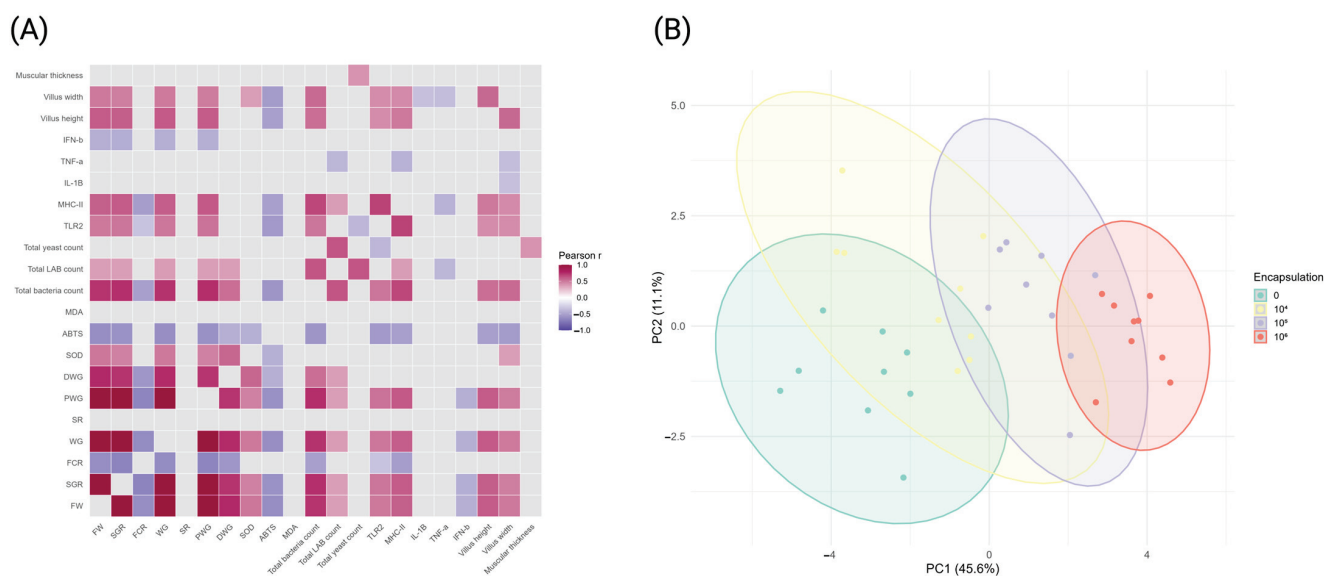


Figure 7. Multivariate relationships among growth performance, antioxidant status, microbiota composition, and immune gene expression in response to encapsulation. **(A)** Pearson correlation heatmap showing significant pairwise correlations ($p < 0.05$) among growth indices, antioxidant parameters, intestinal microbiota, and immune-related genes; correlation coefficients (r) are color-coded from negative (blue) to positive (red). **(B)** Principal component analysis score plot based on all measured variables, showing sample distribution according to encapsulation levels (0, 10^4 , 10^5 , and 10^6 CFU/g); ellipses represent 95% confidence intervals for each group.

3.7. Post-Challenge Survival Following *A. hydrophila* Infection

Encapsulation concentration significantly affected survival following *A. hydrophila* challenge (Figure 8). Survival in the negative control remained at 100% throughout the 14-day post-challenge period. In contrast, survival in the positive control declined rapidly after day 4, reaching 63.33% on day 5 and stabilizing at 45.00% from day 7 to day 14. Fish receiving encapsulated *Lysinibacillus* treatments showed a delayed and attenuated mortality pattern compared with the positive control. In the 10^6 CFU/g group, mortality increased modestly around days 4–5 but stabilized earlier (by day 6), reaching a final survival rate of 75.00%. Notably, the mortality peak in the 10^6 CFU/g group occurred within the same early window (days 4–5) but ended more rapidly and at a lower magnitude than in the positive control, suggesting improved early-phase resistance to bacterial proliferation. Similarly,

final survival rates were 68.33% and 61.67% for the 10^5 and 10^4 CFU/g groups, respectively, all exceeding that of the positive control (45.00%). The progressive improvement in survival from 10^4 to 10^6 CFU/g demonstrates a dose-dependent protective effect, although the survival curves converged after day 6, indicating that the principal benefit was exerted during the acute infection phase.

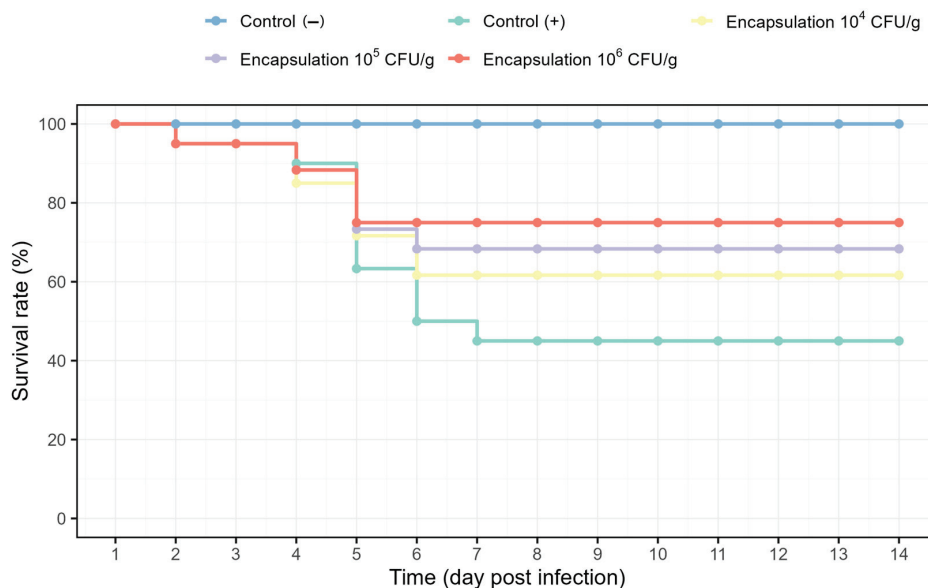


Figure 8. Survival rate (%) of Nile tilapia (*Oreochromis niloticus*) over 14 days following *Aeromonas hydrophila* challenge under different treatments.

In addition, Chi-square analysis showed a significant difference in survival between the positive and negative controls ($\chi^2 = 50.132$, $p < 0.001$; Table 3). Survival in the 10^5 CFU/g ($\chi^2 = 4.226$, $p = 0.040$) and 10^6 CFU/g ($\chi^2 = 5.719$, $p = 0.017$) groups differed significantly from the positive control, whereas the 10^4 CFU/g group did not ($\chi^2 = 1.454$, $p = 0.228$; Table 3). No significant differences were detected among the encapsulation treatments ($\chi^2 \leq 1.398$, $p \geq 0.237$).

Table 3. Pairwise comparisons of post-challenge survival among treatments using the chi-square (χ^2) test following *A. hydrophila* infection.

Treatment A	Treatment B	χ^2 (Chi-Square)	p -Value
Control (+)	Control (−)	50.132	<0.001
Control (+)	Encapsulation 10^4 CFU/g	1.454	0.228
Control (+)	Encapsulation 10^5 CFU/g	4.226	0.040
Control (+)	Encapsulation 10^6 CFU/g	5.719	0.017
Control (−)	Encapsulation 10^4 CFU/g	34.907	<0.001
Control (−)	Encapsulation 10^5 CFU/g	26.893	<0.001
Control (−)	Encapsulation 10^6 CFU/g	23.929	<0.001
Encapsulation 10^4 CFU/g	Encapsulation 10^5 CFU/g	0.714	0.398
Encapsulation 10^4 CFU/g	Encapsulation 10^6 CFU/g	1.398	0.237
Encapsulation 10^5 CFU/g	Encapsulation 10^6 CFU/g	0.118	0.732

4. Discussion

4.1. Growth Performance and Feed Utilization

The present findings demonstrate that encapsulated *Lysinibacillus* sp. PWR01 can significantly enhance fish growth performance, particularly over an extended feeding duration. By week 8, fish fed diets with higher probiotic encapsulation (10^5 – 10^6 CFU/g) showed

greater FW, WG, PWG, and SGR compared to the control, with a clear dose-dependent trend (correlations $r \approx 0.75$, $p < 0.01$). Notably, improvements were modest and statistically non-significant at week 4, suggesting that the probiotic's benefits manifest more strongly after sustained administration. This time-lag may reflect the period required for the probiotic to colonize the gut or modulate host physiology. Similar delayed growth enhancements have been observed in other studies. For example, feeding *L. sphaericus* to zebrafish over 33 days led to increasing WG and SGR with rising dose [30]. Notably, even at the highest tested concentration (10^7 CFU/mL), growth parameters did not differ statistically from controls. By contrast, the present study achieved statistically significant growth enhancement at 10^6 CFU/g, indicating that effective dose thresholds may be reduced through encapsulation, likely due to improved gastrointestinal stability and delivery efficiency. Similarly, Mani, et al. [33] reported that dietary supplementation with *L. macroides* at 10^7 – 10^8 CFU/mL significantly improved growth and feed conversion ratio in *Cyprinus carpio* fingerlings. However, the probiotic was administered in pelletized form without protective encapsulation, and relatively higher microbial loads were required to achieve statistical significance. When contrasted with these findings, the current encapsulated 10^6 CFU/g treatment achieved comparable improvements in FW and SGR at a lower concentration range. In Nile tilapia, diets supplemented related spore-formers (*Bacillus* spp.) over 8–10 weeks have consistently improved WG and feed conversion efficiency. For instance, *Bacillus subtilis* at 10^{10} CFU/g halved the FCR and boosted WG by approximately 40% relative to un-supplemented fish [59]. In the current study, FCR likewise tended to decrease, although this drop was not statistically significant. The overall improvement in growth metrics without adverse effects on survival or body condition aligns with a broad literature reporting that probiotics enhance nutrient utilization and growth in diverse aquaculture species [14,60]. The mechanisms likely involve probiotics contributing exogenous digestive enzymes and vitamins, as well as stimulating the host's digestive capacity [61]. Indeed, encapsulated probiotics have been shown to increase intestinal villi height and digestive enzyme activity, thereby increasing nutrient absorption in species like tilapia [62,63]. Enhanced feed intake or appetite regulation may also play a role; one study on encapsulated *Lactobacillus* noted upregulation of appetite-related genes (e.g., ghrelin) in fish fed the probiotic [64]. Collectively, the growth promotion seen with *Lysinibacillus* sp. PWR01 treatment is consistent with these reports. The lack of significant differences at early time points and the convergence of growth curves by week 8 in the high-dose groups further suggest that while low doses may be insufficient for measurable impact, an adequate concentration sustained over several weeks is required to realize probiotic growth benefits in aquaculture.

4.2. Antioxidant Status and Lipid Peroxidation of Blood Serum

Dietary *Lysinibacillus* encapsulation elicited notable changes in the antioxidant defense parameters of the fish. Serum SOD activity was markedly elevated in probiotic-fed groups, reaching nearly 3-fold higher levels at 10^6 CFU/g compared to controls. This positive correlation between dose and SOD activity ($r = 0.48$, $p < 0.01$) indicates an enhanced enzymatic capacity to neutralize superoxide radicals. Concomitantly, MDA concentration remained unchanged across treatments, suggesting that higher probiotic levels prevented any rise in oxidative stress (and may have helped maintain MDA at baseline levels). These outcomes align with recent probiotic studies reporting bolstered antioxidant defenses in fish. For instance, zebrafish receiving *L. sphaericus* exhibited significantly increased SOD and catalase activities coupled with a linear decrease in MDA content as the probiotic dose rose [30]. Likewise, tilapia fed high-dose *B. subtilis* showed elevated SOD, catalase, and glutathione peroxidase activities and significantly lower MDA than controls [59]. The general interpre-

tation is that probiotics can induce the host's antioxidant enzyme systems, thereby limiting lipid peroxidation and cellular damage under physiological or stress conditions [65,66]. Interestingly, in the present study the total radical scavenging capacity (ABTS assay) of serum was lower in the high-dose group. This inverse relationship with probiotic dose ($r \approx -0.80$) is somewhat counterintuitive, as one might expect overall antioxidant capacity to increase. A possible explanation is that the heightened SOD activity rapidly neutralized free radicals [67–69], thus leaving fewer circulating antioxidant compounds detectable by the ABTS assay. Regardless, the fish did not show elevated MDA, implying no net increase in oxidative damage despite the ABTS reduction. Previous work has noted that probiotic-fed fish often experience less oxidative stress during challenges or high metabolic demand. For example, probiotic-driven activation of the Nrf2 antioxidant pathway has been suggested as a mechanism in carp and trout, leading to strengthened endogenous antioxidant responses [70]. The present results support the view that *Lysinibacillus* supplementation can fortify antioxidant defenses (notably SOD), potentially conferring greater cellular protection. This antioxidant boost is beneficial for overall health and may contribute to the improved growth and disease resistance observed, since oxidative stress is known to impair growth and immune function in fish.

4.3. Intestinal Microbial Counts

Encapsulation of *Lysinibacillus* sp. PWR01 had a pronounced impact on the gut microbiota, as reflected by increased counts of total cultivable bacteria and LAB in the intestine. Total bacterial counts rose steadily with probiotic dose, which was nearly an order of magnitude higher at 10^6 CFU/g than in the control. In addition, LAB counts also increased significantly. These shifts suggest that the probiotic either directly contributed to these populations (as *Lysinibacillus* itself is a Gram-positive fermentative bacterium) or created gut conditions favorable to indigenous beneficial microbes. No changes were seen in intestinal yeast counts, indicating the effect was specific to the bacterial microbiota. The enrichment of LAB is particularly noteworthy, as lactic acid bacteria are often associated with improved gut health and pathogen resistance in fish [71]. A number of studies have likewise reported that probiotic supplements can modulate the gut microbial community toward a more favorable profile. Qiu, et al. [30] found that zebrafish fed *L. sphaericus* exhibited higher abundances of beneficial genera (e.g., *Cetobacterium*, *Lactobacillus*) and a significant reduction in the relative abundance of the pathogenic genus *Aeromonas*. In our challenge experiment, fish receiving *Lysinibacillus* had improved survival to *Aeromonas* infection, and it is tempting to link this to potential suppression of *Aeromonas* in the gut microbiome as seen in Qiu, et al. [30] work. The likely mechanisms for these microbiota changes include competitive exclusion and antimicrobial production. Probiotic *Lysinibacillus* are known to inhibit pathogens by secreting antagonistic compounds and by outcompeting harmful bacteria for nutrients and adhesion sites on the intestinal mucosa. A recent isolation study in marine fish showed a *Lysinibacillus* strain could strongly inhibit *Vibrio* pathogens and co-aggregate with pathogenic bacteria, thereby preventing their colonization [31]. In the present context, *Lysinibacillus* sp. PWR01 may have directly suppressed opportunistic gut pathogens, allowing total bacterial counts to rise and beneficial commensals (like LAB) to flourish.

4.4. Histological Characteristics

Histological observations demonstrated that dietary encapsulated *Lysinibacillus* influenced intestinal morphology while maintaining normal hepatic structure. It can be seen that in the anterior intestine, fish receiving higher encapsulation levels exhibited increased villus height and width, particularly in the 10^6 CFU/g treatment. These structural modifications

indicate an expansion of the absorptive surface area, which is widely recognized as a key determinant of nutrient uptake efficiency in fish [72–74]. Enlarged villi increase the contact interface between digested nutrients and the intestinal epithelium, thereby facilitating more efficient absorption and assimilation [72,75]. Similar morphological responses have been reported in several probiotic studies in aquaculture. For example, dietary supplementation with *Bacillus subtilis* in Nile tilapia significantly increased villus height and intestinal fold development, resulting in improved digestive efficiency and growth performance [59,76]. Likewise, probiotics based on *Lactobacillus* or *Bacillus* species have been shown to enhance intestinal villus architecture in carp and tilapia, which was associated with improved feed utilization and growth outcomes [59,76–78]. The positive correlations observed between encapsulation concentration and villus dimensions in the present study therefore align with the improved growth performance recorded during the feeding trial. Interestingly, muscular layer thickness did not differ significantly among treatments. This indicates that the probiotic primarily affected mucosal and absorptive structures rather than deeper muscular tissues. The mechanisms underlying these morphological improvements may involve several probiotic-mediated processes. Beneficial bacteria can stimulate epithelial cell proliferation, enhance mucosal integrity, and modulate gut-associated immune responses [79–81]. In particular, spore-forming bacteria such as *Lysinibacillus* and *Bacillus* are known to produce extracellular enzymes and bioactive metabolites that promote digestive activity and intestinal health [32,82]. Additionally, probiotics may regulate gut microbiota composition, leading to increased populations of beneficial bacteria such as lactic acid bacteria, which in turn produce short-chain fatty acids and other metabolites that support intestinal epithelial development [83–85]. The increase in LAB counts observed in the present study is therefore consistent with these morphological findings and suggests that microbial modulation may have contributed to the enhanced intestinal structure.

In addition to intestinal effects, liver histology revealed well-preserved hepatic architecture across all treatments. Hepatocytes were arranged in typical radial cords surrounding the central vein, and sinusoidal spaces were clearly defined. No signs of hepatocellular degeneration, necrosis, inflammatory infiltration, or lipid accumulation were observed. These findings indicate that dietary encapsulated *Lysinibacillus* did not exert hepatotoxic effects even at the highest supplementation level. The preservation of liver integrity may also be linked to the enhanced antioxidant status observed in probiotic-fed fish. Increased SOD activity suggests improved protection against oxidative stress [86], which is known to contribute to hepatocellular damage in fish exposed to environmental or metabolic stressors. By strengthening antioxidant defenses, probiotics may help maintain cellular stability and prevent oxidative injury to hepatic tissues [86,87]. The stable MDA levels observed in the present study further support the absence of oxidative damage in hepatic cells. Another possible explanation for the preserved liver morphology is the indirect effect of improved intestinal health. A well-developed intestinal barrier reduces the translocation of harmful bacteria and endotoxins into the bloodstream, thereby lowering hepatic inflammatory burden. Probiotics that stabilize gut microbiota and enhance mucosal integrity are therefore often associated with improved liver condition in fish [81,84]. In the present study, the increase in beneficial bacterial populations and the improved intestinal structure likely contributed to maintaining hepatic homeostasis.

4.5. Gene Expression Analysis

Dietary *Lysinibacillus* significantly influenced the expression of immune-related genes in the intestine, indicating an immunomodulatory role. Transcript levels of *TLR2* and *MHC II* increased progressively with higher probiotic doses. *TLR2* is a pattern-recognition receptor that detects Gram-positive bacterial ligands [88], so its upregulation likely reflects

the immune system's recognition of *Lysinibacillus* cell wall components and a resultant priming of innate immunity. Elevated *MHC II* suggests enhanced antigen presentation capacity, potentially improving the host's ability to activate specific immune responses (e.g., priming T cells against pathogens) [89–91]. In contrast, proinflammatory cytokine genes (*IL-1 β* , *TNF- α* , *IFN- β*) were not induced by a probiotic diet and *TNF- α* was slightly downregulated at the highest dose. The increase in pathogen-recognition and antigen presentation markers, without a surge in inflammatory cytokines, implies that *Lysinibacillus* sp. PWR01 triggered a measured, non-pathological immune stimulation. This immunomodulation is desirable as it suggests the probiotic can bolster immune readiness while avoiding chronic inflammation that could harm growth [14,92]. These findings mirror observations in other probiotic studies. Rwezawula, et al. [93] reported that tilapia fed *L. fusiformis* showed modulation of immune gene expression; for example, proinflammatory markers (*IL-1 β* , *IL-6*, *CXCL8*) were moderated and the complement gene *C3* was upregulated, indicative of enhanced mucosal immunity coupled with reduced systemic inflammation. Similarly, in our study the lack of *IL-1 β* /*TNF* surge and the improvement in survival post-infection point to an immune system that is both activated and balanced. It is noteworthy that not all probiotics behave identically in this regard. For example, a recent trial in common carp using a halophilic *Bacillaceae* probiotic (*Virgibacillus salarius*) found significant upregulation of *IL-1 β* and *TNF- α* in probiotic-fed fish, which the authors associated with an acute immune activation contributing to pathogen defense [94]. By contrast, the *Lysinibacillus* in our study appears to have achieved protective effects without eliciting a strong inflammatory cytokine response. This difference could be due to species-specific immune dynamics or varying properties of the probiotic strains (e.g., differences in cell wall components such as lipoteichoic acids that drive cytokine responses). Mechanistically, probiotics are thought to act as immune modulators by interacting with gut-associated lymphoid tissue as they can stimulate toll-like receptors on intestinal immune cells, leading to enhanced phagocytic activity, local antibody production, and release of regulatory cytokines [95–97]. Many spore-forming bacilli also produce metabolites like short-chain fatty acids, peptidoglycans that have systemic immunological effects, including anti-inflammatory properties [98]. In practical terms, the upregulation of *TLR2* and *MHC II* observed here may translate to faster recognition of invading bacteria and more robust activation of adaptive immunity during infection, thereby improving disease outcomes. Indeed, the positive correlations noted between these immune genes and growth performance metrics in our multivariate analysis suggest a linkage between immune competency and overall vigor of the fish.

4.6. Disease Resistance Against *A. hydrophila*

The ultimate test of the probiotic's efficacy was the challenge with the pathogen *A. hydrophila*. Fish that had been fed *Lysinibacillus*-supplemented diets showed markedly higher survival following infection compared to the positive control (infected but no probiotic). By day 14 post-challenge, the highest dose yielded the highest survival, and both 10^5 and 10^6 groups had significantly better survival than the control. These results provide strong evidence that encapsulated *Lysinibacillus* enhanced the fish's resistance to bacterial infection. This finding is in agreement with multiple recent studies documenting probiotic-mediated disease protection in aquaculture. Qiu, et al. [30] similarly observed that zebrafish fed *L. sphaericus* (particularly at 10^7 CFU/mL) had significantly improved survival when challenged with *A. hydrophila*, attributable to the probiotic's immune-boosting effects. In Nile tilapia, *Bacillus* and *Lysinibacillus* supplementation has conferred protection against various pathogens; for instance, probiotic-fed tilapia challenged with *Providencia* experienced higher survival and reduced infection signs relative to controls [93]. Mechanistically, the enhanced survival can be explained by a combination of the probiotic's direct antagonism

of the pathogen and the primed host immunity discussed above. *Lysinibacillus* sp. PWR01 may produce antimicrobial compounds or bacteriocins that inhibit *A. hydrophila* either in the gut or systemically. Additionally, an improved gut microbiota (with more LAB and overall bacteria) could outcompete or inhibit *A. hydrophila* colonization. The significant positive correlations we observed between the abundance of gut microbiota and immune markers (*TLR2*, *MHC II*) suggest that a probiotic-enriched microbiome was associated with an immune status that is better prepared to fight infections. This is supported by other work showing that probiotics can suppress pathogen virulence factors and reduce pathogen load in hosts [28,99,100]. Furthermore, immune-enhancing effects such as higher lysozyme activity, complement activity, and Ig levels have been documented in probiotic-treated fish, contributing to more effective clearance of invading bacteria [101]. It is also worth noting that the survival curves in our study plateaued after day 6–7 in all probiotic groups, indicating the acute phase of infection was better managed in those fish, after which mortality ceased. In contrast, the positive control continued to suffer losses up to day 7 and ended at a much lower survival. This pattern is emblematic of an effective immunoprophylactic intervention, where the probiotic-treated hosts quickly control the infection. The outcome is highly relevant for aquaculture, as *Aeromonas* species cause septicemia and ulcers in many freshwater fish, and a 30% improvement in survival as seen with the high-dose *Lysinibacillus* could translate to significantly reduced economic losses. It also underscores that *Lysinibacillus* sp. PWR01 is a promising candidate for a biotherapeutic feed additive aimed at disease prevention.

4.7. Limitations and Future Directions

Beyond the applied outcomes, the study contributes novelty at both the strain-discovery and delivery-technology levels. *Lysinibacillus* sp. PWR01 was isolated from rubber latex nodules with evidence of chemically defended, enzyme and secondary metabolites. This rich microhabitat is increasingly recognized to harbor specialized endophytic communities [102]. Such latex-associated niches are plausibly selective for stress-tolerant, resilient bacteria, including endospore formers, because successful persistence requires coping with fluctuating osmotic pressure, oxidative stress, and antimicrobial plant compounds [103]. This ecological provenance therefore provides a biologically grounded rationale for the observed robustness of PWR01 during feed processing and gastrointestinal transit when combined with encapsulation. In parallel, recent aquaculture literature has begun to highlight *Lysinibacillus* spp. as emerging probiotics with measurable effects on host metabolism, intestinal function, and disease resistance [29–31], supporting the relevance of exploring non-traditional reservoirs for candidate strains.

Another innovation lies in positioning latex-derived *Lysinibacillus* as a complementary biocontrol option relative to conventional *Bacillus*-based probiotics. Although *Bacillus* probiotics remain effective and widely deployed, their antagonistic profiles can be strain-specific and sometimes targeted to a narrower set of pathogens. Recent studies demonstrate that individual *Bacillus* isolates may show broad-spectrum activity against *A. hydrophila* and other aquatic pathogens, but these effects are not universal across all commercial strains [104]. In this context, the present work provides academic value by demonstrating that an encapsulated *Lysinibacillus* isolate can confer meaningful protection against *A. hydrophila* in vivo while also improving performance traits under a practical feeding duration. Moreover, the results complement recent findings in zebrafish showing that *L. sphaericus* supplementation can modulate gut physiology and improve resistance to *A. hydrophila* [30], reinforcing the plausibility of *Lysinibacillus* as a disease-mitigating genus in aquatic hosts.

Despite these promising outcomes, several limitations should be acknowledged. First, the current study did not identify the fate of the encapsulated *Lysinibacillus* in the gut

like levels of probiotic recovery from intestine or whether it became a persistent colonizer. Future work could use molecular tracing or selective plating to confirm that *Lysinibacillus* sp. PWR01 successfully colonizes or at least transiently proliferates in the fish gut at effective levels. Second, while key immune transcripts were measured, we did not assess protein-level or functional immune parameters such as phagocytic activity, lysozyme, or specific antibody levels. Including those in future studies would provide a more comprehensive picture of immunological changes. Third, the antioxidant findings (discrepancy between SOD and ABTS results) warrant deeper investigation into oxidative stress pathways; additional markers like catalase, glutathione, or expression of antioxidant genes could clarify how the probiotic influences redox homeostasis. Another limitation is that our trial was conducted in controlled laboratory conditions; performance and health benefits might differ in commercial pond or cage environments where fish are exposed to a more complex array of stressors and pathogens. Field trials will be important to validate the consistency of these benefits in real-world farming scenarios. We also note that the optimal dose observed here was in the 10^5 – 10^6 CFU/g range for feed which remains to be tested whether even higher doses (10^7 – 10^9) yield further gains or simply plateau, as some evidence suggests a saturation effect beyond a certain probiotic level. Additionally, while *Lysinibacillus* sp. PWR01 was effective alone, combining it with other beneficial microbes or prebiotics (synbiotic approaches) could be explored to amplify effects. There is growing interest in multi-strain probiotic formulations that target different aspects of host physiology. For instance, a mix of *Lysinibacillus* for immunity and a yeast or *Lactobacillus* for gut health. Finally, long-term safety and any potential trade-offs should be assessed. Thus far, no adverse effects were observed, consistent with other studies where *Lysinibacillus* strains have been deemed safe at high doses in fish, but thorough evaluations will strengthen the case for regulatory approval and industry adoption.

5. Conclusions

Dietary supplementation with encapsulated *Lysinibacillus* sp. PWR01 improved growth performance, antioxidant capacity, intestinal microbiota composition, immune gene expression, and disease resistance in a dose-dependent manner. Among the tested concentrations, 10^5 – 10^6 CFU/g emerged as the optimal supplementation range, achieving maximal growth enhancement and feed efficiency while significantly strengthening host antioxidant defense and immune responsiveness. Histological examination indicated that fish in encapsulated groups improved intestinal morphology by increasing villus height and width while maintaining normal muscular layer thickness and liver architecture, supporting enhanced intestinal structural integrity without observable tissue damage. Multivariate analyses showed strong positive associations between growth indices, beneficial microbiota, and immune activation, and clearly discriminated encapsulation treatments. These enhancements significantly improved survival rates following an *A. hydrophila* challenge, particularly at concentrations of 10^5 – 10^6 CFU/g. This finding underscores the practical application of encapsulated *Lysinibacillus* sp. PWR01 as a functional feed additive to enhance disease resistance in aquaculture. Collectively, this study provides the first evidence that encapsulated *Lysinibacillus* sp. PWR01 can serve as an effective probiotic in Nile tilapia, offering a practical strategy for optimizing probiotic dosage and delivery technology in aquaculture systems. The findings contribute mechanistic insight into microbiota-immune-growth interactions and support the application of encapsulated spore-forming probiotics as sustainable alternatives to antibiotic use in intensive fish farming.

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curation, L.T.P.K. and N.V.L.; writing—original draft preparation, L.T.P.K., P.S., K.P., M.S.-a., P.P., N.D.-H. and N.V.L.; writing—review and editing, L.T.P.K., P.S. and N.V.L.; supervision, P.P. and N.V.L.; project administration, N.V.L.; funding acquisition, N.V.L. All authors have read and agreed to the published version of the manuscript.

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Article

Dietary Probiotic *Bacillus subtilis* AAHM-BS2360 and Its Postbiotic Metabolites Enhance Growth, Immunity, and Resistance to Edwardsiellosis in *Pangasianodon hypophthalmus*

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Abstract: Edwardsiellosis, caused by *Edwardsiella tarda*, poses a significant threat to the aquaculture industry, particularly in pangasius farming. This study investigates the effects of probiotic *Bacillus subtilis* AAHM-BS2360 and its postbiotic metabolites on growth performance, immune responses, antioxidative activity, and disease resistance against *E. tarda* infection. A total of 240 healthy pangasius (37.0 ± 4.9 g) were divided into four treatment groups with four replicate tanks each, as follows: (1) the Control group, which received feed top-dressed with 100 mL of 0.85% NaCl/kg diet; (2) the Probiotic group, which received feed supplemented with 100 mL of *B. subtilis* AAHM-BS2360 cells at the concentration of 1×10^{12} CFU/kg diet; (3) the Postbiotic group, which received feed supplemented with *B. subtilis* AAHM-BS2360 cell-free supernatant 100 mL/kg diet; and (4) the Pro + Post group, which received a combination of *B. subtilis* AAHM-BS2360 cells and cell-free supernatant. After 30 days of feeding treatment, biochemical serum analysis revealed a significant increase in the AST/ALT ratio in the Postbiotic group. The Probiotic and Postbiotic treatments increased lysozyme activity in mucus, indicating an innate immune response to pathogens. The Pro + Post group exhibited the highest levels of catalase (CAT) in serum and upregulated antioxidant-related genes. All treatment groups receiving *B. subtilis* AAHM-BS2360, metabolites, and their combinations showed significant upregulation of immune-related genes, like *lygl1*, *tgfb*, *b2ml*, and *tnf*. The expression of proinflammatory genes (*lita*, *ifngr1l*, *c3*, *il13*, and *il1b*) increased, with the most pronounced effects observed in the Pro + Post group. The Probiotic group showed significant upregulation of the growth-related gene *igf1*. Meanwhile, the Pro + Post group showed significantly higher values in SGR and ADG parameters, with values of $3.29 \pm 0.98\%$ /day and 1.42 ± 0.52 g/day respectively ($p < 0.05$).

Survival rates were significantly higher in the Pro + Post (87.5%), Postbiotic (84.37%), and Probiotic (81.25%) groups when challenged with *E. tarda*. Dietary supplementation with *B. subtilis* AAHM-BS2360, its metabolites, and their combination enhanced immune response, reduced oxidative stress, and improved growth performance in pangasius, highlighting its potential as a functional feed additive for sustainable aquaculture.

Keywords: pangasius; *Bacillus subtilis*; *Edwardsiella tarda*; probiotic; postbiotic; immune response; proinflammatory activity; antioxidative activity; growth performance

1. Introduction

Pangasianodon hypophthalmus, commonly known as striped catfish, iridescent shark catfish, and pangasius, holds a prominent position in the global whitefish market. It contributes over 3.3 million tons annually and a value of USD 4.03 billion in 2022. Pangasius production is expected to grow up to 66.7% by 2030 to meet global demand, an increasing trend starting from December 2023, where the level of consumer confidence index in the United States for seafood products increased by 2.3%, as well as China's growing market demand by establishing cooperation with several major catfish production countries [1–3]. The increasing demand for pangasius is likely to exert significant pressure on the aquafeed industry, with forecasts predicting an annual growth rate of 30% [4]. These developments present opportunities for increased pangasius production and market expansion, leading to the establishment of a thriving domestic and export pangasius farming industry. Pangasius farming not only enhances sustainability and competitiveness in national aquaculture but also serves as a crucial livelihood strategy for millions of people worldwide, and accessibility to low-income communities contributes to food security [5,6].

Pangasius production's rapid expansion has caused health challenges, especially infectious diseases like Edwardsiellosis caused by *Edwardsiella tarda*. *E. tarda* is a Gram-negative bacterium in the family Enterobacteriaceae that causes systemic infections like hemorrhagic septicaemia, white nodules on internal organs, and has been confirmed zoonotic to humans [7,8]. Mass mortality caused by *E. tarda* has been reported in several pangasius farms in Kerala and Andhra Pradesh, India [9,10]. The overuse of antibiotics beyond the recommended dosage has been identified as a contributing factor to the rising prevalence of antibiotic resistance in Pangasius, a phenomenon observed in Bangladesh and Indonesia [11,12].

An environmentally friendly approach is needed to enhance pangasius' immune response against Edwardsiellosis. Functional feed additives like probiotics, prebiotics, postbiotics, and synbiotics maintain a healthy gut microbiome, producing antimicrobial compounds, stimulating growth, and strengthening disease resistance in aquatic organisms [13–15]. Recent studies have underscored the potential of *Bacillus* spp. in aquaculture by showcasing its ability to develop the immune system (nonspecific and specific immunity) and induce different types of cytokines, namely *tumor necrosis factor (tnf)*, *interleukin (il6, il1b, il8, il10)*, and *myd88* [16]. In addition to the utilization of viable microorganisms belonging to *Bacillus* spp., the metabolite components of these microorganisms have been shown to enhance gastrointestinal health, reduce inflammation, and act as antibacterial and antifungal agents. The composition of bioactive metabolites termed postbiotics encompasses cell-free supernatant (CFS), cell-free spent media (CFSM), short-chain fatty acids (SCFAs), vitamins, various amino acids, enzymes, exopolysaccharides, and bacterial lysates [17,18].

Bacillus subtilis AAHM-BS2360 isolates, derived from the intestinal tract of healthy pangasius, have been found to contain bioactive components, including viable microorganisms (probiotics) as well as inert substrates or cell-free supernatants (CFSs or postbiotics). These components provide various advantages, serving as natural enhancers for the health and production of pangasius. Despite promising findings on probiotics and postbiotics of *Bacillus* spp. in aquaculture, a comprehensive understanding of their impacts on pangasius growth, antioxidative responses, proinflammatory responses, and immunity against *E. tarda* infections is lacking. This study investigates the impact of administering probiotic *Bacillus subtilis* AAHM-BS2360 and its bioactive components on pangasius growth and immune defenses. It specifically examines bactericidal and lysozyme activity and evaluates fish health through serum biochemical analysis. Furthermore, the study analyzes growth performance and explores the expression of proinflammatory, stress antioxidant, and immune-related genes of treated fish. These novelties provide key insights into current advancements in aquaculture additives, helping to foster a modern and thriving aquaculture industry.

2. Materials and Methods

2.1. Ethical Statement

The aquatic animal experiments were conducted following the Ethical Principles and Guidelines for the Use of Animals outlined by the National Research Council of Thailand, which governs the care and use of animals for research purposes. This protocol received approval from the Animal Ethics Committee at Kasetsart University in Thailand (Approval ID: ACKU67-FIS-015) on 20 May 2024.

2.2. Pangasius Husbandry

A total of 1000 pangasius fingerlings, with an average weight of 3.0 ± 0.5 g, were purchased from a local hatchery in Nakhon Pathom, Thailand, and subsequently transported to 3000 L freshwater nursery tank at the Center of Excellence in Aquatic Animal Health Management (CE AAHM), Faculty of Fisheries, Kasetsart University, Bangkok, Thailand. During the nursery stage, pangasius fingerlings were fed Higade 9006T (Charoen Pokphand Foods PCL, Bangkok, Thailand) commercial floating pellets three times daily with a feeding rate of 8.0% of body weight. The pellets contained 42.0% crude protein, 6.0% fat, 3.0% fiber, and 10.0% moisture. After a three-week nursery stage, the pangasius was transitioned to CP 9920D feed (Charoen Pokphand Foods PCL, Thailand) thrice daily at 5% of their body weight. The fish feed has a nutritional composition of 25% crude protein, 3% fat, 8% fiber, and 12% moisture.

A total of 240 pangasius from the nursery tank, with an average weight of 37.0 ± 4.9 g, were equally distributed among sixteen experimental tanks of 200 L and maintained with full aeration. The water quality was closely monitored and controlled weekly to ensure its optimal level for fish, with pH value within the range of 7.56–7.66, dissolved oxygen levels within the range of 3–5 ppm, temperature within the range of 28.69–28.97 °C, alkalinity values within the range of 111.4–113.3 mg/L as CaCO₃, and nitrite levels within the range of 0.09–0.12 ppm. The initial biomass and average weight per tank were recorded before the experiment.

2.3. Bacterial Cultivation and Preparation of Experimental Diets

The *Bacillus subtilis* AAHM-BS2360 was obtained and initially identified by the Centre of Excellence in Aquatic Animal Health Management (CE AAHM), Faculty of Fisheries, Kasetsart University, Thailand. Initially isolated from the intestinal tract of a healthy pangasius from an earthen pond, it demonstrates significant in vitro antagonistic activity against *E. tarda*.

B. subtilis AAHM-BS2360 was routinely cultivated in tryptic soy broth (TSB, Difco™, Baltimore, MD, USA) at pH 7.4 and incubated at 37 °C for 18–24 h. Bacterial density was standardized to 1×10^{12} CFU/mL using a UV-Vis spectrophotometer (600 nm, Thermo Fisher Scientific, Waltham, MA, USA), reaching an absorbance value of 1.4 for the combination of Probiotics and Postbiotics (Pro + Post) group. The culture was centrifuged at $3500 \times g$ for 10 min to obtain two fractions: the cell-free supernatant or postbiotic metabolite (Postbiotic group) and the pellet (Probiotic group). The supernatant was directly used for diet supplementation, while the pellet was washed, resuspended in 0.85% NaCl with the same initial concentration of 1×10^{12} CFU/mL, and subsequently used to supplement the diets of the Probiotic treatment group.

The experimental diet was prepared daily by enriching the commercial feed (CP 9920D) following a completely randomized design with four treatment groups, each consisting of four replicates containing 15 fish per replicate (60 fish/group). The groups were as follows: (1) the Control group, fed with regular feed top-dressed with 100 mL of 0.85% NaCl/kg diet; (2) the Probiotic group, which received feed supplemented with 100 mL of *B. subtilis* AAHM-BS2360 cells at the concentration of 1×10^{12} CFU/kg diet; (3) the Postbiotic group, which received feed supplemented with *B. subtilis* AAHM-BS2360 cell-free supernatant 100 mL/kg diet; and (4) the Pro + Post group, which received feed supplemented with a combination of *B. subtilis* AAHM-BS2360 cells and cell-free supernatant. The feeding experiment was conducted for 30 days, with a feeding rate of 4% body weight.

2.4. Growth Performance

The fish growth rates were determined through the calculation of the average daily growth rate (ADG) and the specific growth rate (SGR). The fish samples were weighed on a two-digit electronic scale. Daily records of feed consumption were diligently maintained, allowing for the computation of the feed conversion ratio (FCR). The detailed formulas for these calculations were provided by Bunnoy et al. [19].

2.5. Sample Collection

Following thirty days of dietary treatment, samples of tissue, including gills, skin mucus, whole blood, intestine, liver, and head kidney, were collected from eight fish/group (two fish/tank). These tissues were preserved in TriZOL™ reagent (Thermo Fisher Scientific, Waltham, MA, USA) for gene expression assays and stored at −80 °C. Additionally, samples of tissues were placed in 1.5 mL tubes for immune parameter and antioxidant analyses, and stored at −20 °C. The whole blood (without anticoagulant) was collected in 1.5 mL tubes and allowed to clot at ambient temperature. After clotting, serum was separated by centrifugation at $3500 \times g$ for 15 min, then stored at −80 °C for further analysis. Eight fish per group were also sampled 24 h after the challenge test (according to Section 2.11), with head kidney, intestine, and whole blood collected for gene expression analysis.

2.6. Parameter of Serum Biochemical

The instrument utilized for serum biochemical assay was the Pushkang veterinary coagulation and chemistry combo analyzer, model MSC100V (Zhejiang PushKang Biotechnology Co., Ltd., Shaoxing, China). The reagent panel employed was the veterinary general chemistry 23 panel (CAT. no VE60010). The serum samples were immediately analyzed for the measurement of 23 biochemical parameters: albumin (ALB), total protein (TP), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), direct bilirubin (DBIL), total bilirubin (TBIL), globulin (GLB), indirect bilirubin (IBIL), urea, calcium (Ca), creatinine (Crea), cholesterol, glucose (GLU), amylase (AMY), total bile acids (TBA), creatine kinase (CK), total carbon dioxide

(tCO₂), inorganic phosphorus (P), albumin/globulin ratio (ALB/GLB), urea/creatinine ratio (Urea/Crea), and AST/ALT ratio (AST/ALT).

2.7. Humoral Innate Immune Responses Assays

2.7.1. Bactericidal Activity

The bactericidal activity assay was performed according to the previously published report [20]. A virulent freshwater fish pathogen *E. tarda* was routinely cultured in TSB medium using the conditions described in Section 2.3. This pathogen was utilized to evaluate the bactericidal activity of the mucus and serum samples. The concentration of *E. tarda* in the bacterial suspension was adjusted to 1×10^5 CFU/mL in sterile phosphate-buffered saline (PBS), pH 7.4, by measuring the absorbance at 600 nm. Subsequently, 40 µL of each sample was gently mixed with 10 µL of 1×10^5 CFU/mL *E. tarda* at room temperature for 90 min, and the survival of *E. tarda* was determined by counting the number of colonies on trypticase soy agar (TSA) (Merck KGaA, Darmstadt, Germany) plates after 24 h. After the incubation period, the bacterial colonies were counted as presented on the TSA plates. The bactericidal activity was determined using the following formula: $BA (\%) = [(T_0 - T_{24})/T_0] \times 100$, where T_0 is the total initial bacterial count and T_{24} is the number of bacteria remaining on the plate after 24 h.

2.7.2. Lysozyme Activity

The lysozyme activity was quantified using the approach adapted from the methodology originally proposed by Parry et al. [21]. A solution *Micrococcus lysodeikticus* (Sigma-Aldrich, Darmstadt, Germany) was prepared at a concentration of 0.2 mg/mL in phosphate-buffered saline (PBS) with pH 6.2. Subsequently, 10 µL of fish serum and mucus were added to a flat-bottomed microtiter plate, followed by the addition of 250 µL of *M. lysodeikticus* solution to each well. The decrease in absorbance at 540 nm was measured using a microplate spectrophotometer reader (iMark™ Microplate Absorbance Reader, BIO-RAD, Hercules, CA, USA), followed by incubation of the plate at room temperature for a period of 0 and 5 min. The units of lysozyme activity were calculated, where one unit is defined as the quantity of enzyme required to reduce the absorbance by 0.001 per minute, as described by Sukhsangchan et al. [22].

2.8. Determination of Oxidative Stress and Antioxidant Status of Target Organs

The tissues selected for antioxidative analysis included the serum, liver, intestine, skin, and gills. Fifty milligrams of each organ was accurately weighed and combined with 1 mL of PBS (0.1 M, pH 7.4). The mixture was then homogenized and centrifuged at $1100 \times g$ for 10 min at 4 °C. The supernatant was carefully transferred to a microcentrifuge tube and stored at −20 °C to ensure optimal conditions for subsequent analysis.

2.8.1. Measurement of Catalase (CAT) Activity

CAT activity was examined according to the method adapted from Maehly [23]. One hundred microliters of the sample supernatant was mixed with 2.5 mL of phosphate buffer (50 mM, pH 5.0) and 0.4 mL of hydrogen peroxide (5.9 mM). The change in absorbance at 240 nm was recorded using a spectrophotometer every 30 s for 2 min. CAT activity in the sample was calculated by comparing it to the activity of the CAT standard. The CAT activity in the sample was expressed as units per minute per milligram of protein.

2.8.2. Measurement of Glutathione Peroxidase (GPx) Activity

The measurement of GPx enzyme activity was conducted following the method adapted from Mohandas et al. [24]. A reaction mixture was prepared by combining the following components: 100 µL of sample supernatant, 1.49 mL of 0.1 M phosphate buffer

(pH 7.4), 100 μL of 1 mM EDTA, 100 μL of 1 mM sodium azide, 50 μL of glutathione reductase (1 U/mL), 50 μL of 1 mM glutathione, 100 μL of 0.2 mM NADPH, and 10 μL of 0.25 mM H_2O_2 . GPx activity was measured by monitoring the oxidation of NADPH at a wavelength of 340 nm at 25 °C. Enzymatic activity was expressed as μM of NADPH oxidized per minute per milligram ($\text{min}^{-1}\text{mg}^{-1}$) of protein, with a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

2.8.3. Measurement of Glutathione Reductase (GR) Enzyme Activity

The activity of the GR enzyme was measured using the method modified by Sahreen et al. [25]. A reaction mixture was prepared by combining 100 μL of the sample supernatant with 100 μL of phosphate buffer (0.1 M, pH 7.6), 100 μL of oxidized glutathione (1 mM), and 100 μL of NADPH (0.1 mM). The mixture was incubated at 25 °C, and the absorbance was recorded at 340 nm. The GR enzyme activity was expressed as μM of NADPH oxidized $\text{min}^{-1}\text{mg}^{-1}$ of protein, calculated using a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

2.8.4. Measurement of Reduced Glutathione (GSH) Content

The glutathione (GSH) content was determined using the method adapted from Jollow et al. [26]. The reaction mixture consisted of 1 mL of 4% *w/v* sulfosalicylic acid and 1 mL of the sample supernatant. After mixing thoroughly, the mixture was incubated at 4 °C for 1 h. Following incubation, the mixture was centrifuged at $1100 \times g$ at 4 °C for 20 min, and the supernatant was collected. A 100 μL aliquot of the supernatant was then mixed with 2.9 mL of a reaction solution containing 100 mM phosphate buffer (pH 7.4) and 100 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). The absorbance of the resulting yellow chromogen was determined at 412 nm. A calibration curve for GSH was then generated, and the GSH content in the sample was expressed as nmol/mg of protein.

2.8.5. Measurement of Glutathione-S-Transferase (GST) Activity

GST activity was assessed using the method described by Habig et al. [27]. A reaction mixture was prepared by combining 300 μL of the sample supernatant with 1.475 mL of 0.1 M phosphate buffer (pH 6.5), 200 μL of reduced glutathione (1 mM), and 25 μL of 1-Chloro-2,4-dinitrobenzene (CDNB) (1 mM). The absorbance of the resulting mixture at 340 nm was measured. Enzyme activity was calculated as nmol of CDNB conjugate in units per minute per milligram of protein, with a molar extinction coefficient of $9.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

2.8.6. Measurement of Malondialdehyde (MDA) Content

The MDA content in the samples was assessed using the thiobarbituric acid reactive substances (TBARS) assay, as described in the previous studies [28,29]. A 100 μL aliquot of the sample supernatant was mixed with 450 μL normal saline solution (0.85% *w/v*), 1000 μL of trichloroacetic acid (TCA, 10% *w/v*), and 200 μL of thiobarbituric acid (TBA, 0.67% *w/v*). The reaction mixture was boiled for 30 min and cooled. Subsequently, 2 mL of distilled water was added, and the mixture was centrifuged at $1100 \times g$ for 10 min. The supernatant was collected, and the absorbance was measured using a spectrophotometer at 532 nm. The resulting value was used to calculate the MDA content by comparing it with a standard curve of tetramethoxypropane (TMP). The MDA amount in the sample is expressed as nmol/mg of protein.

2.8.7. Measurement of Superoxide Dismutase (SOD) Activity

SOD activity was measured using the modified method obtained from Takada et al. [29,30]. A 100 μL aliquot of the sample supernatant was mixed with 1 mL of a

solution containing 0.1 mM xanthine, 0.025 mM nitro blue tetrazolium salt, 0.1 mM disodium ethylenediaminetetraacetate dihydrate, 60 mM sodium carbonate buffer (pH 10.2), and xanthine oxidase. The change in absorbance at 560 nm was measured every 30 s for 2 min, and the calibration curve for the SOD standard was plotted. The SOD activity in the sample was expressed as units per minute per milligram of protein.

2.9. cDNA Preparation and Gene Expression Analysis Using Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA was extracted from head kidney, gills, intestine, whole blood, liver, and spleen samples according to TriZOLTM manual protocol (Thermo Fisher Scientific, Waltham, MA, USA). The total RNA was quantified using a NanoDropTM spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A total of 1 µg of total RNA was used as a template to synthesize first-strand complementary DNAs (cDNAs) using MaximeTM RT PreMix (iNtRON Biotechnology, Seongnam-si, Republic of Korea). The synthesized first-strand cDNA was stored at −20 °C for further analysis.

The quantitative real-time PCR (qRT-PCR) assay was performed using 2× RealMODTM Green W² 2× qPCR Mix-SYBR[®] (iNTRON Biotechnology, Republic of Korea) in an Azure CieloTM real-time PCR (Azure biosystems, Dublin, CA, USA). The qPCR cycling conditions consisted of an initial condition of one cycle of 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 90 s, and a final extension at 72 °C for 10 min. The housekeeping genes, including *actb1* and *rna18s*, were used to standardize mRNA and cDNA quantities, to ensure consistency and reliability of the results. The target genes were analyzed using qRT-PCR to assess non-specific immune-related genes, proinflammatory genes, antioxidant-related genes, and growth-related genes.

All primers were standardized to ensure amplification efficiency before the qRT-PCR analysis. The relative expression levels of genes in the fish tissues were calculated using the $2^{-\Delta\Delta CT}$ analysis according to the protocol of Livak and Schmittgen [31]. The primer sequences of all targeted genes are detailed in Table 1.

Table 1. Primers used in qRT-PCR analysis of gene expression.

Gene Group	Genes	Nucleotide Sequences (5' → 3')	Tm (°C)	Efficiency (%)	Accession Number
Housekeeping gene	<i>Actin, beta 1 (actb1)</i>	F-5'-GAGCGCAAGTACTCTGTATGGA-3' R-5'-CTGTGGTGGTTACAGTCCTGTT-3'	60	98.5	XM_026928832.3
	<i>18S rRNA (rna18s)</i>	F-5'-TGACTCAACACGGGAAACCTC-3' R-5'-CAGACAAATCGCTCCACCAAC-3'	60	102.4	XR_004577708
Stress oxidative and Antioxidant-related gene	<i>Copper-only SOD repeat protein (cusr)</i>	F-5'-GTCCATCTTACCCGGTGCC-3' R-5'-CGAGAGAAGACCCGGAACGC-3'	60	99	XM_034299545.1
	<i>Catalase (cat)</i>	F-5'-AGCAGGCGGAGAAGTACCCA-3' R-5'-GCTGCTCCACCTCAGCGAAA-3'	60	104.7	XM_026919141.2
	<i>Glutathione peroxidase 3 (gpx3)</i>	F-5'-GTCAGTGCAGGATGCAACAC-3' R-5'-TTGGAATTCCGCTCATTGAT-3'	60	106.3	XM_026947312.2
	<i>Heat shock protein 70 family, member 13 (hspa13)</i>	F-5'-CTCCTCCTAAACCCGAGTC-3' R-5'-CCACCAGCACGTTAAACACA-3'	60	100.4	XM_026934573.2
	<i>Nitric oxide synthase 1 (neuronal) (nos1)</i>	F-5'-ACACCACGGAGTGTGTTCTGT-3' R-5'-GGATGCATGGGACGTTGCTG-3'	60	99.6	XM_026931613.2
Immune-related gene	<i>Lysozyme g-like 1 (lygl1)</i>	F-5'-TTTTGGAGACGTCACAAAGATCG-3' R-5'-TGGTGATGATGTTCTTGACTGCT-3'	60	101.3	XM_026940542.3
	<i>Transforming growth factor, beta (tgfb)</i>	F-5'-GAACACTGTCTCTTGTCCTC-3' R-5'-TCGTATTTGGTGGTGAGGATGG-3'	60	93.4	XM_026909968.3
	<i>Tumor necrosis factor (tnf)</i>	F-5'-AGTGCAAAGTCAAAAAGCGAGG-3' R-5'-TGATACTTGGAGCCAGAGCATC-3'	60	100.2	XM_026921878.3
	<i>Beta-2-microglobulin, like (b2ml)</i>	F-5'-AGAGAACACACTGATCTGCCAC-3' R-5'-GCCACGCTCTTAGTCAGATGAA-3'	60	94.6	XM_026925487.3

Table 1. Cont.

Gene Group	Genes	Nucleotide Sequences (5' → 3')	T _m (°C)	Efficiency (%)	Accession Number
Proinflammatory-related gene	<i>Lipopolysaccharide-induced TNF factor (litaf)</i>	F-5'-TGCATTATTGGCTGTATGTATGGC-3' R-5'-GTGGATATGTGCTCAGTTCCTGTT-3'	60	95.1	XM_026930110.3
	<i>Interferon gamma receptor 1-like (ifngr1l)</i>	F-5'-TACATAACAGTGGGAAGCTCAACCA-3' R-5'-AAGTTTGTGTTGTTGGACGGAAAT-3'	60	90.2	XM_026939395.3
	<i>Complement C3 (c3)</i>	F-5'-GAATAGCTCCTGACTGTCCAC-3' R-5'-ATGTGTAGCCAGTCTGTTCTG-3'	60	98.3	XM_034300087.2
	<i>Interleukin 13 (il13)</i>	F-5'-GTTTATATCCGGCTTTCATGTGCA-3' R-5'-AGAAAAACAACCACGACCTTCATC-3'	60	102.3	XM_034311451.2
	<i>Interleukin 1, beta (il1b)</i>	F-5'-CTATTCTGCTGGCCATTACTCTGA-3' R-5'-ATGAGAGAAAGAGGTTGCTCTTCA-3'	60	100.8	XM_034312378.2
Growth-related gene	<i>Growth hormone 1 (gh1)</i>	F-5'-CCCAGCAAGAACCTCGGCAA-3' R-5'-GCGGAGCCAGAGAGTCGTTC-3'	60	94.1	GQ859589.1
	<i>Insulin-like growth factor 1 (igf1)</i>	F-5'-GCAACGGCACACAGACACGC-3' R-5'-CAGACGTTCCCTCACCATCCTCT-3'	60	96.2	XM_034313382.2

2.10. Disease Resistance and Survival Rate (SR) After *E. tarda* Challenges

At the end of the experiment, 40 fish were randomly selected from each group (10 fish per replicate) and transferred to 250 L fiberglass tanks with full aeration. These fish were then subjected to intraperitoneal (*i.p.*) injection with *E. tarda*. The cultivation of *E. tarda* was routinely carried out in TSB, and the concentration was adjusted using a spectrophotometer as described in Section 2.3.

Each fish was injected with a 100 µL solution containing *E. tarda* at a concentration of 1×10^8 CFU/mL (1×10^7 CFU/fish). The optimum concentrations and dosages were determined based on preliminary 14-day LD₅₀ tests conducted on the fish. At 24 h post-infection, eight surviving fish from each group were selected for sampling gene expression parameters as described in the previous section. Daily mortality rates were recorded for 14 days, and the survival rate (SR) was analyzed using the Kaplan–Meier method [32].

2.11. Statistical Data Analysis

The statistical analysis was performed using GraphPad Prism 10 version 10.4.1 for macOS (GraphPad Software, Boston, MA, USA), employing one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test after passing the Shapiro–Wilk test to assess the normality of data distribution. Survival curves were created using GraphPad Prism 10 to visualize the survival rate profile over time. Data are shown as means ± standard deviations (SD). Letter superscripts and asterisks (*) indicate statistical significance differences among groups, with a threshold of $p < 0.05$. Statistical significance was determined at p -values of * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

3. Results

3.1. Serum Biochemical Analysis

Most serum biochemical analysis results showed non-significant values for all treatments across all biochemical test parameters ($p > 0.05$), except for the AST/ALT ratio parameter, which exhibited significant values in the postbiotic *B. subtilis* AAHM-BS2360 treatment compared to the control ($p < 0.05$). The serum biochemical values are presented in Figure 1A–W.

The albumin parameters in all treatment groups exhibited mean values ranging from 0.633 to 4.5 g/dL, while the globulin levels demonstrated mean values from 29.17 to 78.33 g/L. The ALB/GLB ratio attained a mean value of 0.18. The mean concentrations of bilirubin in serum samples, comprising DBIL, IBIL, and TBIL, were found to be 0.475 mg/dL, 1.499 mg/dL, and 17.541 mg/dL, respectively. Furthermore, a similar pat-

tern was observed across all treatments for the parameters of urea, creatine, and inorganic phosphorus, with values of 5.415 mg/dL, 0.5 mg/dL, and 0.62 mg/dL, respectively. The urea/creatinine ratio obtained a mean value of 2.65.

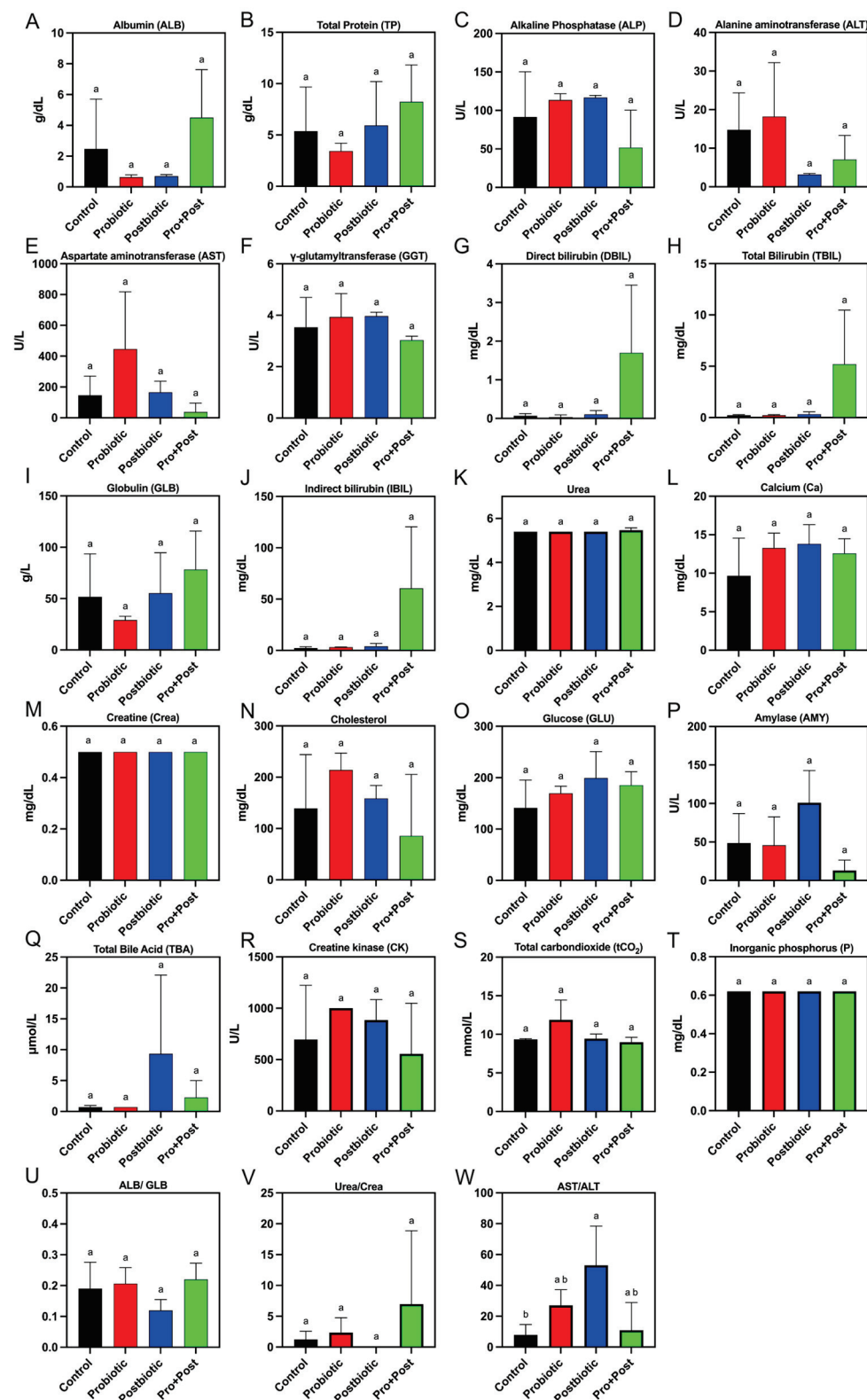


Figure 1. (A–W) Biochemistry of *P. hypophthalmus* following 30-day supplementation with probiotic, postbiotic, and their combination derived from *B. subtilis* AAHM-BS2360. Data are represented as mean $\pm$ SD ($n = 8$). Data in each graph with different letters denote statistically significant differences ($p < 0.05$).

The mean value of total protein parameters ranged from 3.43 g/dL to 8.23 g/dL, while the GGT content demonstrated a mean value of 3.61 U/L. The tCO₂ levels exhibited an average value of 9.9 mmol/L, and alkaline phosphatase levels exhibited a range of 51.67–116.8 U/L. The cholesterol and glucose levels in serum samples exhibited an average range of 85.70–214.0 mg/dL and 141.1–199.3 mg/dL, respectively. Calcium and amylase levels demonstrated mean values of 12.32 mg/dL and 52.01 U/L.

The range of mean values of total bile acid levels across all treatments was found to be between 0.7 and 9.3 µmol/L, while the mean value of creatine kinase was recorded as 783.9 U/L. Furthermore, the range of mean values of ALT and AST in all treatments was determined to be 3.167–18.2 U/L and 37.5–446.6 U/L, respectively. The AST/ALT ratio exhibited a range of mean values from 7.9 to 53.0, indicating that Postbiotic treatment resulted in a significant increase compared to the control treatment ($p < 0.05$).

3.2. Nonspecific Humoral Immune Responses

3.2.1. Bactericidal Activity

The investigation revealed no statistically significant differences in bactericidal activity parameters between the serum and mucus samples among groups ($p > 0.05$). The bactericidal activity values for each group are presented in Figure 2A,B.

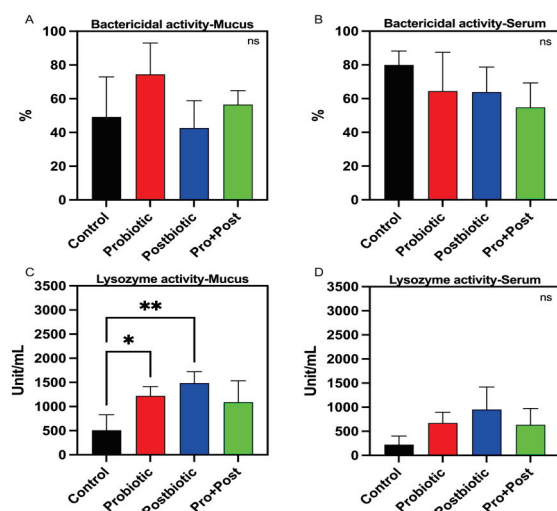


Figure 2. Bactericidal activity (%) response in mucus (A) and serum (B), and lysozyme activity (Unit/mL) in mucus (C) and serum (D) of *P. hypophthalmus* following 30-day supplementation with probiotic, postbiotic, and their combination derived from *B. subtilis* AAHM-BS2360. Data are represented as mean $\pm$ SD ($n = 8$). Asterisks indicate statistically significant differences (ns = not significant). * ($p < 0.05$), ** ($p < 0.01$).

3.2.2. Lysozyme Activity

Statistical analysis of lysozyme activity in mucus samples revealed a substantial increase in both Probiotic and Postbiotic treatments in comparison to the Control group ($p < 0.05$; $p < 0.01$), with values recorded at 1220 ± 194.1 units/mL and 1487 ± 234.7 units/mL, respectively, as depicted in Figure 2C. The serum samples exhibited comparable lysozyme activity parameters across all treatment groups ($p > 0.05$), as illustrated in Figure 2D.

3.3. Responses of Oxidative Stress and Antioxidant Activity

3.3.1. Liver

The GPx, GR, and SOD activities and the GSH level in the liver showed no significant differences among the treatments ($p > 0.05$). CAT activity was significantly

higher in the Probiotic group compared to the Control ($p < 0.05$), with a peak of 39.12 ± 6.45 units/min/mg protein. The Control group had the highest GST and MDA levels. GST was significantly lower in the Probiotic group compared to the Control, Postbiotic, and Pro + Post groups ($p < 0.01$; $p < 0.05$). All *B. subtilis* AAHM-BS2360-treated groups showed significant reduction in MDA levels compared to the Control group ($p < 0.05$). The graphical details are presented in Figure 3(Aa–Ag).

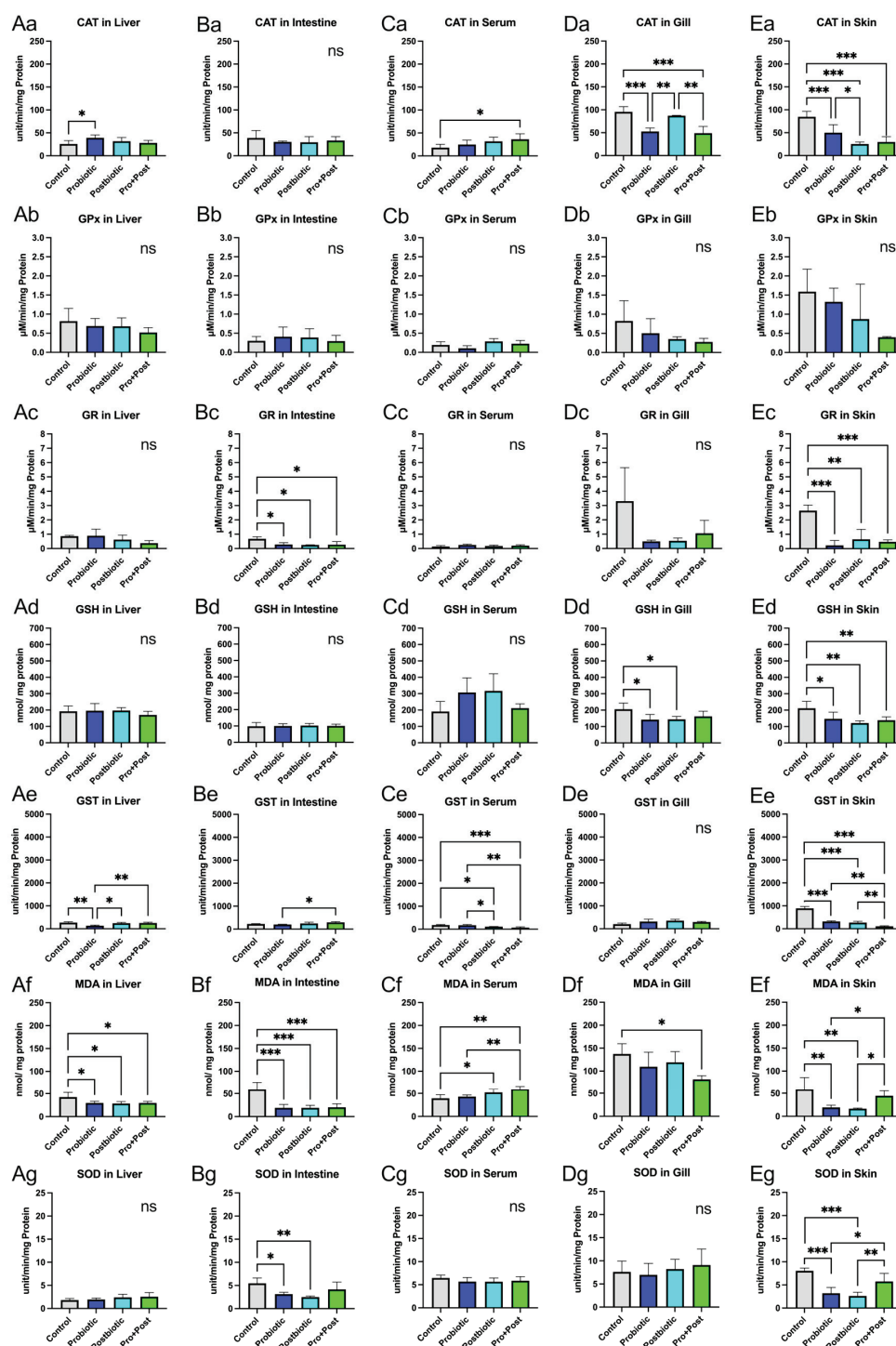


Figure 3. Levels of MDA and GSH, and activities of CAT, SOD, GPx, GR, and GST in liver (Aa–Ag), intestine (Ba–Bg), serum (Ca–Cg), gills (Da–Dg), and skin (Ea–Eg) of *P. hypophthalmus* following 30-day supplementation with probiotic, postbiotic, and their combination derived from *B. subtilis* AAHM-BS2360. Data are represented as mean $\pm$ SD ($n = 8$). Asterisks indicate statistically significant differences (ns = not significant). * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

3.3.2. Intestine

The MDA levels in the intestine were highest in the Control group (59.28 ± 16.37 nmol/mg protein) and significantly differed from all treatments ($p < 0.001$). The GR and SOD levels were also highest in the Control group, with significant differences from other groups ($p < 0.05$; $p < 0.01$). The Pro + Post group showed a significant increase in GST activity (294.1 ± 31.26 units/min/mg protein) compared to the Probiotic group ($p < 0.05$). No significant differences were found in CAT and GPx activities and GSH levels across the treatments ($p > 0.05$) (Figure 3(Ba–Bg)).

3.3.3. Serum

The GPx, GR, and SOD activities and GSH level showed no significant differences among groups ($p > 0.05$). The Pro + Post group showed the highest CAT activity and MDA level (36.3 ± 11.93 units/min/mg protein; 59.97 ± 6.68 nmol/mg protein), significantly higher than the Control ($p < 0.05$; $p < 0.01$) and Probiotic groups ($p < 0.01$). The Postbiotic group also showed higher MDA level than the Control group ($p < 0.05$). For GST, the Control group had significantly higher values than the Postbiotic and Pro + Post groups ($p < 0.05$; $p < 0.001$), while the Probiotic group showed the highest GST activity (173.5 ± 29.31 nM/min/mg protein), significantly exceeding Postbiotic and Pro + Post treatments ($p < 0.05$; $p < 0.01$) (Figure 3(Ca–Cg)).

3.3.4. Gills

The Control group showed the highest CAT activity (95.43 ± 11.31 units/min/mg protein), significantly exceeding those in the Probiotic and Pro + Post groups ($p < 0.001$). The Postbiotic group also had significantly higher CAT activity than the Probiotic and Pro + Post groups ($p < 0.01$). GSH levels in the Control group differed significantly from the Probiotic and Postbiotic groups ($p < 0.05$), while MDA was significantly higher than in the Pro + Post group ($p < 0.05$). No significant differences were found in GPx, GR, GST, and SOD across the treatments ($p > 0.05$) (Figure 3(Da–Dg)).

3.3.5. Skin

Oxidative status in the skin showed that the Control group had the highest values for most parameters, except GPx, which showed no significant differences across the treatments (0.39 – 1.58 μ M/min/mg protein; $p > 0.05$). CAT activities were significantly higher in the Control group compared to all groups ($p < 0.001$), with the Probiotic group also higher than the Postbiotic group ($p < 0.05$).

GR activity and GSH level followed a similar trend, with the Control group significantly higher than all other treatments ($p < 0.05$; $p < 0.01$; $p < 0.001$). For GST, the Control group (888.2 ± 86.23 nM/min/mg protein) was significantly higher than all *B. subtilis* AAHM-BS2360-treated groups ($p < 0.001$), and the Probiotic and Postbiotic groups differed from the Pro + Post group ($p < 0.01$). In MDA and SOD, the Control and Pro + Post group showed significantly higher values than the Probiotic and Postbiotic groups ($p < 0.05$; $p < 0.01$; $p < 0.001$) (Figure 3(Ea–Eg)).

3.4. Oxidative Stress and Antioxidant-Related Gene Expression

3.4.1. Following a 30-Day Supplementation with Probiotic, Postbiotic, and Their Combination Derived from *B. subtilis* AAHM-BS2360

The Pro + Post group showed significant upregulation of *cusr* gene expression in the gill and whole blood compared to the Control and Postbiotic group ($p < 0.05$; $p < 0.001$), as well as significantly higher in the spleen compared to the Control group ($p < 0.05$). The Probiotic group showed increased *cusr* expression in the gills and spleen compared to the

Control group ($p < 0.05$) (Figure 4(Aa,Ba,Ea)). No significant differences were observed in the head kidney, intestine, or liver across treatments ($p > 0.05$) (Figure 4(Ca,Da,Fa)).

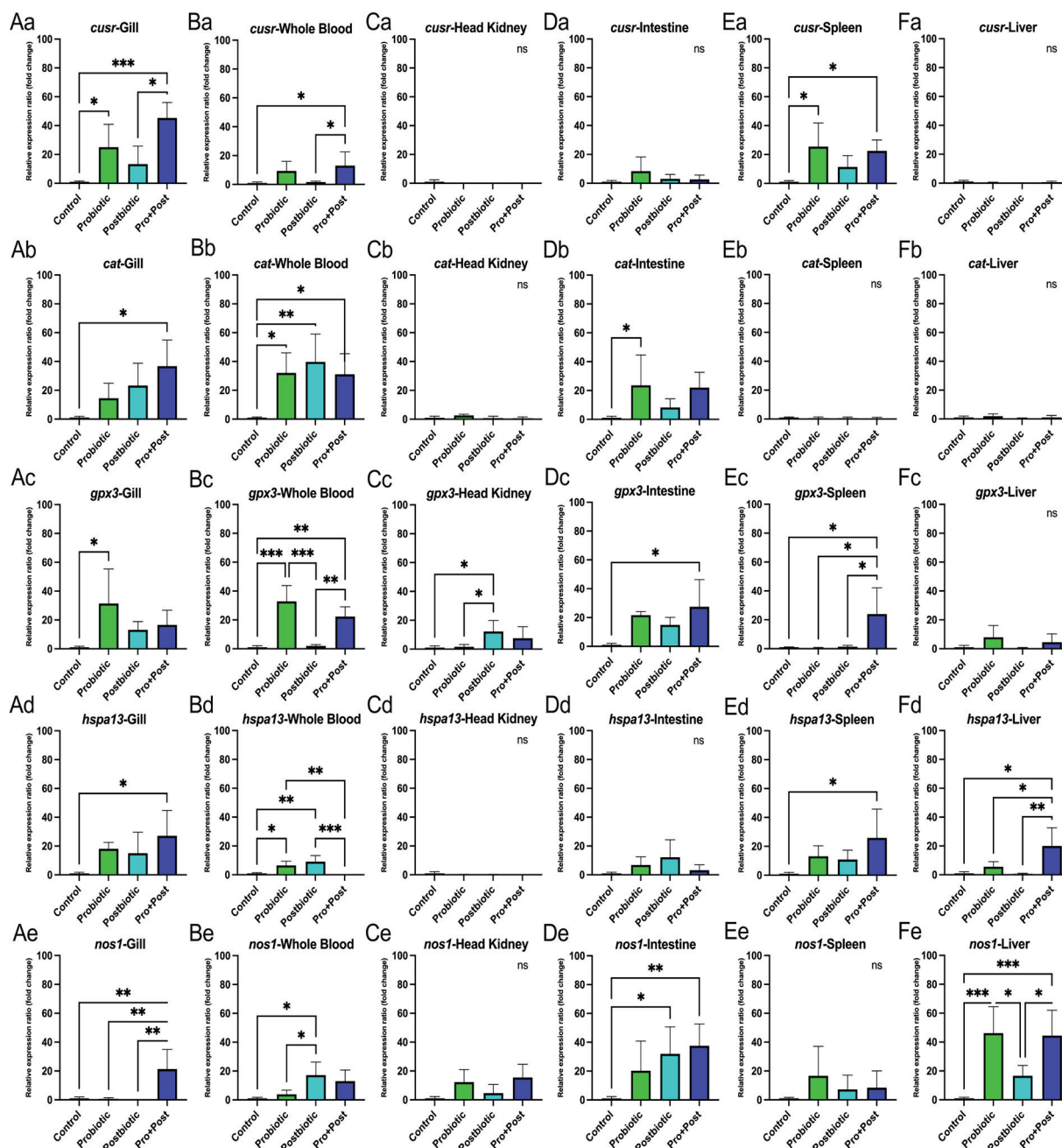


Figure 4. Relative stress oxidative and antioxidant-related gene expression (fold change) including *cur*, *cat*, *gpx3*, *hspa13*, and *nos1* in gills (Aa–Ae), whole blood (Ba–Be), head kidney (Ca–Ce), intestine (Da–De), spleen (Ea–Ee), and liver (Fa–Fe) of *P. hypophthalmus* following 30-day supplementation with probiotic, postbiotic, and their combination derived from *B. subtilis* AAHM-BS2360. Data are represented as mean $\pm$ SD ($n = 8$). Asterisks indicate statistically significant differences (ns = not significant). * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

In whole blood, all *B. subtilis* AAHM-BS2360-based treatments (Probiotic, Postbiotic, Pro + Post) showed higher *cat* expression compared to the Control group ($p < 0.05$; $p < 0.01$). In the gills, the Pro + Post group showed a significant increase of *cat* gene over the Control ($p < 0.05$), while in the intestine, the Probiotic group exhibited significant upregulation compared to the Control group ($p < 0.05$) (Figure 4(Ab,Bb,Db)). No significant differences were observed in the head kidney, spleen, or liver ($p > 0.05$) (Figure 4(Cb,Eb,Fb)).

Expression of the antioxidant-related *gpx3* gene showed no significant differences in the liver across treatments ($p > 0.05$) (Figure 4(Fc)). However, the Probiotic group showed significant *gpx3* upregulation in the gills ($p < 0.05$) compared to the Control group and in whole blood compared to the Control and Postbiotic groups ($p < 0.001$). The Pro + Post group also showed higher *gpx3* expression in whole blood compared to the Postbiotic and Control groups ($p < 0.01$). In the head kidney, the Postbiotic group had significantly higher *gpx3* expression than the Control and Probiotic groups ($p < 0.05$). The Pro + Post group showed elevated *gpx3* expression in the intestine and spleen compared to the Control group and other treatments, respectively ($p < 0.05$) (Figure 4(Ac,Bc,Cc,Dc,Ec)).

The Pro + Post group showed significant upregulation of the *hsa13* gene in the gill, spleen, and liver compared to the Control group ($p < 0.05$). In the liver, *hsa13* expression was also significantly higher in the Pro + Post group than in the Probiotic and Postbiotic groups ($p < 0.05$; $p < 0.01$). In whole blood, the Probiotic and Postbiotic groups had significantly higher *hsa13* expression than both the Control and Pro + Post groups ($p < 0.05$; $p < 0.001$) (Figure 4(Ad,Bd,Ed,Fd)). No significant differences were observed in the head kidney and intestine ($p > 0.05$) (Figure 4(Cd,Dd)).

As shown in Figure 4(Ce,Ee), *nos1* expression showed no significant differences in the head kidney and spleen across treatments ($p > 0.05$). In the gills, the Pro + Post group showed significantly higher *nos1* expression than all other groups ($p < 0.01$). In whole blood, the Postbiotic group had the highest *nos1* expression, significantly exceeding the Control and Probiotic groups ($p < 0.05$). In the intestine, both Pro + Post and Postbiotic groups showed elevated *nos1* expression compared to the Control group ($p < 0.01$; $p < 0.05$). In the liver, *nos1* was significantly upregulated in the Probiotic and Pro + Post groups compared to the Control and Postbiotic groups ($p < 0.05$; $p < 0.001$) (Figure 4(Ae,Be,De,Fe)).

3.4.2. Post-Challenge with *E. tarda*

In whole blood, *cusr* expression was significantly higher in all treated groups compared to the Control group ($p < 0.05$). Similarly, *gpx3* and *nos1* were significantly upregulated in the Postbiotic group compared to all other treatments ($p < 0.05$; $p < 0.01$; $p < 0.001$). For *hsa13*, the Postbiotic group showed significantly higher expression than the Pro + Post and Control groups, while the Probiotic group also showed increased *hsa13* expression compared to the Control group ($p < 0.05$; $p < 0.01$) (Figure 5A,G,J,M). Expression of the *cat* gene showed no significant differences among treatments ($p > 0.05$) (Figure 5D).

In the intestine, only the *hsa13* gene was significantly upregulated in the Postbiotic group compared to the Control and Probiotic groups ($p < 0.05$; $p < 0.01$), while the Pro + Post group also showed higher *hsa13* expression than Control ($p < 0.05$) (Figure 5K). No significant differences were observed in *cusr*, *cat*, *gpx3*, or *nos1* expression in the intestine across treatments ($p > 0.05$) (Figure 5B,E,H,N).

In the head kidney, *cusr* and *cat* gene expression showed no significant differences among treatments ($p > 0.05$) (Figure 5C,F). However, the *gpx3* gene was significantly upregulated in the Postbiotic group compared to the Probiotic and Control groups ($p < 0.05$). Both Postbiotic and Pro + Post groups showed increased *hsa13* expression compared to the Control group ($p < 0.01$; $p < 0.001$), with the Pro + Post group also differing significantly from the Probiotic group ($p < 0.01$). Notably, *nos1* expression was significantly elevated in the Pro + Post group compared to all other treatments ($p < 0.01$, $p < 0.001$) (Figure 5I,L,O).

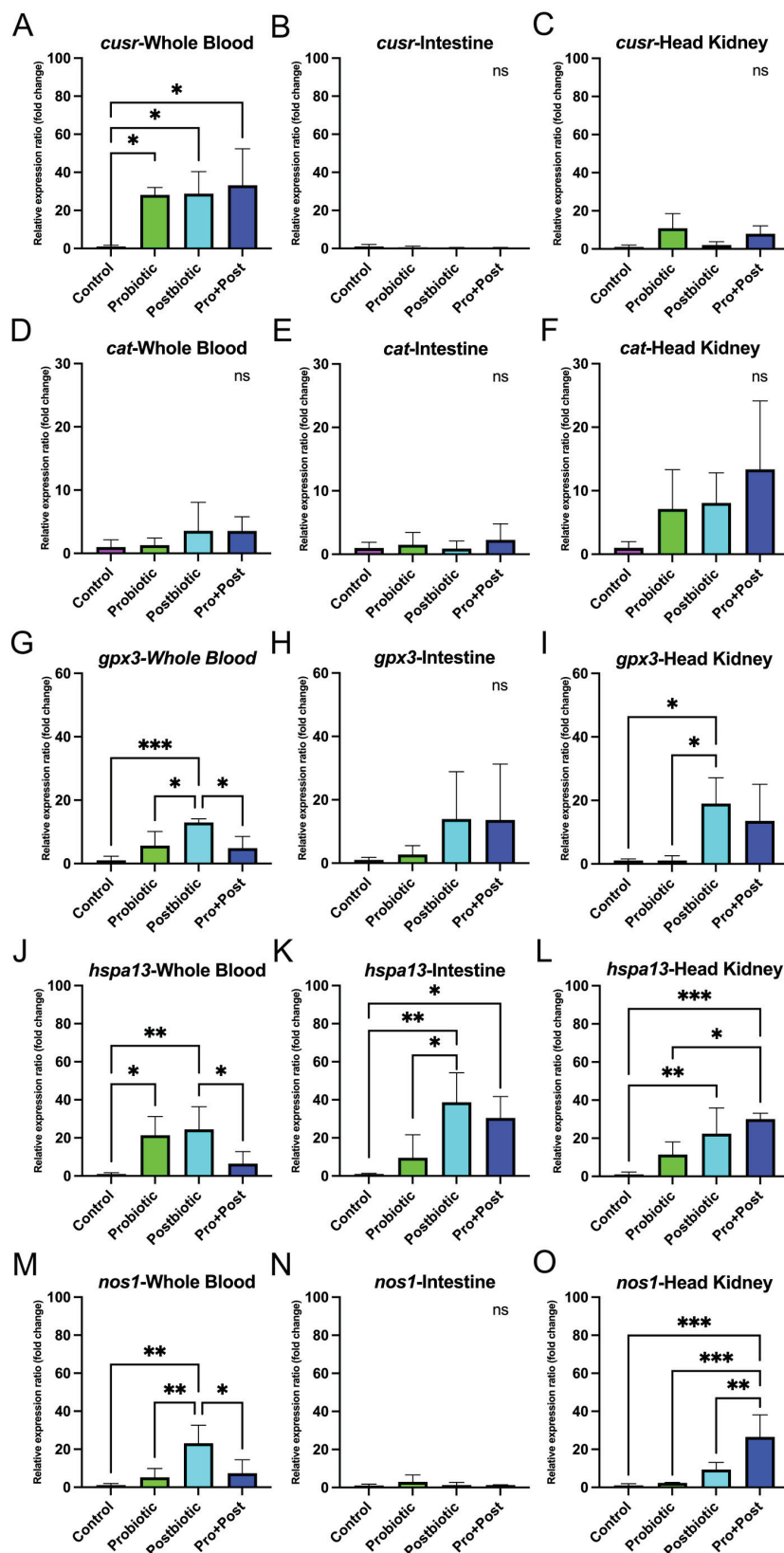


Figure 5. Relative antioxidant-related gene expression (fold change) including *cusr* (A–C), *cat* (D–F), *gpx3* (G–I), *hspa13* (J–L), and *nos1* (M–O) in whole blood, intestine, and head kidney of *P. hypophthalmus* following 30-day supplementation with probiotic, postbiotic, and their combination derived from *B. subtilis* AAHM-BS2360 after being challenged with *E. tarda*. Data are represented as mean $\pm$ SD ($n = 8$). Asterisks indicate statistically significant differences (ns = not significant). * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

3.5. Non-Specific Immune-Related Gene Expression

3.5.1. Following a 30-Day Supplementation with Probiotic, Postbiotic, and Their Combination Derived from *B. subtilis* AAHM-BS2360

All treatments containing *B. subtilis* AAHM-BS2360 significantly upregulated *lyg1* genes in the gills compared to the Control group ($p < 0.01$; $p < 0.001$). The Pro + Post group showed the strongest activation of *tgfb* and *tnf* compared to all treatment groups ($p < 0.05$; $p < 0.01$; $p < 0.001$). For *b2ml*, the Pro + Post group showed significantly higher expression than the Control and Probiotic group ($p < 0.01$). The Postbiotic group also upregulated *tnf* gene compared to the Control group ($p < 0.05$) (Figure 6A,G,M,S).

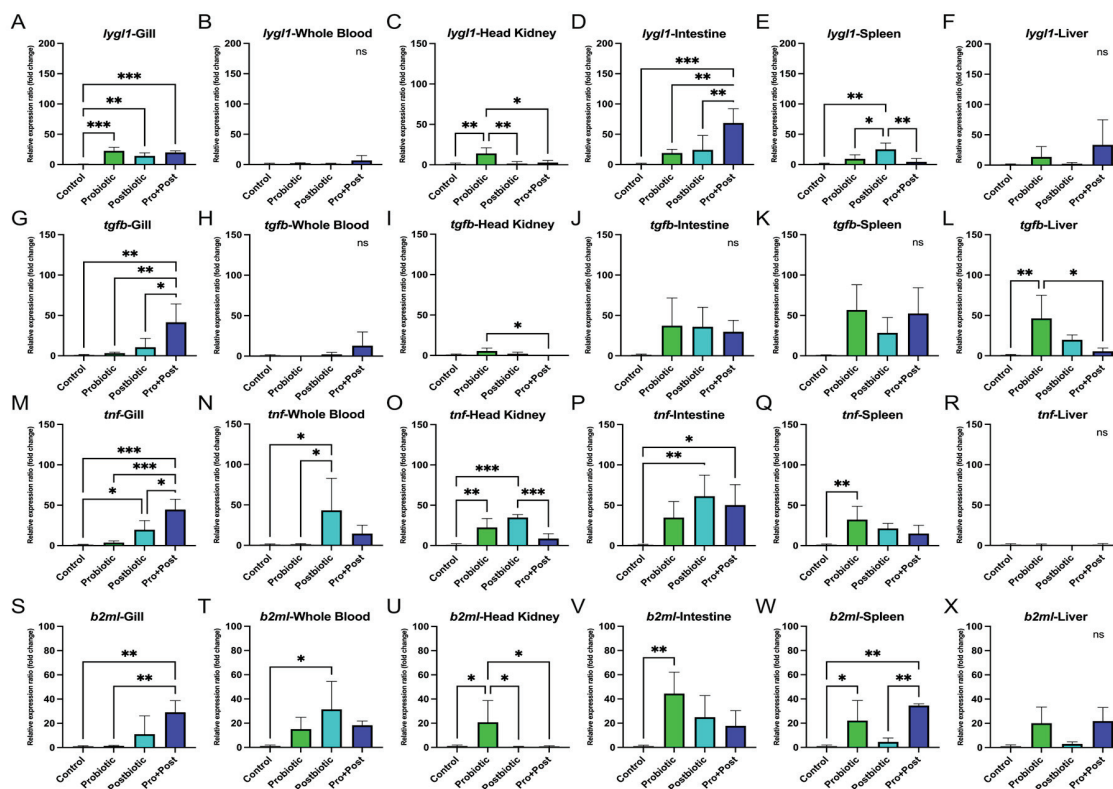


Figure 6. Relative immune-related gene expression (fold change) including *lyg1* (A–F), *tgfb* (G–L), *tnf* (M–R), and *b2ml* (S–X) in gill, whole blood, head kidney, intestine, spleen, and liver of *P. hypophthalmus* following 30-day supplementation with probiotic, postbiotic, and their combination derived from *B. subtilis* AAHM-BS2360. Data are represented as mean $\pm$ SD ($n = 8$). Asterisks indicate statistically significant differences (ns = not significant). * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

No significant differences in *lyg1* expression were observed in whole blood and liver across treatments ($p > 0.05$). In the intestine, the Pro + Post group showed the highest *lyg1* expression compared to all groups ($p < 0.01$; $p < 0.001$). In the head kidney, the Probiotic group had the highest *lyg1* expression ($p < 0.05$; $p < 0.01$), while in the spleen, the Postbiotic group showed significantly elevated *lyg1* gene expression compared to all other treatments ($p < 0.05$; $p < 0.01$) (Figure 6B–F).

For the *tgfb* gene, the Probiotic group showed significantly higher expression in the head kidney compared to the Pro + Post group ($p < 0.05$). A similar pattern was seen in the liver, where *tgfb* expression was higher in the Probiotic group than in the Control and Pro + Post groups ($p < 0.01$; $p < 0.05$). No significant differences were observed in whole blood, intestine, or spleen across treatments ($p > 0.05$) (Figure 6I–L).

In the liver, no significant differences were found in *tnf* and *b2ml* expression across treatments ($p > 0.05$) (Figure 6R,X). The Postbiotic group showed significant upregulation of *tnf* in whole blood, head kidney, and intestine compared to the Control group ($p < 0.05$;

$p < 0.01$; $p < 0.001$), and exceeded the Probiotic group in whole blood ($p < 0.05$) and the Pro + Post group in the head kidney ($p < 0.001$). The Probiotic and Pro + Post groups showed significant upregulation of *tnf* gene in head kidney, spleen, and intestine compared to each Control group, respectively ($p < 0.01$; $p < 0.05$) (Figure 6N–Q).

The *b2ml* gene expression was significantly higher in the Postbiotic group compared to the Control group in whole blood ($p < 0.05$). The Probiotic group showed upregulated *b2ml* in the head kidney and intestine compared to the Control group ($p < 0.05$; $p < 0.01$) and was also significantly higher than both Postbiotic and Pro + Post groups in the head kidney ($p < 0.05$). In the spleen, *b2ml* was significantly upregulated in the Probiotic group compared to the Control group ($p < 0.05$), while the Pro + Post group showed the highest *b2ml* expression, significantly exceeding the Control and Postbiotic groups ($p < 0.01$) (Figure 6T–W).

3.5.2. Post-Challenge with *E. tarda*

In whole blood, *lygl1* expression was significantly higher in the Postbiotic group compared to the Control and Probiotic groups ($p < 0.05$), while *tgfb*, *tnf*, and *b2ml* showed no significant differences among treatments (Figure 7A,D,G,J).

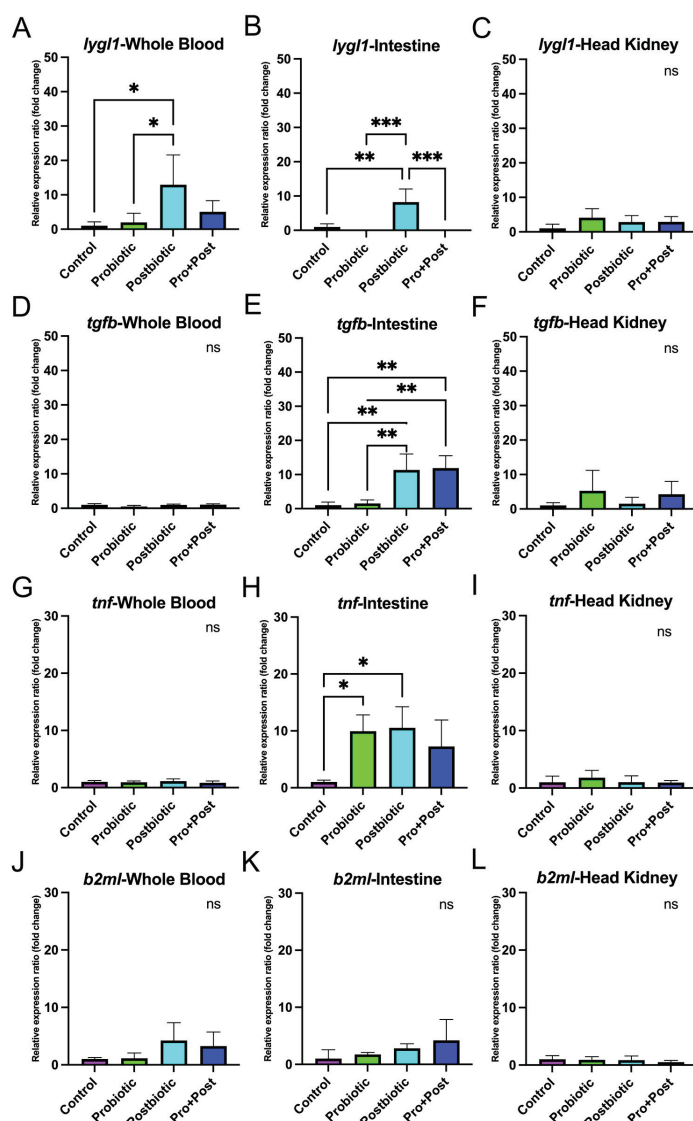


Figure 7. Relative immune-related gene expression (fold change) including *lygl1* (A–C), *tgfb* (D–F), *tnf* (G–I), and *b2ml* (J–L) in head kidney, intestine, and whole blood of *P. hypophthalmus* following 30-day

supplementation with probiotic, postbiotic, and their combination derived from *B. subtilis* AAHM-BS2360 after being challenged with *E. tarda*. Data are represented as mean $\pm$ SD ($n = 8$). Asterisks indicate statistically significant differences (ns = not significant). * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

In the intestine, *lyg11* expression was highest in the Postbiotic group, significantly exceeding all other treatments ($p < 0.01$; $p < 0.001$). The *tgfb* expression was significantly elevated in both the Postbiotic and Pro + Post groups compared to the Control and Probiotic groups ($p < 0.01$). The *tnf* gene expression was also significantly upregulated in the Probiotic and Postbiotic groups compared to the Control group ($p < 0.05$). However, *b2ml* expression showed no significant differences among treatments ($p > 0.05$) (Figure 7B,E,H,K). In the head kidney, no significant changes were observed in any immune gene expression across treatment groups ($p > 0.05$) (Figure 7C,F,I,L).

3.6. Proinflammatory-Related Gene Expression

3.6.1. Following a 30-Day Supplementation with Probiotic, Postbiotic, and Their Combination Derived from *B. subtilis* AAHM-BS2360

No significant differences in expression were observed for *litaf* in whole blood, *ifngr11* in the head kidney, *c3* in the head kidney, intestine, and liver, *il13* in the head kidney and intestine, or *il1b* in the intestine, spleen, and liver ($p > 0.05$) (Figure 8(Ba,Cb–Cd,Dc–De,Ee,Fc,Fe)).

The Pro + Post group showed significantly higher *litaf* expression than the Control group in the gill, intestine, spleen, and liver ($p < 0.05$; $p < 0.01$; $p < 0.001$). Compared to the Probiotic group, *litaf* was also significantly upregulated in the Pro + Post group in the gill, intestine, and spleen ($p < 0.05$; $p < 0.01$). In the spleen, *litaf* expression in the Pro + Post group was significantly higher than in the Postbiotic group ($p < 0.05$). In the head kidney, the Probiotic group dominated significantly *litaf* expression compared to other groups ($p < 0.05$; $p < 0.01$), while in the intestine, the Postbiotic group exhibited substantially higher *litaf* expression than the Control group ($p < 0.01$). Detailed graphs of *litaf* gene are presented in Figure 8(Aa,Ca,Da,Ea,Fa).

The Pro + Post group showed significantly elevated *ifngr11* expression in the gills compared to other groups ($p < 0.01$; $p < 0.001$) and in the intestine compared to the Control group ($p < 0.01$). The Probiotic group exhibited increased *ifngr11* expression in whole blood, spleen, and liver to the Control group ($p < 0.05$) and showed higher expression in the spleen than the Pro + Post group ($p < 0.05$). Additionally, the Postbiotic group displayed significantly upregulated *ifngr11* expression in whole blood compared to the Control ($p < 0.05$) (Figure 8(Ab,Bb,Db,Eb,Fb)).

In the gills, *c3* expression was significantly elevated in the Probiotic and Pro + Post groups compared to the Control group ($p < 0.01$; $p < 0.001$), with the Probiotic group also showing higher expression than the Postbiotic group ($p < 0.05$). In whole blood, the Postbiotic group exhibited the highest *c3* expression, surpassing to other treatments ($p < 0.001$), while the Pro + Post group also showed significantly higher expression than Control ($p < 0.001$). In the spleen, *c3* expression was significantly upregulated in the Pro + Post group compared to the Probiotic and Control groups ($p < 0.05$; $p < 0.001$) (Figure 8(Ac,Bc,Ec)).

The *il13* gene was significantly upregulated in the gills and liver of the Probiotic group compared to the Control group ($p < 0.05$). In the gills, the Pro + Post group also showed higher *il13* expression than the Control group ($p < 0.01$). In whole blood, the Postbiotic group had significantly elevated *il13* expression compared to the Control group ($p < 0.01$), while the Pro + Post group showed higher expression than both the Control and Postbiotic groups ($p < 0.05$) (Figure 8(Ad,Bd,Ed,Fd)).

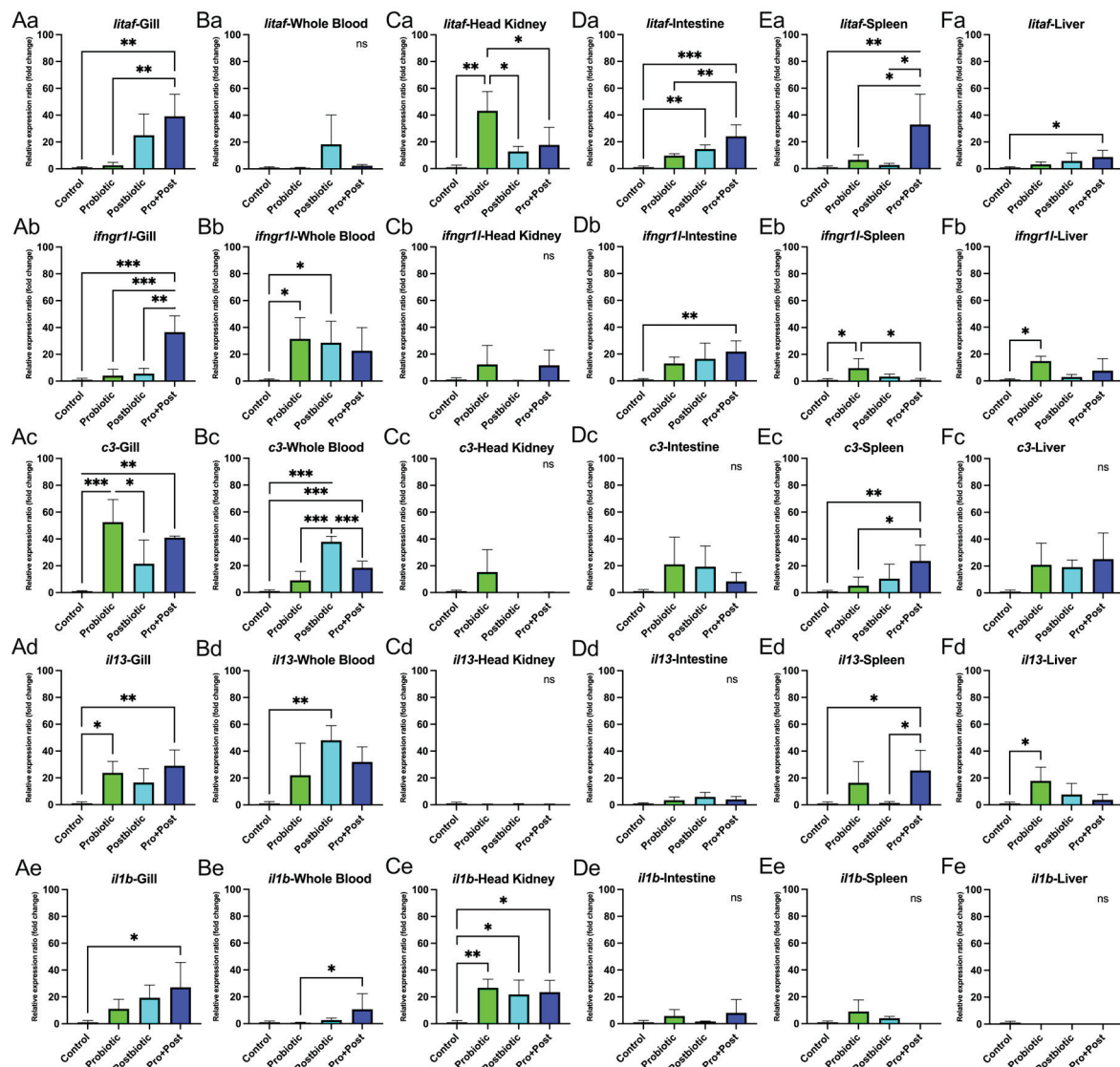


Figure 8. Relative proinflammatory-related gene expression (fold change), including *litaf*, *ifngr1l*, *c3*, *il13*, and *il1b* in gills (Aa–Ae), whole blood (Ba–Be), head kidney (Ca–Ce), intestine (Da–De), spleen (Ea–Ee), and liver (Fa–Fe) of *P. hypophthalmus* following 30-day supplementation with probiotic, postbiotic, and their combination derived from *B. subtilis* AAHM-BS2360. Data are represented as mean $\pm$ SD ($n = 8$). Asterisks indicate statistically significant differences (ns = not significant). * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

In the gills, *il1b* expression was significantly upregulated in the Pro + Post group compared to the Control group ($p < 0.05$). In whole blood, *il1b* expression in the Pro + Post group differed significantly from the Probiotic group ($p < 0.05$). In the head kidney, all *B. subtilis* AAHM-BS2360-based treatments (Probiotic, Postbiotic, and Pro + Post) showed significantly higher *il1b* expression compared to the Control group ($p < 0.05$; $p < 0.01$) (Figure 8(Ae,Be,Ce)).

3.6.2. Post-Challenge with *E. tarda*

In whole blood, the Postbiotic group showed significantly higher *litaf* and *il13* expression than the Control group ($p < 0.05$), and the *il13* gene was also significantly elevated compared to the Probiotic group ($p < 0.01$). The Pro + Post group significantly upregulated *ifngr1l* ($p < 0.01$; $p < 0.05$) and *c3* ($p < 0.01$) compared to other treatments. No significant

differences in *il1b* expression were observed across treatments in whole blood ($p > 0.05$) (Figure 9A,D,G,J,M).

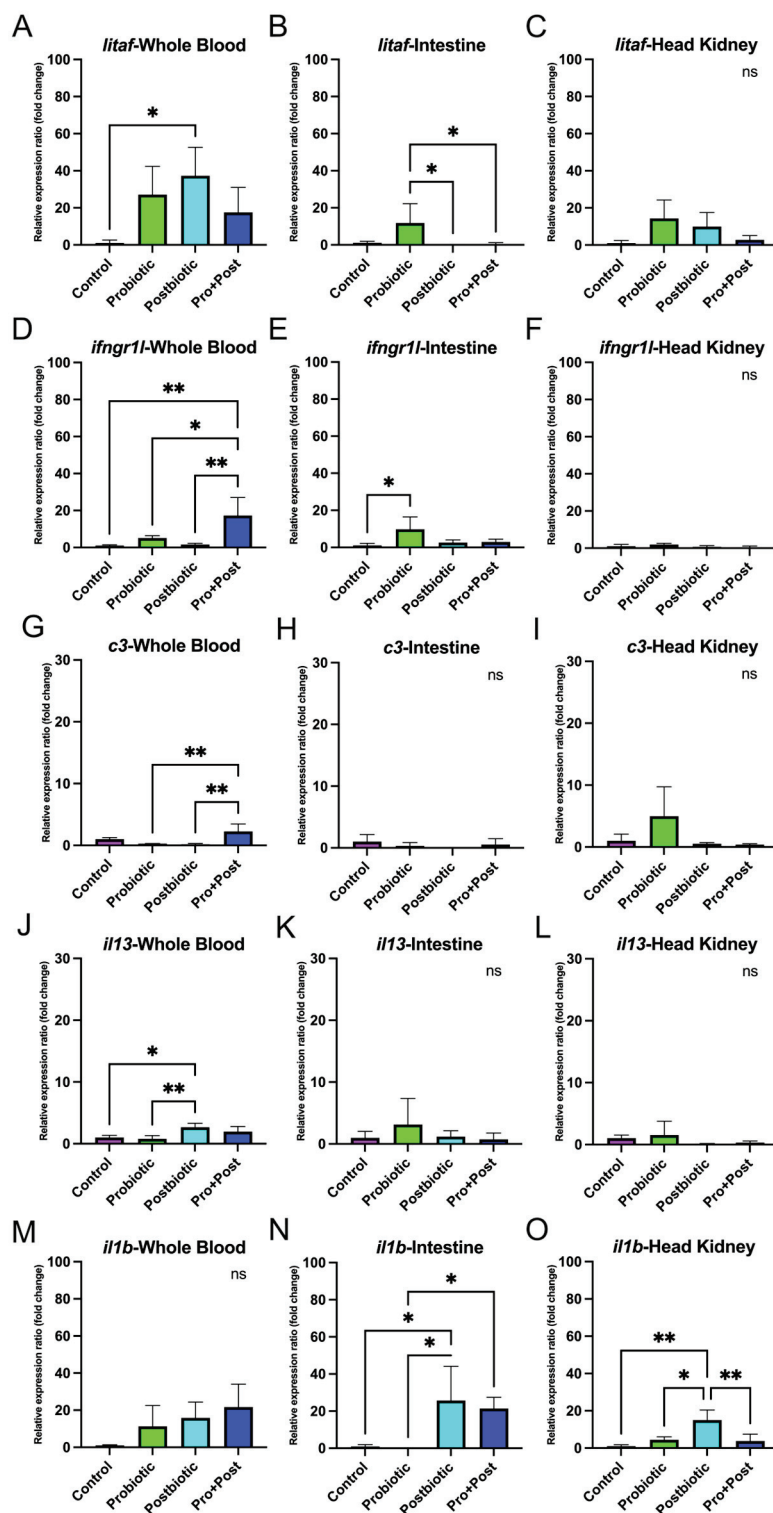


Figure 9. Relative proinflammatory-related gene expression (fold change) including *litaf* (A–C), *ifngr1* (D–F), *c3* (G–I), *il13* (J–L), and *il1b* (M–O) in head kidney, intestine and whole blood of *P. hypophthalmus* following 30-day supplementation with probiotic, postbiotic, and their combination derived from *B. subtilis* AAHM-BS2360 after being challenged with *E. tarda*. Data are represented as mean $\pm$ SD ($n = 8$). Asterisks indicate statistically significant differences (ns = not significant). * ($p < 0.05$), ** ($p < 0.01$).

In the intestine, the Probiotic group significantly increased *litaf* expression compared to the Postbiotic and Pro + Post groups ($p < 0.05$), and *ifngr1l* compared to the Control group ($p < 0.05$). The Postbiotic group significantly upregulated *il1b* compared to the Control and Probiotic groups, while the Pro + Post group also showed higher *il1b* expression than the Probiotic group ($p < 0.05$). No significant differences were found in *c3* and *il13* expression in the intestine ($p > 0.05$) (Figure 9B,E,H,K,N). The investigation revealed that the gene expression levels of *litaf*, *ifngr1l*, *c3*, and *il13* in all treatment groups were not significantly different ($p > 0.05$), which was a notable observation in the head kidney. However, a significant upregulation of the *il1b* gene was observed in the Postbiotic treatment group in comparison to all alternative treatments ($p < 0.05$; $p < 0.01$) (Figure 9C,F,I,L,O).

3.7. Growth-Related Gene Expression

The results of the qRT-PCR analysis revealed a significant increase in the activity of the *igf1* gene in the Probiotic treatment group in comparison to the Control and Postbiotic treatments ($p < 0.05$) (Figure 10A). However, the *gh1* gene expression did not show significant differences in all treatments ($p > 0.05$) (Figure 10B).

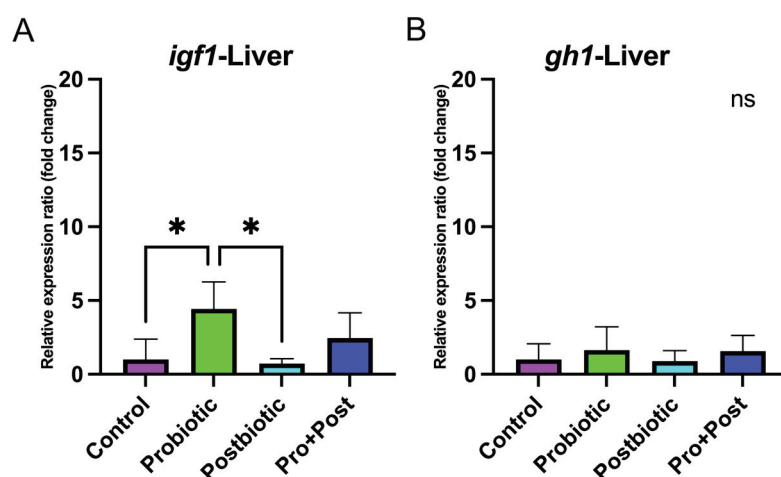


Figure 10. Relative growth-related gene expression (fold change) including *igf1* (A) and *gh1* (B) in the liver of *P. hypophthalmus* following 30-day supplementation with probiotic, postbiotic, and their combination derived from *B. subtilis* AAHM-BS2360. Data are represented as mean $\pm$ SD ($n = 8$). Asterisks indicate statistically significant differences (ns = not significant). * ($p < 0.05$).

3.8. Growth Performance Analysis

The Probiotic and Pro + Post treatment groups significantly increased the SGR value compared with the Control group ($p < 0.05$). The Pro + Post group had a significantly higher ADG value than the Control group ($p < 0.05$). Despite the absence of a statistically significant difference in FCR values across all treatments ($p > 0.05$), the group's treatment containing *B. subtilis* AAHM-BS2360 exhibited superior performance in terms of growth and feed efficiency, as evidenced by the average values. The data are presented in Table 2.

Table 2. Growth performance of *P. hypophthalmus* after 30 days of dietary supplementation with probiotic, postbiotic, and their combination derived from *B. subtilis* AAHM-BS2360.

Growth Parameter	Treatment			
	Control	Probiotic	Postbiotic	Pro + Post
Specific growth rate (SGR); %/day	0.74 $\pm$ 0.43 ^a	2.81 $\pm$ 0.61 ^b	2.61 $\pm$ 0.79 ^{ab}	3.29 $\pm$ 0.98 ^b
Average daily gain (ADG); g/day	0.37 $\pm$ 0.23 ^a	1.31 $\pm$ 0.35 ^{ab}	1.24 $\pm$ 0.40 ^{ab}	1.42 $\pm$ 0.52 ^b
Feed conversion ratio (FCR)	1.06 $\pm$ 0.35 ^a	0.79 $\pm$ 0.04 ^a	0.77 $\pm$ 0.05 ^a	0.81 $\pm$ 0.13 ^a

Data (Mean $\pm$ SD) in same row with different letters are significantly different ($p < 0.05$).

3.9. Histopathology

The histopathological examination of the gills demonstrated the absence of proliferation and hyperplasia of mucous cells, characterized by a normal lamellae structure. (Figure 11A–D). The liver analysis revealed unremarkable hepatocyte nuclei and an absence of congestion in the sinusoids, though low levels of hydropic degeneration were observed (Figure 11E–H). The head kidney exhibited normal conditions devoid of inflammation or necrosis of the hematopoietic tissue (Figure 11I–L). The melano-macrophage centers (MMC) in the spleen exhibited immune response activity in all treatments, devoid of any observed congestion or lymphoid depletion (Figure 11M–P). The intestinal villi were observed to be intact, characterized by a clear epithelial lining and goblet cells. This observation is indicative of optimal tissue health and structural integrity (Figure 11Q–T).

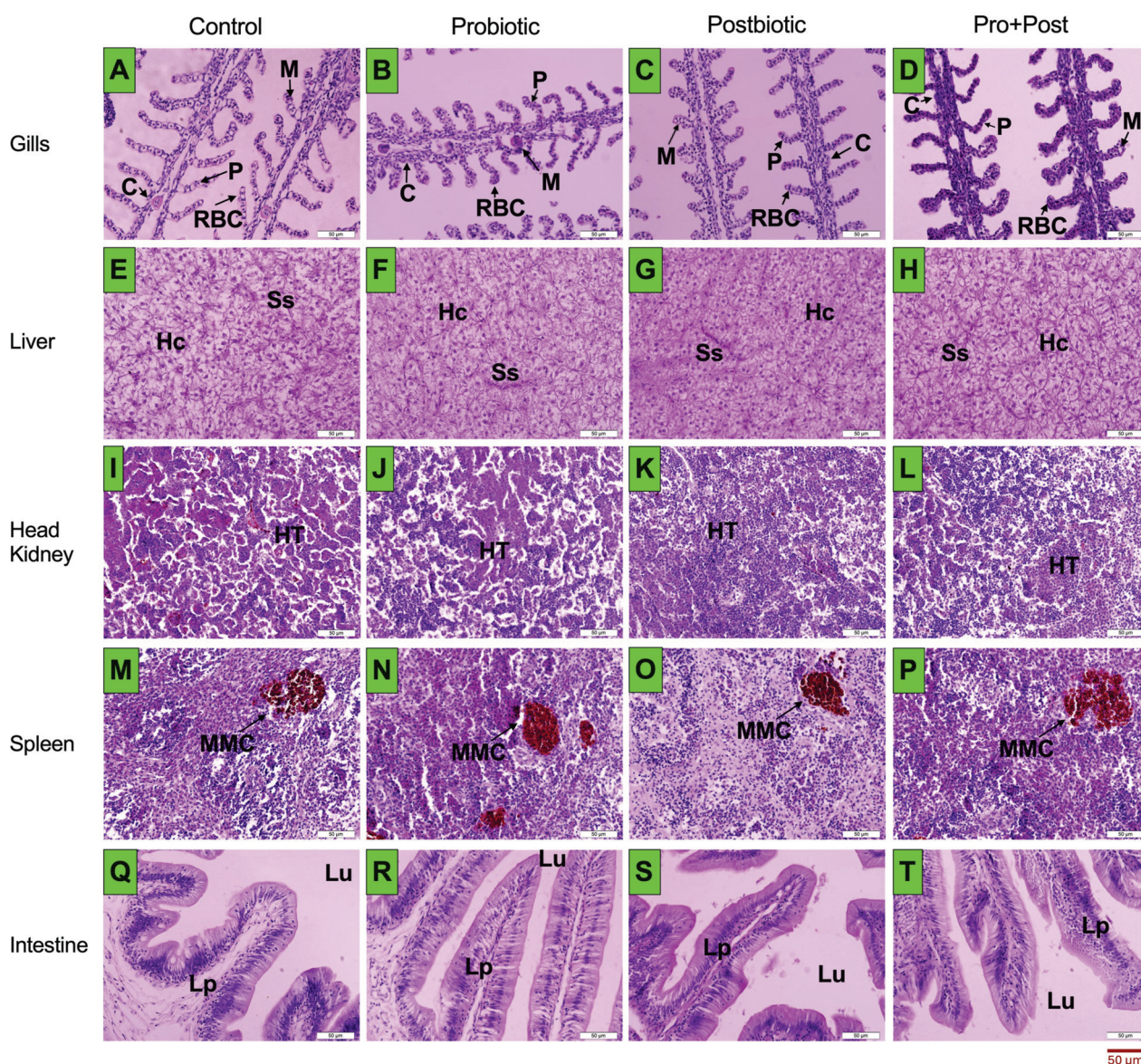


Figure 11. Histopathology of gills (A–D), liver (E–H), head kidney (I–L), spleen (M–P), and intestine (Q–T) of *P. hypophthalmus* following 30-day supplementation with probiotic, postbiotic, and their combination derived from *B. subtilis* AAHM-BS2360. Pillar cells (P), mucous cells (M), chloride cells (C), erythrocytes/red blood cells (RBC), hepatocyte (Hc), sinusoid (Ss), melano-macrophage center (MMCs), hematopoietic tissue (HT), lumen (LU), lamina propria (LP).

3.10. Survival Rate Analysis Following Post-Challenge Against *E. tarda*

The survival rate analysis revealed that the Pro + Post treatment group exhibited the highest survival rate of 87.5%, which was significantly different from the Control group with a value of 62.5% ($p < 0.05$). The Postbiotic and Probiotic groups demonstrated a survival rate of 84.37% and 81.25%, respectively, which was significantly different from the Control group ($p < 0.05$) (Figure 12).

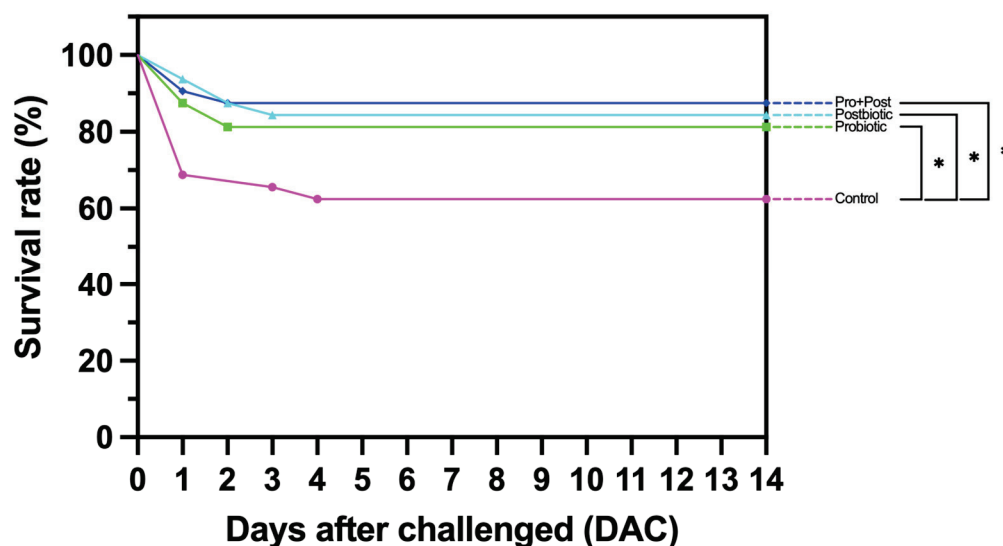


Figure 12. Survival rate (SR) (%) of *P. hypophthalmus* following 30-day supplementation with probiotic, postbiotic, and their combination derived from *B. subtilis* AAHM-BS2360 after intraperitoneal injection with *E. tarda*. Asterisks indicate statistically significant differences ($p < 0.05$).

4. Discussion

This study demonstrates the potential of *Bacillus subtilis* AAHM-BS2360 and its postbiotic metabolites as functional feed additives that improve growth performance, modulate immune responses, and reduce oxidative stress in *P. hypophthalmus* challenged with *Edwardsiella tarda*. No significant alterations in most serum biochemical parameters suggest that *B. subtilis* AAHM-BS2360 and its derivatives are safe and non-toxic. Serum biochemical profiles provide valuable information regarding the health status of pangasius and act as a monitoring tool for significant physiological stress or organ dysfunction in pangasius [33,34]. However, a noteworthy exception was observed for the AST/ALT ratio, which exhibited a considerable increase in the Postbiotic group, primarily attributable to the markedly augmented levels of AST as opposed to the comparatively lower levels of ALT.

In addition, a low range of ALT levels was also found in the 10 mg/kg ethanolic Kratom crude extract treatment in tilapia, the multivalent oral vaccine hydrogel in Asian seabass, and the treatment yeast extract supplemented feed in pangasius [29,35,36]. An increase in the AST/ALT ratio may reflect a transient hepatic response to bioactive compounds. Elevated AST/ALT ratios have been linked to an increase in oxidative stress conditions in the liver of fish and show the accumulation of microvacuoles, indicating lipid deposits in the liver [37,38]. Lipid peroxidase is closely associated with oxidative stress conditions. The extent of damage can be quantified by measuring malondialdehyde (MDA) levels and oxidative stress genes in the target organs [39]. The MDA enzyme analysis in the serum samples of the Postbiotic group showed a significant increase compared to the Control group. However, the MDA levels in the liver of the Control group were more dominant. To verify the physiological condition of the liver, histopathological analysis was also performed, which provided an overview of the health of the fish liver. Excessive lipid consumption can lead to increased lipid deposition in the liver, resulting in cell swelling,

nuclear translocation, accumulation of lipid droplets, and an increase in hepatocyte diameter [40]. In contrast, the benefits of probiotics and their postbiotic metabolites in alleviating hepatic lipid accumulation and oxidative stress by augmenting uridine circulation are noteworthy [41]. Histological analysis of the liver revealed no evidence of lipid droplets or fatty degeneration. In general, the organs were in satisfactory condition and did not exhibit substantial tissue damage.

A significant increase in lysozyme activity in mucus samples from both the probiotic and postbiotic groups indicates enhanced mucosal immunity. Lysozyme is a key innate immune enzyme that hydrolyzes bacterial cell walls, offering first-line protection against pathogens [42]. These findings suggest that the postbiotic metabolites of *B. subtilis* AAHM-BS2360 may enhance the synthesis of antimicrobial peptides, lysozyme, or beneficial microbial metabolites within the mucus thereby impeding the proliferation of pathogens [16]. These mechanisms encompass stimulation of innate immunity factors, production of antimicrobial metabolites, modulation of skin microbiota, activation of mucosal immunity, and enhancement of barrier integrity [43–45]. A comparable enhancement of lysozyme activity in mucus has been observed in the context of probiotic *Pediococcus acidilactici* feeding in tilapia [46]. Although bactericidal activity did not differ significantly among groups, the upregulation of innate immune-related genes such as *lyg11* and *b2ml* suggests immunomodulatory effects at the molecular level [47,48].

The decline in MDA levels across the liver, intestine, gills, and skin samples in the treatment group, in conjunction with the incorporation of *B. subtilis* AAHM-BS2360 components in the feed, signifies a reduction in lipid peroxidation, a process initiated by reactive oxygen species (ROS), including superoxide radicals, hydrogen peroxide, and hydroxyl radicals. MDA, being reactive, can interact with proteins, DNA, and lipids, resulting in cellular damage and apoptosis. Increased MDA levels lead to reduced growth performance of pangasius, attributed to a decrease in antioxidant function and inflammatory response, which are biomarkers of oxidative stress in pangasius [49,50]. Similarly, Liu et al. [51] also showed an increase in MDA in striped catfish due to excessive histamine exposure. In addition to the decrease in MDA levels, the *B. subtilis* AAHM-BS2360 component in the target organ also increased catalase (CAT) activity. The Probiotic group showed increased CAT activity in the liver, the Pro + Post group showed increased CAT in serum, and the Postbiotic group showed increased CAT levels in the gill. Catalase (CAT) functions as an antioxidant enzyme that decomposes H_2O_2 into water (H_2O) and oxygen (O_2), thus preventing oxidative damage. Catalase cooperates with superoxide dismutase (SOD), which converts superoxide radicals (O_2^-) into H_2O_2 , which is then neutralized by catalase. In conditions where catalase is present in low concentrations, glutathione peroxidase (GPX) can also reduce H_2O_2 using glutathione (GSH). Catalase is essential in maintaining cellular redox balance, protecting cells from oxidative damage and maintaining physiological homeostasis [49,52]. In the present study, the treatment group of metabolite components of *B. subtilis* AAHM-BS2360 did not demonstrate a significant increase in the activity of other antioxidant enzymes, such as GPx, GR, GSH, GST, and SOD, when compared to the Control group. This may be attributed to the effective treatment in suppressing ROS production, thereby ensuring that other antioxidant enzymes do not need to be activated excessively, and the physiological regulation of the body adjusts the production of antioxidant enzymes as required, as shown in the study on the effects of probiotic, parabiotic, and postbiotic *B. subtilis* on Asian seabass and tilapia [53,54].

The finding of antioxidant enzyme activity is consistent with the results of the antioxidant gene expression analysis, which also exerted a positive effect on all treatment groups of *B. subtilis* AAHM-BS2360 metabolite components. In response to a subsequent challenge with *E. tarda*, both the Postbiotic and Pro + Post groups exhibited a predominant

modulation of antioxidant gene expression in response to infection. The production of bioactive metabolites, such as peptides, exopolysaccharides, antioxidant enzymes, and surfactants, by *Bacillus* spp. has been demonstrated to enhance the activity of the internal antioxidant system in fish [55,56]. The regulation of key antioxidant genes and oxidative stress responses further supports the role of *B. subtilis* AAHM-BS2360 in providing effective protection in preventing oxidative stress in pangasius, which is consistent with previous reports of probiotic-mediated oxidative stress reduction in several fish species [54,57–59].

The present study investigates the modulatory effects of a dietary treatment containing *B. subtilis* AAHM-BS2360 on the expression of immune-related genes (*lygl1*, *tgfb*, *tnf*, *b2ml*) and proinflammatory genes (*litaf*, *ifngr1l*, *c3*, *il13*, *il1b*) in various target organs. This finding suggests that *B. subtilis* AAHM-BS2360 supplementation enhances both innate and adaptive immunity, exhibiting broad immunomodulatory effects, especially in enhancing non-specific immune responses, immune signaling and cytokine response, both post-feeding and challenge tests with pathogenic bacteria [60]. The upregulation of *lygl1*, *tgfb*, *tnf*, and *b2ml* gene expression in gills tissue in the Pro + Post group highlights the role of *B. subtilis* AAHM-BS2360 in modulating mucosal immunity, which is critical for protecting fish from waterborne pathogens [61]. In addition, the upregulation of immune genes was also seen to be dominant in the Postbiotic treatment after the challenge test with *E. tarda*. The Pro + Post group also demonstrated a marked tendency for proinflammatory gene expression activity. The probiotic strain *Pseudomonas aeruginosa* FARP72, administered at 10^8 cells/g of feed in pangasius, significantly enhanced the innate immune response and proinflammatory gene. This was evident in increased lysozyme activity after 30 days and elevated gene expression of *c3* and *il1b* in liver and kidney organs after 1 day of intraperitoneal injection with *E. tarda* [62]. In summary, potential therapeutic interventions like dietary modification, probiotics, postbiotics, and gut microbiota transplantation may reduce oxidative stress and inflammation linked to metabolic syndrome by altering the gut microbiota and epigenetic changes [63,64].

The present study set out to assess the impact of a feeding formulation containing cell components and metabolites of *B. subtilis* AAHM-BS2360 on the growth of pangasius. Supplementation with *Bacillus subtilis* AAHM-BS2360 metabolite components significantly impacted specific growth rate (SGR), particularly in the Probiotic and Pro + Post groups. This effect was corroborated by the significant upregulation of the *igf1* gene expression in the Probiotic group. The expression of *igf1* gene provides valuable insights into the molecular mechanisms that stimulate tissue growth, offering a comprehensive understanding of successful aquaculture [65]. Furthermore, an increase in % SGR was demonstrated following 60 days of feeding probiotic *B. aerius* B81e to *Pangasius bocourti* [66]. *Bacillus subtilis*-supplemented feed was also reported to have a positive impact on the growth performance of hybrid grouper [67].

Following the challenge posed by the pathogenic bacteria *E. tarda*, the Pro + Post group demonstrated the highest survival rate, reaching an impressive 87.5%. This was closely followed by the Postbiotic group, which achieved a survival rate of 84.37%. The Probiotic group also performed exceptionally well, with a survival rate of 81.25%. To provide a point of comparison with the present study, a challenge test against *Aeromonas hydrophila* FW52 in *Pangasius bocourti* using probiotic *Bacillus aerius* B81e resulted in an SR of 77.3% [66], while probiotic *B. velezensis* JW achieved an SR of 80% against *A. hydrophila* in *Carassius auratus* [68]. Furthermore, a probiotic mixture comprising *Bacillus subtilis*, *Enterococcus faecium*, *Lactobacillus plantarum*, and *Saccharomyces cerevisiae* has been shown to yield an SR of 60% in pangasius fry in response to *A. hydrophila* injection [69]. In a similar study, the use of *Bacillus subtilis* CFS in Asian seabass led to an SR of 69.3% [53]. These comparative data

demonstrate the superior protective effects of the combination of postbiotic metabolite and probiotic *B. subtilis* AAHM-BS2360 against pathogenic infection in pangasius.

5. Conclusions

Overall, these findings underscore the potential of *B. subtilis* AAHM-BS2360 as a functional feed additive in pangasius aquaculture. The supplementation of *B. subtilis* AAHM-BS2360 as probiotics resulted in a significant enhancement of growth performance and immune gene expression. In contrast, the supplementation of the postbiotic metabolite component of *B. subtilis* AAHM-BS2360 demonstrated a remarkable augmentation in lysozyme activity within the mucus, which serves as the fish's innate immune barrier against pathogens. Furthermore, the post-challenge response with *E. tarda* revealed an upregulation of immune and proinflammatory genes. The combination of probiotics and postbiotics (Pro + Post) offered broad-spectrum immunomodulatory benefits, which could be advantageous in disease management strategies. It excelled in modulating proinflammatory and antioxidant gene expression, significantly dominating the challenge test with the highest SR. Future research should explore the long-term effects of *B. subtilis* AAHM-BS2360 supplementation and its potential application to other aquaculture species.

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Institutional Review Board Statement: The aquatic animal experiments were conducted following the Ethical Principles and Guidelines for the Use of Animals outlined by the National Research Council of Thailand, which governs the care and use of animals for research purposes. This protocol received approval from the Animal Ethics Committee at Kasetsart University in Thailand (Approval ID: ACKU67-FIS-015) on 20 May 2024.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in this manuscript.

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Conflicts of Interest: Author N.W. belongs to Company Name PT. Central Proteinaprima. The company had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Article

Effects of Fermented Longan Peel (*Dimocarpus longan*) on Growth Performance, Digestive Enzyme Activity, Intestinal Microstructure, Immune Response, and Gene Expression of Nile Tilapia (*Oreochromis niloticus*) Raised Under Biofloc System

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Abstract

The valorization of agricultural by-products as functional feed additives represents a promising strategy for sustainable aquaculture. This study evaluated the effects of dietary fermented longan peel (FLP), produced through enzymatic hydrolysis and *Lactiplantibacillus plantarum* fermentation, on growth performance, digestive physiology, gut morphology, innate immunity, and gene expression in Nile tilapia (*Oreochromis niloticus*) cultured under a biofloc system. Five experimental diets were formulated with graded FLP levels (0, 5, 10, 20, and 40 g kg⁻¹) and fed to fish for eight weeks. Growth indices, including final weight, weight gain, and specific growth rate, improved significantly in fish receiving 20 g kg⁻¹ FLP, following a strong quadratic response pattern. *In vitro* digestibility assays showed enhanced carbohydrate and protein digestibility, coinciding with increased intestinal amylase and protease activities. Histological analysis indicated that moderate FLP inclusion (10–20 g kg⁻¹) promoted villus height, crypt depth, and epithelial organization. Innate immune parameters, including lysozyme, peroxidase, and alternative complement activity, were markedly elevated in serum and mucus, particularly at 20–40 g kg⁻¹ after eight weeks. Gene expression profiling revealed significant up-regulation of growth-related (*IGF-1*, *GH*, *NPY-α*, *Galanin*), immune-related (*TLR-7*, *TNF-α*, *NFκB*), and antioxidant-related (*hsp70*, *Keap-1*, *nrf-2*, *GST-α*) genes in fish fed higher FLP levels, with responses plateauing beyond 20 g kg⁻¹. Overall, FLP supplementation at 20 g kg⁻¹ optimally enhanced growth, digestive efficiency, intestinal health, and innate immune status. These findings demonstrate the potential of fermented longan peel as a cost-effective, bioactive, and sustainable functional feed ingredient for tilapia and other warm-water aquaculture species.

Keywords: fermented longan peel; Nile tilapia; digestive enzymes; immune response; biofloc

1. Introduction

Aquaculture is the fastest-growing food production sector worldwide and plays a crucial role in ensuring global food security, nutrition, and rural employment [1]. It currently supplies more than half of the fish consumed by humans and is projected to expand further as capture fisheries reach their limits [2]. However, intensive aquaculture production faces significant challenges, including the high cost of conventional feed ingredients, dependence on fishmeal and fish oil, and the increasing occurrence of disease outbreaks [3]. These issues underscore the need for sustainable feed alternatives that can improve growth, immunity, and environmental performance while supporting a circular bioeconomy [4]. Among cultured species, Nile tilapia (*Oreochromis niloticus*) is one of the most economically important freshwater fish due to its rapid growth, omnivorous feeding behavior, and tolerance to various farming conditions [5]. It is widely cultivated in Asia, Africa, and Latin America and represents a major protein source for human consumption [6]. Nonetheless, intensive rearing systems often expose tilapia to environmental stress, poor water quality, and pathogenic infections, leading to impaired immunity and reduced productivity [5,7]. In this context, the use of natural functional feed additives derived from agricultural by-products has been recognized as a promising strategy to enhance fish health and sustainability in tilapia farming.

Longan (*Dimocarpus longan* Lour.) is a tropical fruit widely grown in Thailand, Vietnam, and China [8], with annual global production approximately of 3.44 million tons [9,10]. Longan peel accounts for 12.4–19.6% of the whole fruit weight [11]. During longan pulp processing, tens of thousands of tons of peel and seeds are discarded annually, causing environmental pollution and resource wastage. Thus, the reuse of longan peel and seeds has significant development potential, but there is a lack of a systematic review of the active ingredients, health benefits and applications of longan plants [8]. Studies indicate that the longan peel and seeds are rich in polyphenols, flavonoids, and polysaccharides with antioxidant, antityrosinase, antibacterial, antifungal, antidiabetic, and other activities [12,13]. The longan peel phenolic compounds include ellagic acid, gallic acid and corilagin in free, esterified- and etherified forms [14]. Recent studies demonstrated that longan peel powder improved growth performance, immune activity, and gene expression in Nile tilapia, suggesting its potential as a functional feed additive [15]. Nevertheless, its high fiber and antinutrient contents may limit digestibility and nutrient absorption [8,14]. Fermentation represents an efficient bioprocessing approach to enhance the nutritional and functional value of plant-based ingredients [16,17]. Through microbial metabolism, fermentation degrades complex carbohydrates and antinutritional factors, increases bioactive compound availability, and produces beneficial metabolites such as organic acids and enzymes [18,19]. Fermented plant materials have been shown to improve feed utilization, gut health, and immune status in aquaculture species [20–23]. In particular, fermentation with lactic acid bacteria like *Lactobacillus* enhances antioxidant potential and contributes to the production of probiotics that positively influence the intestinal microbiota of fish [24,25].

In addition to functional feeds, the biofloc technology (BFT) system has gained prominence as an environmentally friendly aquaculture practice that promotes nutrient recycling, microbial biomass formation, and disease resistance [26]. BFT supports beneficial microbial consortia that improve digestion, water quality, and immunity in cultured fish, making it an excellent platform for testing functional additives [27]. Integrating fermented agro-industrial by-products with biofloc systems can provide synergistic benefits, enhancing nutrient bioavailability, stimulating immune responses, and reducing production costs.

Recent studies suggest that combining fermented agricultural or industrial by-products with biofloc systems may produce additive or synergistic benefits [28,29]. Fermented plant substrates introduced into biofloc environments can serve as additional

carbon sources, modulate microbial community structure, and enhance microbial protein production [27,30]. Moreover, fermentation-derived bioactive may interact with biofloc microbial consortia to increase short-chain fatty acid production, improve intestinal epithelial integrity, and stimulate immune signaling pathways [31]. Emerging evidence indicates that such integrated strategies can enhance nutrient bioavailability, digestive enzyme activity, antioxidant capacity, and non-specific immune responses in cultured fish species [25,32–34]. These findings support the concept that dietary fermentation and microbial-based rearing systems should not be considered independent interventions but rather interconnected components of a functional aquaculture microbiome. Despite increasing evidence on the benefits of both longan peel and fermented plant materials in aquaculture, the effects of fermented longan peel supplementation in Nile tilapia cultured under biofloc systems remain unexplored. Understanding how fermentation-derived metabolites and biofloc microorganisms interact to influence fish physiology is essential for optimizing feed strategies that align with sustainable production goals. Therefore, this study aimed to evaluate the effects of dietary fermented longan peel (FLP) supplementation on growth performance, digestive enzyme activity, intestinal microstructure, immune response, and expression of growth-, immune-, and antioxidant-related genes in Nile tilapia reared under a biofloc system. The findings are expected to advance knowledge on the functional application of fruit by-products in aquafeeds and contribute to sustainable aquaculture practices through the combined use of fermentation and biofloc technology.

2. Materials and Methods

2.1. Production of Fermented Longa Peel

2.1.1. Sample Preparation

Longan peel material was first collected and thoroughly washed to remove impurities. The material was then dehydrated in a hot-air oven at 50 °C for 48 h to remove moisture. After drying, the peels were ground into a fine powder and passed through a 100-mesh sieve to ensure uniform particle size. The resulting powder was subsequently stored in sealed plastic bags at 4 °C until further processing.

2.1.2. Pretreatment Process

Longan peel powder (10 g) was mixed with 0.5% sodium hydroxide solution and subjected to high-pressure treatment at 50 MPa for either 5 or 20 min. After pressurization, the material was thoroughly rinsed with deionized water to remove residual alkali. The treated samples were then dried in a hot-air oven at 80 °C for 24 h and subsequently stored at 4 °C until further analysis, following the general procedure described by Sahare, Singh, Laxman and Rao [35].

2.1.3. Enzymatic Hydrolysis

Pressure-treated longan peel (4 g) was suspended in 40 mL of 0.1 M citrate buffer (pH 4.8) and subjected to enzymatic digestion using iKnowZyme PXC (REACH BIOTECHNOLOGY Co., Ltd., Thanyaburi, Thailand) at a dosage of 100 µg per gram of substrate. The mixture was incubated at 55 °C with continued shaking at 150 rpm for 72 h. After hydrolysis, the suspension was centrifuged at 12,000 × g for 10 min, and the resulting supernatant was collected for determination of reducing sugar content and antioxidant properties [36].

2.1.4. Preparation of Starter Cultures for Fermentation

The bacterial starter culture was prepared using *Lactiplantibacillus plantarum* TISTR 2265, obtained from the Thailand Institute of Scientific and Technological Research. This strain has been reported to process significant biotechnological potential, including rapid

growth and the capacity to enhance GABA production, total phenolic content, and antioxidant capacity [37]. The strain was initially cultured in MRS broth at 37 °C for 24 h. Subsequently, 5% (*v/v*) of the activated culture was transferred into fresh MRS broth and incubated under the same condition until optical density at 600 nm reached 0.6–0.8. The bacterial cells were then harvested by centrifugation at 6000× *g* for 15 min at 4 °C, washed twice with 0.9% NaCl, and finally adjusted to a cell density of approximately 7–8 log CFU mL^{−1}.

2.1.5. Production of Fermented Longan Peel

Following the 72 h enzymatic hydrolysis, the longan peel mixtures were sterilized at 121 °C for 15 min. After cooling to room temperature, the substrates were aseptically inoculated with 5% (*w/v*) of the prepared *L. plantarum* TISTR 2265 culture. Fermentation was conducted at 30 °C for 72 h under controlled conditions. After fermentation, the mixtures were centrifuged at 6000 rpm for 15 min, and the resulting solid fraction was collected for further analyses.

The proximate composition and bioactive compound profile of the fermented longan peel (FLP) are presented in Tables 1 and 2. Moisture, dry matter, ash, crude protein, and lipid contents were determined according to AOAC procedures [38]. Quantification of major phenolic compounds was conducted following the methods described by Dhanani, Shah and Kumar [39] and Pereira, Câmara, Cacho and Marques [40]. Antioxidant properties, including ABTS⁺ scavenging ability, FRAP, total flavonoids, DPPH (IC₅₀), and total phenolics, was evaluated using established protocols [41,42].

Table 1. Proximate analysis of fermented longan peel (FLP).

Parameter	FLP
Moisture (%)	8.2
Protein (%)	12.4
Fat (%)	1.19
Fiber (%)	48.3
Ash (%)	9.64

Table 2. Phenolic compounds of fermented longan peel (mg/100 g dry weight).

Compound	Longan Peel	Fermented Longan Peel (FLP)	Methods
Gallic acid	29.59	29.57	HPLC-PDA
Catechin	203.11	Nd.	HPLC-PDA
Epicatechin	161.34	Nd.	HPLC-PDA
Caffeic acid	Nd.	Nd.	HPLC-PDA
Epicatechin gallate	Nd.	Nd.	HPLC-PDA
p-Coumaric acid	Nd.	Nd.	HPLC-PDA
Naringin	105.70	Nd.	HPLC-PDA
Rosmarinic acid	59.35	11.07	HPLC-PDA
o-Coumaric acid	14.65	8.52	HPLC-PDA
Quercetin	Nd.	10.19	HPLC-PDA

Nd. = Not detected.

2.2. Experiment Diets

A basal diet was formulated according to the established nutrient requirements of Nile tilapia, following the guidelines described by Wannavijit, Outama, Le Xuan, Lumsangkul, Lengkidworraphiphat, Tongsiri, Chitmanat and Doan [43] (Table 3). Experimental diets were prepared by supplementing the basal formulation with graded levels of fermented longan peel (FLP). All dry ingredients were finely ground, accurately weighed, and thoroughly

mixed using a mechanical mixer to ensure homogeneity. Subsequently, fish oil and soybean oil were gradually incorporated to achieve uniform distribution within the feed matrix. Distilled water (approximately 300 mL per kg of diet) was then slowly added to obtain an appropriate consistency for pellet formation. The resulting dough was processed through a pelletizer (Siam Farm Services Co., Ltd., Lampang 52130, Thailand) to produce uniform pellets. The pellets were subsequently dried and stored under appropriate conditions until use.

Table 3. Feed ingredients and proximate composition (g kg^{−1}) of fermented longan peel (FLP).

	FLP 0	FLP 5	FLP 10	FLP 20	FLP 40
Fish meal	150	150	150	150	150
Corn meal	200	200	200	200	200
Soybean meal	390	390	390	390	390
Wheat flour	70	70	70	70	70
Rice bran	150	145	140	130	110
FLP	0	5	10	20	40
Binder	20	20	20	20	20
Soybean oil	2	2	2	2	2
Premix ¹	10	10	10	10	10
Vitamin C ²	8	8	8	8	8
Proximate composition of the experimental diets (% of dry matter basis)					
Dry matter	94.18	93.99	94.19	94.18	93.79
Crude protein	31.39	32.01	31.32	31.56	31.23
Crude lipid	1.49	1.51	1.40	1.59	1.52
Ash	8.67	8.41	8.52	8.48	8.14
Fiber	4.32	4.67	4.75	5.34	5.93
GE (kcal/g) ³	4.03	4.04	4.02	4.03	4.01

¹ Vitamin and trace mineral mix supplemented as follows (IU kg^{−1} or g kg^{−1} diet): retinyl acetate 1,085,000 IU; cholecalciferol 217,000 IU; D, L- α -tocopherol acetate 0.5 g; thiamin nitrate 0.5 g; pyridoxine hydrochloride 0.5 g; niacin 3 g; folic 0.05 g; cyanocobalamin 10 g; Ca pantothenate 1 g kg^{−1}; inositol 0.5 g; zinc 1 g; copper 0.25 g; manganese 1.32 g; iodine 0.05 g; sodium 7.85 g; ² Vitamin C 98% 5 g; ³ GE = gross energy.

2.3. Experiment Design

Male Nile tilapia (*Oreochromis niloticus*) fingerlings were obtained from PC Farm, Thailand. Upon arrival, the fish were acclimated for 30 days and fed a commercial diet (CP 9950). This was followed by a two-week adaptation period during which the fish were fed the control experimental diet. After acclimation, a total of 300 fish with an initial weight of 15.04 ± 0.03 g were randomly distributed into 15 aerated tanks (150 L each), with 20 fish per tank. The experiment was conducted using a Completely Randomized Design (CRD) consisting of five dietary treatments, each with three replicates. Fish were fed their respective experimental diets to apparent satiation twice daily at 08:30 and 16:30 for eight weeks.

2.4. Biofloc Water Preparation and Management

Two weeks prior to fish stocking, biofloc formation was initiated in each tank by adding 2 g of fish feed, 400 g of salt, 5 g of dolomite, and 5 g of molasses as a carbon source to stimulate heterotrophic bacterial proliferation. Tanks were continuously aerated to maintain suspended microbial aggregates and prevent sedimentation. Visible floc formation and increased water turbidity were observed within the conditioning period, indicating successful establishment of microbial aggregates before the start of the feeding trial.

Throughout the experiment, the carbon-to-nitrogen (C:N) ratio was maintained at 15:1 by daily supplementation of molasses (40% carbon), following the protocol of Avnimelech [44]. The C:N ratio was calculated based on dietary nitrogen input and estimated

residual nitrogen in each tank, as described by Cardona, Lorgeoux, Chim, Goguenheim, Le Delliou and Cahu [45]. Water quality parameters, including ammonium, pH, dissolved oxygen, and floc volume were monitored regularly and remained within acceptable ranges ($\text{NH}_3 = 0.19 \pm 0.01 \text{ mg L}^{-1}$, $\text{pH} = 7.86 \pm 0.68$, dissolved oxygen = $5.73 \pm 0.48 \text{ mg L}^{-1}$, and floc volume = $8.97 \pm 0.46 \text{ mL L}^{-1}$) for tilapia culture throughout the experimental period.

2.5. Growth Performance Measurements

Growth performance and survival of Nile tilapia were evaluated using standard aquaculture performance indicators. The following parameters were calculated: Weight gain (WG) = final weight (g) – initial weight (g); Specific growth rate (SGR %) = $100 \times (\ln \text{final weight} - \ln \text{initial weight}) / \text{total duration of experiment}$; Feed conversion ratio (FCR) = feed given (dried weight)/weight gain (wet weight), and Survival rate (%) = (final fish number/initial fish number) $\times 100$.

2.6. In Vitro Digestibility and Digestive Enzyme Activity

2.6.1. Crude Enzyme Extraction

Crude enzyme extracts were prepared from small intestinal tissue by homogenizing samples in 0.2 M phosphate buffer (pH 8.0) at a 1:3 (*w/v*) tissue-to-buffer ratio, following the method described by Rungruangsak-Torrissen [46]. The homogenate was then centrifuged at $15,000 \times g$ for 15 min at 4 °C, and the resulting supernatant was carefully collected while avoiding the upper lipid layer. The collected supernatant served as the crude enzyme extract. All samples were subsequently stored at –80 °C until further analysis [47].

2.6.2. Digestive Enzyme Assays

Amylase activity was determined according to the method described by Areekijserree, Engkagul, Kovitvadhi, Thongpan, Mingmuang, Pakkong and Rungruangsak-Torrissen [48], using 5% soluble starch as the substrate. The absorbance was measured at 540 nm, and enzyme activity was calculated based on a maltose standard curve, expressed as $\text{mg maltose h}^{-1} \text{ mg}^{-1} \text{ protein}$. Lipase activity was measured following the procedure of Rungruangsak-Torrissen [46], using 0.01 M p-nitrophenyl palmitate as the substrate. The absorbance was recorded at 410 nm, and activity was quantified using a p-nitrophenol standard curve, expressed as $\mu\text{mol p-nitrophenol h}^{-1} \text{ mg}^{-1} \text{ protein}$. Total protease activity was assayed using 5% azocasein according to Areekijserree, Engkagul, Kovitvadhi, Thongpan, Mingmuang, Pakkong and Rungruangsak-Torrissen [48]. One unit of enzyme activity (U) was defined as the amount of enzyme producing a 1.0-unit increase in absorbance at 440 nm under assay conditions, and the activity was expressed as $\text{U h}^{-1} \text{ mg}^{-1} \text{ protein}$. Trypsin activity was determined using 1.25 mM benzoyl-DL-arginine-p-nitroanilide as the substrate [46]. The absorbance was measured at 410 nm, and the enzyme activity was calculated using a p-nitroanilide calibration curve, expressed as $\mu\text{mol p-nitroaniline h}^{-1} \text{ mg}^{-1} \text{ protein}$.

2.6.3. In Vitro Digestibility

Crude enzyme extracts were dialyzed overnight in 50 mM phosphate buffer (pH 8.0) prior to use in the *in vitro* digestibility assay. Experimental diets were finely ground to serve as substrates. Protein and carbohydrate digestibility were evaluated using crude intestinal enzymes from fish, following a modified protocol of Rungruangsak-Torrissen, Rustad, Sunde, Eiane, Jensen, Opstvedt, Nygård, Samuelsen, Mundheim and Luzzana [49].

For each assay, 5 mg of dried diet powder was mixed with 10 mL of 50 mM phosphate buffer (pH 8.0), 50 μL of 0.5% chloramphenicol, and 125 μL of dialyzed crude enzyme extract. The mixture was incubated at 25 °C in a shaking incubator for 24 h to mimic digestion in tropical fish. A baseline control (T0) was obtained by withdrawing 0.5 mL of

the reaction mixture before enzyme addition, immediately heating it at 100 °C for 5 min to stop all enzymatic activity, and storing it at −80 °C for subsequent analysis. Digestion was initiated by adding 0.5 mL of dialyzed enzyme extract with standardized trypsin activity. After 24 h of incubation, 1 mL of the digested suspension was collected, heat-inactivated (100 °C, 5 min), and preserved at −80 °C for further measurements.

Protein digestibility was quantified using the trinitrobenzene sulphonic acid (TNBS) method, which detects free amino groups released during hydrolysis. A 200 µL aliquot of the digested sample was mixed with 2 mL of phosphate buffer (50 mM, pH 8.0) and 1 mL of 0.1% TNBS, followed by incubation in the dark at 60 °C for 1 h. The reaction was stopped with 1 mL of 1 M HCl, and absorbance was measured at 420 nm. Values were calculated using a DL-alanine standard curve and expressed as µmol DL-alanine per g of feed per unit of trypsin activity, correcting for enzyme variability [50].

Carbohydrate digestibility was determined by measuring reducing sugars using the dinitrosalicylic acid (DNS) assay. One milliliter of digested sample was reacted with 500 µL of DNS reagent, boiled for 5 min, cooled, and read at 540 nm. Reducing sugar concentrations were derived from a maltose standard curve and expressed as mg maltose per g of feed per unit of amylase activity, enabling comparisons across treatments [50].

2.7. Intestinal Microstructure

During sampling, mid-intestinal segments were collected from six fish in each treatment group (2 fish per tank). Tissues were gently rinsed with phosphate-buffered saline (PBS; pH 7.4) and fixed in 10% neutral-buffered formalin for 24 h. After fixation, samples were processed for routine histology: they were dehydrated, embedded in paraffin, sectioned at 4 µm thickness, and stained with hematoxylin and eosin (H&E) following the procedure outlined by Ruiz, Owatari, Yamashita, Ferrarezi, Garcia, Cardoso, Martins and Mourinho [51]. Prepared slides (3 replication per sample) were examined under a compound microscope (Leica DM750, Leica Microsystems, Wetzlar, Germany) at 20× magnification. Morphometric parameters, including villus height (VH), villus width (VW), and crypt depth (CD), were quantified using Leica LAS X imaging software version 3.9.0 (Leica Microsystems, Wetzlar, Germany).

2.8. Evaluation of Innate Immune Responses

2.8.1. Sample Preparation

Fish samples (three per tank) were collected at weeks 4 and 8 for analyses of skin mucus and serum immune parameters.

Skin mucus collection followed a modified version of Quade and Roth [52] as adapted by Van Doan, Hoseinifar, Naraballoh, Paolucci, Wongmaneeprateep, Charoenwattanasak, Dawood and Abdel-Tawwab [53]. Fish were anaesthetized with clove oil (5 mL L^{−1}) and placed in polyethylene bags containing 10 mL of 50 mM NaCl. Gentle downward strokes along the body surface for approximately one minute facilitated mucus release. The solution was transferred to 15 mL tubes and centrifuged at 1500× g for 10 min at 4 °C. About 1 mL of the supernatant was aliquoted into Eppendorf tubes and stored at −80 °C until analysis.

Serum samples were obtained following Van Doan, Wannavijit, Tayyamath, Quynh, Sumon, Linh, Seesuriyachan, Phimolsiripol, Esteban and Gisbert [25]. Blood was drawn from the caudal vein using 1 mL syringes, left to clot at room temperature for 1 h, and then refrigerated at 4 °C for several hours. Serum was separated and stored at −80 °C for later assays.

2.8.2. Lysozyme Activity

Lysozyme activity in both skin mucus and serum (3 replications per fish) was quantified following the method of Parry Jr, Chandan and Shahani [54], with modifications described by Wannavijit, Outama, Le Xuan, Lumsangkul, Lengkidworraphiphat, Tongsir, Chitmanat and Doan [43].

2.8.3. Peroxidase Activity

Peroxidase activity (3 replications per fish) was quantified following Quade and Roth [52] with slight adjustments from Wannavijit, Outama, Le Xuan, Lumsangkul, Lengkidworraphiphat, Tongsir, Chitmanat and Doan [43].

2.8.4. Alternative Complement Pathway Activity (ACH50)

ACH50 activity (2 replications per fish) was measured using a modified hemolytic assay based on Yano, Ando and Nakao [55] with some adjustments as described in [25].

2.9. Genes Expression Analysis

Relative expression levels of immune- and antioxidant-related genes were evaluated using two fish from each tank. Fish were anesthetized with clove oil (100 mg mL⁻¹), after which the liver, hindgut, and head kidney were dissected and immediately processed for RNA extraction. Total RNA was isolated using either TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) or the PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA, USA). RNA concentration and purity were assessed with a NanoDrop™ One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by measuring absorbance at 260 and 280 nm.

A total of 1000 ng RNA from each sample was reverse-transcribed into cDNA using the iScript™ cDNA Synthesis Kit (BIO-RAD, Laboratories, Hercules, CA, USA). The primers used in this study were adopted from previously published studies and have been validated for gene expression analysis in Nile tilapia (Table 4). Quantitative real-time PCR (qRT-PCR) was performed in triplicate using 100 ng cDNA, 400 µM of each primer, and iTaq Universal SYBR Green Supermix (2×) on a CFX96 Touch Deep Well Real-Time PCR System (BIO-RAD, USA). The PCR cycling parameters followed Le Xuan, Vu Linh, Wannavijit, Outama, Lubis, Machimbirike, Chromkaew, Phimolsiripol and Van Doan [56]; an initial denaturation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s, and a melt-curve stage consisting of 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. Relative gene expression was calculated using the 2^{-ΔΔCt} method Livak and Schmittgen [57], supported by standard-curve validation for amplification efficiency. by, with standard curve analysis.

Table 4. Primer sequences, amplicons, and associated information for quantitative real-time PCR of fermented longan peel.

Gene	Sequence	Accession Number	Reference
<i>Beta-actin</i>	F: CAGCAAGCAGGAGTACGATGAG R: TGTGTGGTGTGTGGTTGTTTTG	XM_003443127.4	[58]
<i>IGF-I: Insulin-growth factor 1</i>	F: GTCTGTGGAGAGCGAGGCTTT R: CACGTGACCGCCTTGCA	NM_001279503	[58]
<i>GH: Growth hormone</i>	F: TCGGTTGTGTGTTTGGGCGTCTC R: GTGCAGGTGCGTGACTCTGTTGA	XM_003442542	[58]
<i>Ghrelin</i>	F: GTGGTGCAAGTCAACCAAGTG R: CATGGCTTGCGCACCAATTC	AB104859.1	[59]
<i>NPY-α F</i>	F: TCTCGCTCACTGCTGTCCC R: CAGAGGCGTGGTGTTCGTT	XM_003448854.5	[60]

Table 4. Cont.

Gene	Sequence	Accession Number	Reference
<i>Galanin</i>	F: TGTTAGGGCCCCATGGACTA R: GAAGTCCTCCTCCTGGCCTA	XM_003453581.5	[59]
<i>HSP70 = Heat Shock Protein 70</i>	F: TTCAAGGTGATTTTCAGACGGAG R: CTTTCATCTTCACCAGGACCATG	XM_019357557.1	[61]
<i>keap1</i>	F: CTTCCGCATCATGAACGAGC R: CACCAACTCCATACCGCACT	XM_003447926.3	[61]
<i>Nrf2</i>	F: CTGCCGTAAACGCAAGATGG R: ATCCGTTGACTGCTGAAGGG	XM_003447296.4	[61]
<i>GST-a</i>	F: ACTGCACACTCATGGGAACA R: TTAAGAGCCAGCGGATTGAC	NM_001279635	[62]
<i>EF-α</i>	F: CTACAGCCAGGCTCGTTTCG R: CTTGTCACTGGTCTCCAGCA	AB075952	[63]
<i>TLR-7</i>	F: TCAGCAGGGTGAGAGCATAC R: ACATATCCCAGCCGTAGAGG	XM_005477981.1	[64]
<i>TNFα</i>	F: CCAGAAGCACTAAAGGCGAAGA R: CCTTGGCTTTGCTGCTGATC	NM_001279533.1	[58]
<i>nf-κB</i>	F: GAACATCAGACCGACGACCA R: TCTCCGCCAGTTTCTTCCA	XM_003457469.4	[61]

2.10. Statistical Analysis

Data normality was examined using the Kolmogorov–Smirnov test. One-way ANOVA was performed to assess treatment effects, and significant differences among means were identified using Duncan’s multiple range test. All analyses were conducted using GraphPad Prism version 10 (GraphPad Software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

The optimal dietary inclusion level of fermented longan peel (FLP) was estimated using quadratic regression analysis based on growth performance indicators, including final weight (FW), weight gain (WG), specific growth rate (SGR), and feed conversion ratio (FCR), as shown in Figure 1. The quadratic model used in the analysis was: $Y = a + bX + cX^2$. Here, Y represents the response variable (FW, WG, SGR, or FCR), X represents the dietary FLP inclusion level, and a, b, and c are regression coefficients estimated using GraphPad Prism version 10 (GraphPad Software, San Diego, CA, USA).

The optimal inclusion level was calculated from the vertex of the quadratic equation using the formula: $X_{opt} = -b/2c$, where X_{opt} represents the estimated optimal dietary level. Based on this analysis, the optimal FLP inclusion levels were estimated to be 21.87 g kg^{−1} for FW, 21.91 g kg^{−1} for WG, 21.83 g kg^{−1} for SGR, and 20.64 g kg^{−1} for FCR, as illustrated in Figure 1.

In addition, prior to statistical analysis, data normality was tested using the Kolmogorov–Smirnov test. Differences among treatments were evaluated using one-way ANOVA, followed by Duncan’s multiple range test for mean comparisons. All analyses were performed using GraphPad Prism version 10, and statistical significance was considered at $p < 0.05$.

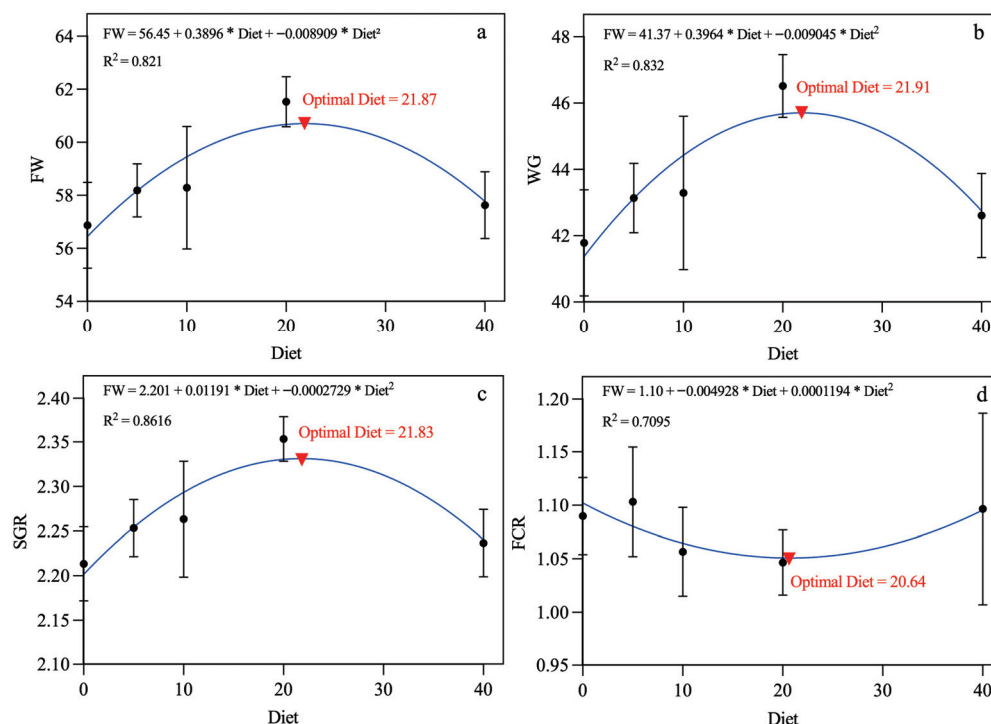


Figure 1. Growth performance and feed utilization of Nile tilapia fed with different dietary levels of fermented longan peel. Polynomial regression curves show the relationship between diet and (a) final body weight, (b) weight gain, (c) specific growth rate, and (d) feed conversion ratio. Optimal dietary levels are indicated by red arrows.

3. Results

3.1. Growth Parameters

The growth performance and feed utilization indices of Nile tilapia (*O. niloticus*) fed diets containing graded levels of fermented longan peel (FLP) for eight weeks are summarized in Table 5 and Figure 1. Initial body weights (IW) were comparable among all groups, ranging from 15.00 to 15.08 g, indicating uniformity at the start of the trial.

Table 5. Growth performance of utilization of Nile tilapia fed with different dietary levels of fermented longan peel.

	FLP 0	FLP 5	FLP 10	FLP 20	FLP 40
IW (g)	15.08 ± 0.02 ^a	15.05 ± 0.03 ^a	15.00 ± 0.00 ^a	15.02 ± 0.02 ^a	15.02 ± 0.02 ^a
FW (g)					
4 weeks	30.72 ± 1.05 ^a	32.53 ± 0.32 ^a	30.95 ± 0.93 ^a	31.97 ± 0.88 ^a	29.9 ± 2.06 ^a
8 weeks	56.87 ± 0.94 ^b	58.19 ± 0.58 ^{ab}	58.29 ± 1.34 ^{ab}	61.53 ± 0.55 ^a	57.63 ± 0.73 ^{ab}
WG (g)					
4 weeks	15.63 ± 1.05 ^a	17.48 ± 0.30 ^a	15.95 ± 0.93 ^a	16.95 ± 0.88 ^a	14.88 ± 2.07 ^a
8 weeks	41.78 ± 0.92 ^b	43.14 ± 0.61 ^{ab}	43.29 ± 1.34 ^{ab}	46.52 ± 0.55 ^a	42.61 ± 0.73 ^{ab}
SGR (%/day)					
4 weeks	2.37 ± 0.11 ^a	2.57 ± 0.03 ^a	2.41 ± 0.10 ^a	2.52 ± 0.09 ^a	2.28 ± 0.24 ^a
8 weeks	2.21 ± 0.02 ^b	2.25 ± 0.02 ^{ab}	2.26 ± 0.04 ^{ab}	2.35 ± 0.01 ^a	2.24 ± 0.02 ^b
FCR					
4 weeks	0.72 ± 0.02 ^a	0.66 ± 0.02 ^a	0.66 ± 0.00 ^a	0.64 ± 0.03 ^a	0.68 ± 0.04 ^a
8 weeks	1.09 ± 0.02 ^a	1.10 ± 0.03 ^a	1.06 ± 0.02 ^a	1.05 ± 0.02 ^a	1.10 ± 0.05 ^a
SR (%)					
4 weeks	93.33 ± 1.67 ^a	91.67 ± 3.33 ^a	96.67 ± 3.33 ^a	96.67 ± 1.67 ^a	96.67 ± 1.67 ^a
8 weeks	95.00 ± 2.89 ^a	91.67 ± 3.33 ^a	96.67 ± 3.33 ^a	93.33 ± 3.33 ^a	93.33 ± 6.67 ^a

IW = initial fish weight, FW: final fish weight, WG: weight gain, SGR: specific fish growth rate⁻¹, FCR: feed conversion ratio, SR: survival rate. Different letters in the same row indicate significant differences ($p < 0.05$).

After eight weeks, final body weight (FW) increased progressively with higher dietary FLP inclusion, with the FLP20 group achieving the greatest weight (61.53 ± 0.55 g), compared with the control group (56.87 ± 0.94 g). This improvement exhibited a significant quadratic response ($p < 0.05$, $R^2 = 0.821$), and the estimated optimal inclusion level was 21.87 g kg^{-1} .

A similar trend was observed for weight gain (WG), which increased from 41.78 ± 0.92 g in the control diet to 46.52 ± 0.55 g in the FLP20 treatment. WG also followed a significant quadratic pattern ($p < 0.05$, $R^2 = 0.8318$), with an estimated optimum of 21.91 g kg^{-1} dietary inclusion.

Specific growth rate (SGR) improved correspondingly, reaching a maximum of $2.35 \pm 0.01\%/ \text{day}$ in the FLP20 group, compared with $2.21 \pm 0.02\%/ \text{day}$ in the control. This parameter also demonstrated a significant quadratic effect ($p < 0.05$, $R^2 = 0.8616$), with the optimal inclusion level estimated at 21.83 g kg^{-1} .

Feed conversion ratio (FCR) showed a numerical reduction from 1.09 ± 0.02 (control) to 1.05 ± 0.02 (FLP20), following a quadratic trend ($R^2 = 0.7095$) with an estimated optimum of 20.64 g kg^{-1} , although the differences were not statistically significant ($p > 0.05$).

Survival rate (SR) remained consistently high across all treatments, ranging from 91.67% to 96.67%, with no significant differences among groups ($p > 0.05$).

3.2. In Vitro Digestibility

The *in vitro* digestibility results are presented in Table 6. After the 8-week feeding trial, significant differences in intestinal carbohydrate digestibility were detected among treatments, with values ranging from 2.35 ± 0.05 to $2.91 \pm 0.03 \text{ } \mu\text{mol maltose/g feed}$ per unit of amylase activity. Fish fed the FLP20 diet exhibited the highest carbohydrate digestibility (2.91 ± 0.03), followed by the FLP40 (2.72 ± 0.05); both were significantly greater than that of the control group (FLP0; 2.35 ± 0.05) ($p < 0.05$). Protein digestibility showed a similar pattern, increasing significantly with FLP supplementation. The FLP20 treatment showed the highest protein digestibility (28.62 ± 1.10), which was significantly greater than that of the control (23.54 ± 0.13) ($p < 0.05$). Although the FLP5 and FLP40 groups also exhibited higher protein digestibility than the control, their values were not significantly different from those observed in the FLP20 group.

Table 6. *In vitro* digestibility of carbohydrate ($\mu\text{mol maltose g feed}^{-1} \text{ amylase activity}^{-1}$) and protein ($\mu\text{mol DL-alanine equivalent g feed}^{-1} \text{ trypsin activity}^{-1}$) in intestine of Nile tilapia after eight weeks of feeding with diets containing different levels of fermented longan peel (FLP).

	FLP 0	FLP 5	FLP 10	FLP 20	FLP 40
Carbohydrate	2.35 ± 0.05^c	2.69 ± 0.05^b	2.39 ± 0.03^c	2.91 ± 0.03^a	2.72 ± 0.05^{ab}
Protein	23.54 ± 0.13^c	26.8 ± 0.26^{ab}	25.49 ± 0.44^{bc}	28.62 ± 1.10^a	28.07 ± 0.72^{ab}

Different letters in a row denote significant differences ($p < 0.05$) and means $\pm$ SE.

3.3. Digestive Enzyme Activity

The intestinal digestive enzyme activities of Nile tilapia fed diets containing different levels of fermented longan peel (FLP) for eight weeks are presented in Table 7. Amylase activity was significantly affected by dietary treatment ($p < 0.05$), increasing from $48.05 \pm 2.22 \text{ } \mu\text{mol maltose/hr./mg protein}$ in the control group to a maximum of $57.15 \pm 1.88 \text{ } \mu\text{mol maltose h}^{-1} \text{ mg}^{-1} \text{ protein}$ in the FLP20 group. Fish fed the FLP5, FLP10, and FLP40 diets showed intermediate amylase activities that were not significantly different from either the control or the FLP20 group. Lipase activity did not differ significantly among treatments ($p > 0.05$), with values ranging from 0.13 ± 0.01 to $0.15 \pm 0.01 \text{ } \mu\text{mol}$

p-nitrophenol/hr./mg protein, indicating that dietary FLP supplementation had no detectable effect on lipid digestion.

Table 7. Intestinal digestive enzymes activities of Nile Tilapia fed diets with different levels of fermented longan peel (FLP) for eight weeks. Different letters in the same row indicate significant differences ($p < 0.05$).

	FLP 0	FLP 5	FLP 10	FLP 20	FLP 40
Amylase activity	48.05 ± 2.22 ^b	54.85 ± 2.52 ^{ab}	51.98 ± 1.32 ^{ab}	57.15 ± 1.88 ^a	54.77 ± 1.35 ^{ab}
Lipase activity	0.15 ± 0.01 ^a	0.14 ± 0.00 ^a	0.13 ± 0.01 ^a	0.13 ± 0.01 ^a	0.14 ± 0.01 ^a
Protease activity	2.77 ± 0.08 ^{bc}	2.73 ± 0.14 ^{bc}	2.58 ± 0.03 ^c	3.24 ± 0.06 ^a	3.06 ± 0.06 ^{ab}
Trypsin activity	8.31 ± 0.14 ^b	8.48 ± 0.11 ^b	8.30 ± 0.20 ^b	9.58 ± 0.06 ^a	8.46 ± 0.09 ^b

Amylase activity (μmol maltose/hr./mg protein), Lipase activity (μmol p-nitrophenol/hr./mg protein), Protease activity (U/hr./mg protein), and Trypsin activity (μmol p-nitroaniline/hr./mg protein).

Protease activity varied significantly among treatments ($p < 0.05$). The highest activity was observed in the FLP20 group (3.24 ± 0.06 U/hr./mg protein), followed by the FLP40 group (3.06 ± 0.06); both values were significantly higher than that of the control group (2.77 ± 0.08 U h⁻¹ mg⁻¹ protein). The FLP10 group exhibited the lowest protease activity (2.58 ± 0.03 h⁻¹ mg⁻¹ protein) among treatments. Trypsin activity was also significantly influenced by dietary FLP supplementation ($p < 0.05$). Fish fed the FLP20 diet showed the highest trypsin activity (9.58 ± 0.06 μmol p-nitroaniline/hr./mg protein), whereas the other treatments showed lower but statistically similar values ranging from 8.30 to 8.48 μmol p-nitroaniline h⁻¹ mg⁻¹ protein.

3.4. Intestinal Microstructure

Histological examination revealed well-preserved intestinal structures across all treatments, characterized by normal villi and crypt organization (Figure 2 and Table 8). Fish fed FLP-supplemented diets showed improved gut morphology relative to the control group. Notably, the FLP10 and FLP20 treatments exhibited longer, more orderly villi, deeper crypts, and denser enterocyte arrangement. In contrast, the control (FLP0) and the highest inclusion level (FLP40) displayed shorter and less compact villi. These observations suggest that moderate FLP inclusion (10–20 g kg⁻¹) promotes favorable intestinal structural development in Nile tilapia.

Table 8. Impact of dietary supplementation with fermented longan peel on intestinal microstructure.

	FLP 0	FLP 5	FLP 10	FLP 20	FLP 40
VH (μm)	445.40 ± 13.29 ^b	485.80 ± 11.37 ^{ab}	449.40 ± 25.36 ^b	500.60 ± 3.50 ^{ab}	523.60 ± 14.62 ^a
VW (μm)	88.51 ± 0.57 ^b	92.88 ± 0.63 ^{ab}	90.81 ± 1.84 ^{ab}	93.73 ± 0.65 ^a	93.05 ± 1.01 ^{ab}
CD (μm)	33.74 ± 0.92 ^a	30.08 ± 3.15 ^a	31.39 ± 2.85 ^a	28.11 ± 1.28 ^a	30.32 ± 0.79 ^a
VH:CD	13.34 ± 0.68 ^b	16.53 ± 1.36 ^{ab}	14.5 ± 0.60 ^{ab}	18.06 ± 1.06 ^a	17.53 ± 0.12 ^a

VH: villus height; VW: villus width; CD: crypt depth; VH:CD: villus height per crypt depth ratio. Different letters in the same row indicate significant differences ($p < 0.05$).

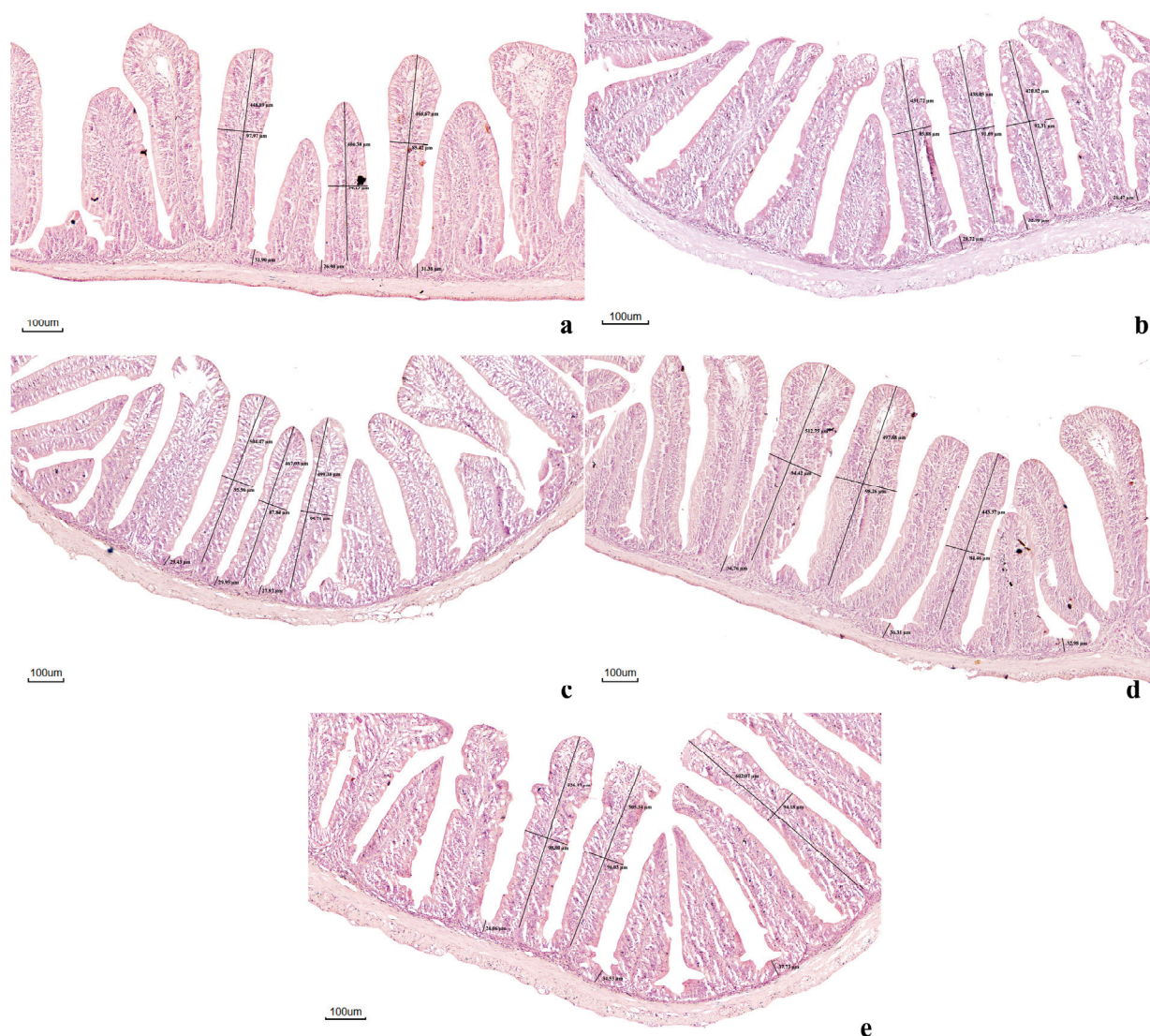


Figure 2. Histological representations of the H&E-stained intestinal sections of Nile tilapia. (a) Control group (FLP0), (b) FLP5: fermented longan peel at 5 g kg^{−1} diet, (c) FLP10: fermented longan peel at 10 g kg^{−1} diet, (d) FLP20: fermented longan peel at 20 g kg^{−1} diet, and (e) FLP40: fermented longan peel at 40 g kg^{−1} diet. Morphological features including villus height and crypt depth were observed across treatments. Scale bars represent 100 μm.

3.5. Innate Immune Response

3.5.1. Mucosal Immune Response

Dietary supplementation with FLP did not produce significant changes in skin mucus lysozyme activity (MLA) or peroxidase activity (MPA) during the first 4 weeks of feeding (Table 9). However, after 8 weeks, both immune parameters increased markedly in fish receiving higher FLP inclusion levels (FLP20 and FLP40) compared with the control group ($p < 0.05$; Table 9). At week 4, MLA and MPA values remained comparable across all treatments ($p > 0.05$). By week 8, MLA reached its highest level in fish fed FLP40 ($17.32 \pm 0.62 \mu\text{g mL}^{-1}$), significantly surpassing the control group ($12.03 \pm 0.33 \mu\text{g mL}^{-1}$) ($p < 0.05$), whereas fish in the FLP10 and FLP20 groups showed intermediate but statistically similar responses. Likewise, although MPA did not vary among diets at week 4, a significant increase was recorded in the FLP20 group at week 8 (0.52 ± 0.02), more than doubling the activity observed in control fish (0.25 ± 0.02) ($p < 0.05$).

Table 9. Skin mucus lysozyme and peroxidase activities of Nile tilapia after 4 and 8 weeks feeding with experimental fermented longan peel (FLP).

		FLP 0	FLP 5	FLP 10	FLP 20	FLP 40
4 weeks	SMLA	12.47 ± 1.13 ^a	11.27 ± 1.56 ^a	9.99 ± 0.66 ^a	11.38 ± 0.99 ^a	11.72 ± 1.36 ^a
	SMPA	0.29 ± 0.03 ^a	0.25 ± 0.04 ^a	0.32 ± 0.03 ^a	0.38 ± 0.04 ^a	0.33 ± 0.05 ^a
8 weeks	SMLA	12.03 ± 0.33 ^b	11.62 ± 1.29 ^b	13.67 ± 1.39 ^{ab}	13.29 ± 1.30 ^{ab}	17.32 ± 0.62 ^a
	SMPA	0.25 ± 0.02 ^b	0.44 ± 0.07 ^{ab}	0.32 ± 0.08 ^{ab}	0.52 ± 0.02 ^a	0.39 ± 0.04 ^{ab}

SMLA: Skin mucus lysozyme activity ($\mu\text{g mL}^{-1}$); SMPA: Skin mucus peroxidase activity ($\mu\text{g mL}^{-1}$). Different letters in the same row indicate significant differences ($p < 0.05$).

3.5.2. Serum Immune Responses

Dietary supplementation with fermented longan peel (FLP) led to significant improvements in several serum innate immune parameters in Nile tilapia after both 4 and 8 weeks of feeding ($p < 0.05$; Table 10). After 4 weeks, serum lysozyme activity (SLA) was markedly higher in fish receiving the 40 g kg⁻¹ FLP diet compared with the control group ($p < 0.05$), while the remaining treatments showed intermediate, non-significant differences. By week 8, SLA was significantly elevated in fish fed 20 and 40 g kg⁻¹ FLP relative to the control, whereas other FLP levels produced moderate responses without statistical differences.

Table 10. Serum lysozyme and peroxidase activities of Nile tilapia after 4 and 8 weeks feeding with experimental fermented longan peel (FLP).

		FLP 0	FLP 5	FLP 10	FLP 20	FLP 40
4 weeks	SLA	6.68 ± 0.65 ^b	7.88 ± 0.2 ^{ab}	7.69 ± 0.36 ^{ab}	8.46 ± 0.33 ^{ab}	9.15 ± 0.73 ^a
	SPA	0.12 ± 0.04 ^b	0.37 ± 0.07 ^{ab}	0.33 ± 0.07 ^{ab}	0.43 ± 0.04 ^a	0.29 ± 0.10 ^{ab}
	ACH50	282.60 ± 22.20 ^{bc}	253.60 ± 44.57 ^c	306.70 ± 49.20 ^{ab}	446.10 ± 12.97 ^a	423.10 ± 23.74 ^{ab}
8 weeks	SLA	5.65 ± 0.21 ^b	7.12 ± 0.39 ^{ab}	6.97 ± 0.17 ^{ab}	7.46 ± 0.43 ^a	7.13 ± 0.22 ^a
	SPA	0.12 ± 0.05 ^b	0.49 ± 0.02 ^a	0.35 ± 0.13 ^{ab}	0.44 ± 0.02 ^{ab}	0.18 ± 0.09 ^{ab}
	ACH50	280.90 ± 27.92 ^b	357.70 ± 15.59 ^{ab}	300.90 ± 39.71 ^b	491.50 ± 26.94 ^a	354.00 ± 34.93 ^{ab}

SLA: Serum lysozyme activity ($\mu\text{g mL}^{-1}$); SPA: Serum peroxidase activity ($\mu\text{g mL}^{-1}$); ACH50: Alternative complement activity. Different letters in the same row indicate significant differences ($p < 0.05$).

Serum peroxidase activity (SPA) also responded to FLP inclusion. At week 4, SPA was significantly increased in the 20 g kg⁻¹ group compared with the control fish ($p < 0.05$). At week 8, however, only the 5 g kg⁻¹ FLP group exhibited a significantly higher SPA value relative to the control group.

Alternative complement pathway activity (ACH50) showed a consistent enhancement, with the 20 g kg⁻¹ FLP diet producing significantly higher ACH50 titres at both sampling points than their respective controls ($p < 0.05$). Other FLP treatments generally yielded intermediate responses, although the 5 g kg⁻¹ group at week 4 was significantly lower than the 20 g kg⁻¹ group.

Overall, FLP supplementation, especially at 20 g kg⁻¹, substantially improved non-specific serum immune responses, demonstrating a clear immunostimulatory effect towards the end of the feeding trial.

3.6. Intestinal Growth, Immune, and Antioxidant-Related Gene Expression

The influence of dietary fermented longan peel (FLP) on the transcription of genes associated with growth regulation, immune function, and antioxidant defense in the intestinal tissue of Nile tilapia is presented in Figure 3. Overall, fish receiving FLP-supplemented diets, particularly those fed 20 and 40 g kg⁻¹, showed pronounced elevations in several target genes ($p < 0.05$). Marked increases were detected for *IGF-1*, *GH*, *Ghrelin*, *NPY- α* , *Galanin*, *hsp70*, *Keap-1*, *nrf-2*, *GST- α* , *Ef- α* , *TLR-7*, *TNF- α* , and *NF- κ B*, demonstrating broad

enhancement of physiological pathways associated with growth, immune competence, and oxidative stress mitigation.

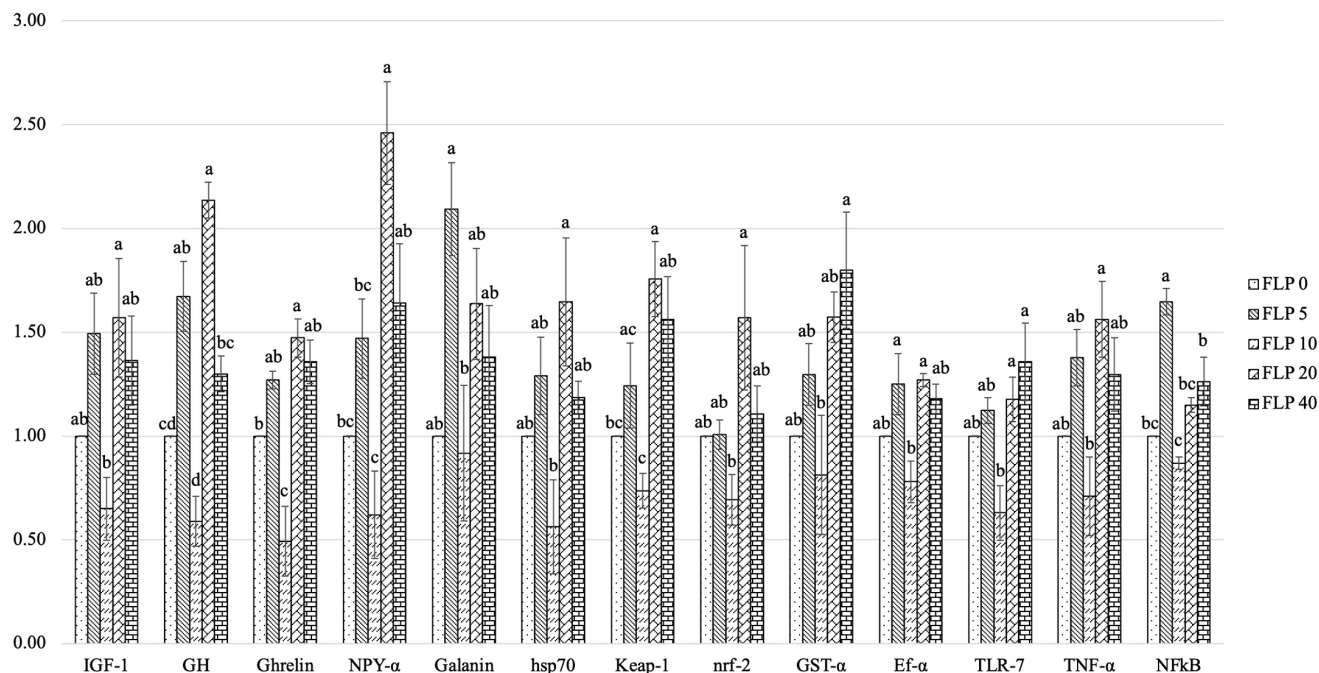


Figure 3. Relative transcript expression levels of growth, immune, and antioxidant-related genes in the intestine of Nile tilapia after 8 weeks feeding trial with fermented longan peel (FLP). B-actin was used as an internal reference gene. Data are presented as mean $\pm$ SEM. Different superscript letters indicate statistically significant differences ($p < 0.05$).

Growth-related markers such as *NPY-α* and *Galanin* exhibited the strongest responses, with transcript abundance in FLP 20 and FLP 40 groups approximately 1.5–2.1 times greater than that of the control, and significantly higher than responses observed in fish fed lower inclusion levels (FLP 5 and FLP 10). Antioxidant-related genes (*hsp70*, *Keap-1*, *nrf-2*, *GST-α*, and *Ef-α*) also showed clear upregulation in the FLP 20 and FLP 40 treatments, reflecting enhancements of roughly 1.2–1.7-fold over the control group, indicating improved cellular protective capacity. In contrast, fish receiving 5 or 10 g kg^{−1} FLP generally did not exhibit significant changes relative to unsupplemented fish.

Immune-regulatory genes, namely *TLR-7*, *TNF-α*, and *NFκB*, likewise increased by approximately 1.3–1.6-fold in the FLP 20 and FLP 40 groups, whereas expression levels in FLP 0, FLP 5, and FLP 10 remained statistically similar. Moderate, non-significant improvements were observed for certain genes (e.g., *IGF-1*, *GH*, *Ghrelin*) in fish fed 10 g kg^{−1} FLP, but these responses did not differ meaningfully from either the control or FLP 5 groups.

Notably, no substantial differences were detected between the FLP 20 and FLP 40 groups for most evaluated genes, suggesting that the transcriptional response tends to plateau beyond 20 g kg^{−1} dietary inclusion. The lowest gene expression levels consistently occurred in the control and FLP 5 treatments.

In summary, incorporating 20–40 g kg^{−1} FLP into Nile tilapia diets markedly stimulated intestinal gene expression related to growth regulation, innate immune function, and antioxidative defense, reaching increases of up to ~2.5-fold relative to the control, while lower supplementation levels produced limited or negligible effects.

4. Discussion

Sustainable aquaculture increasingly depends on innovative nutritional strategies that enhance fish growth, health, and resilience while supporting environmental and

economic sustainability goals [65]. One promising approach is the valorization of agro-industrial by-products as functional feed additives, which contributes to waste reduction and promotes a circular bioeconomy. Among various strategies, fermentation of plant residues has gained growing attention because it can improve the nutritional quality, digestibility, and biological activity of feed materials. Fermented products often showed enhanced antioxidant and immunomodulatory properties due to the degradation of complex polysaccharides, reduction of antinutritional factors, and increased availability of bioactive compounds [66]. In the present study, dietary supplementation with fermented longan peel (FLP) significantly improved growth performance, digestive enzyme activity, immune responses, and the expression of growth-, immune-, and antioxidant-related genes in Nile tilapia (*Oreochromis niloticus*).

Growth performance is a key indicator of feed efficiency and overall physiological status in aquaculture species [67]. In the present study, fish receiving 20 g kg⁻¹ of FLP showed significant improvements in growth performance and feed efficiency compared with the control group. Although research on fermented longan peel in fish nutrition remains limited, similar growth-promoting effects have been reported with other fermented agro-industrial by-products. For example, fermented corn cob improved growth performance and feed efficiency in Nile tilapia [22], while fermented sweet potato residue enhanced nutrient utilization and immune status in common carp (*Cyprinus carpio*) [68]. Comparable results have also been reported in European seabass (*Dicentrarchus labrax*) [69] fed solid-state fermented brewer's spent grain; Nile tilapia fed fermented rice bran [70], fermented *Sargassum muticum* [21], fermented soybean meal [71], and fermented corn husk [25]. Fermentation can improve the nutritional value of plant ingredients by reducing fiber and antinutritional compounds while increasing the availability of nutrients and bioactive components [72]. These improvements may enhance nutrient digestibility and utilization, thereby supporting better growth performance [24]. Moreover, the presence of lactic acid bacteria, such as *Lactiplantibacillus plantarum*, may also contribute to the modulation of gut microbiota, fostering a balanced microbial community that supports nutrient absorption and intestinal health [73,74]. In the present study, enhanced digestive enzyme activities, particularly amylase, protease, and trypsin, were observed in fish fed the FLP20 diet. Similar enhancements in digestive physiology have been reported in fish fed fermented plant-based feed additives and phytochemical ingredients [75,76]. The fermentation process may increase the availability of nutrients and bioactive compounds that support digestive function and intestinal physiology [17,77].

Histological observations further supported these findings. Nile tilapia fed FLP-supplemented diets exhibited clear improvements in intestinal microstructure compared with the control group. Fish in the FLP10 and FLP20 groups displayed taller and more organized villi, deeper crypts, and well-aligned enterocytes, indicating improved intestinal health and absorptive capacity. Increased villus height expands the absorptive surface area of the intestine, facilitating more efficient nutrient uptake, while deeper crypts indicate active epithelial renewal and mucosal integrity [78]. The improved intestinal microstructure observed in the FLP-fed groups may be associated with the antioxidant properties of polyphenols and flavonoids present in fermented longan peel [14]. These compounds are known to help maintain epithelial integrity and protect intestinal tissues from oxidative stress [79,80]. Similar improvements in intestinal microstructure have been reported in African catfish (*Clarias gariepinus*) fed fermented spent coffee ground [81], spotted seabass (*Lateolabrax maculatus*) fed compound probiotics fermented soybean meal [71], and Nile tilapia (*Oreochromis niloticus*) fed fermented rice hulls [82] and fermented *Sargassum muticum* [21]. In the present study, the improved intestinal microstructure observed in the

FLP10 and FLP20 groups is consistent with the enhanced digestive enzyme activities and growth performance.

Fish rely heavily on their skin mucus and serum innate immune components as the first line of defense against environmental and pathogenic challenges [83,84]. Dietary supplementation with functional feed additives has been reported to enhance immune responses and improve disease resistance in aquaculture species [85,86]. In the present study, dietary FLP supplementation significantly increased lysozyme, peroxidase, and alternative complement (ACH50) activities in both serum and skin mucus of Nile tilapia. These parameters play important roles in nonspecific immune defense mechanisms. Similar immunostimulatory effects have been reported with fermented feed additives, including fermented *Sargassum muticum* [21], fermented corn husk and corn cob [22,25], fermented soybean meal [71], and pomelo (*Citrus grandis*) peel and soybean meal co-fermented protein [87]. The improved immune responses observed in the present study may be associated with the polyphenol and flavonoid content of fermented longan peel (Table 1), as these compounds possess antioxidant and immunomodulatory properties [88]. Second, the fermentation process generates bioactive metabolites, such as lactic acid, peptides, and exopolysaccharides, which may serve as immunostimulants by activating macrophages, complement pathways, and pattern-recognition receptors [89–91]. Moreover, probiotic microorganisms like *Lactiplantibacillus plantarum* present in the fermented substrate may directly modulate gut-associated lymphoid tissue (GALT) and promote mucosal immune responses through improved microbial balance and short-chain fatty acid production [92,93]. Gene expression analysis provided further insight into the physiological mechanisms underlying the observed responses [94]. Dietary FLP supplementation significantly upregulated genes associated with growth regulation (*IGF-1*, *GH*, *Ghrelin*, *NPY- α* , and *Galanin*), immune responses (*TLR-7*, *TNF- α* , *NF κ B*), and antioxidant defense (*hsp70*, *Keap-1*, *nrf-2*, *GST- α* , and *Ef α*). It should also be noted that the transcriptional responses observed in the FLP10 group did not consistently follow a linear dose–response pattern. Such variations are commonly reported in nutritional gene expression studies, as gene regulation is influenced by complex physiological feedback mechanisms and individual biological variability. In the present study, the most consistent transcriptional activation occurred in the FLP20 group, which corresponded with the superior growth performance, digestive enzyme activity, and immune responses observed at the physiological level. The upregulation of *GH* and *IGF-1* corresponds with the improved growth performance observed in FLP-fed fish, as these genes play key roles in somatic growth regulation and protein synthesis in fish [95–97]. Similar transcriptional responses have been reported in fish fed fermented plant-based feed additives, including fermented rice bran [70], fermented corn husk and corn cob [25], fermented *Sargassum muticum* [21], and fermented spent coffee ground [98]. Increased expression of immune-related genes such as *TLR-7*, *TNF- α* , and *NF- κ B* indicates activation of innate immune signaling pathways [99,100], which is consistent with the elevated immune parameters observed in serum and mucus. Moreover, the upregulation of antioxidant-related genes including *nrf-2*, *Keap-1*, *GST- α* , and *hsp70* suggests enhanced antioxidant defense capacity and improved tolerance to oxidative stress [101].

An important contextual factor of this study is that Nile tilapia was cultured within a biofloc technology (BFT) system, which is characterized by complex microbial processes that contribute to nutrient recycling and water quality management. Biofloc systems stimulate heterotrophic bacterial growth through carbon supplementation and manipulation of the carbon-to-nitrogen ratio [102]. The microbial aggregates generated in BFT systems contain proteins, enzymes, vitamins, and immunologically active compounds that can contribute to fish nutrition and health [103,104]. Therefore, the physiological responses observed in this study likely reflect an interaction between dietary FLP supplementation and the

biofloc culture environment. The polyphenols and flavonoids present in fermented longan peel may complement the nutritional and immunological benefits associated with biofloc systems, thereby contributing to improved digestion, immune responses, and antioxidant capacity. The absence of additional benefits at the highest FLP inclusion level (40 g kg⁻¹) suggests that excessive inclusion of fermented plant materials may reduce dietary balance or limit nutrient utilization efficiency. Similar threshold responses have been reported for other phytogetic feed additives in aquaculture diets [24]. These findings indicate that moderate inclusion levels of FLP provide the most favorable physiological outcomes.

5. Conclusions

The present results demonstrate that dietary supplementation with fermented longan peel at an inclusion level of 20 g kg⁻¹ can enhance growth performance, digestive function, intestinal microstructure, immune responses, and antioxidant defense in Nile tilapia cultured under biofloc conditions. These findings support the potential application of fermented longan peel as a sustainable phytogetic feed additive for improving fish health and productivity while contributing to the valorization of agricultural by-products in aquaculture systems.

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Informed Consent Statement: All experimental procedures involving Nile tilapia (*Oreochromis niloticus*) were conducted in accordance with the ethical guidelines for animal care and use established by the Faculty of Agriculture, Chiang Mai University, Thailand. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Chiang Mai University (Approval No.: [RAGIACUC001/2567]). Fish were handled with utmost care to minimize stress, and anesthesia was applied prior to sampling to ensure animal welfare.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Article

Shrimp White Spot Viral Infections Are Attenuated by Organic Acids by Regulating the Expression of HO-1 Oxygenase and β -1,3-Glucan-Binding Protein

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Abstract: The absence of efficient on-farm interventions against white spot syndrome viral (WSSV) infections can cause significant economic losses to shrimp farmers. With this exploratory study we aimed to test, both in vitro and in vivo, the efficacy of an organic acid mixture (Aq) against WSSV infections in shrimp. In vitro, using shrimp gut primary cells (SGP), 2% Aq significantly reduced WSSV infection and the amounts of H₂O₂ released but had no impact on CAT and SOD expression. In vivo, in a shrimp challenge test, 2% Aq significantly downregulated the expression of proteins involved in WSSV virulence, such as the lipopolysaccharide- β -1,3-glucan-binding protein (LGBP) and the TLR signalling pathway (LvECSIT), and increased the expression of HO-1 oxygenase. Additionally, at 2% Aq, the expression of the digestive-related enzyme carboxypeptidase B was upregulated in the gut, alongside a significant decrease in IL-22 expression, a cytokine usually increased during WSSV infection in shrimp. A low mortality rate (7.33%) was recorded in infected shrimp treated with 2% Aq compared to the 96.66% mortality in the absence of Aq. The peritrophic membrane (PM) was proven essential to ensure Aq efficacy, as the infected and treated PM deficient shrimp (PM[−]) had a mortality rate of 27.8%, compared to only 9.34% mortality in the infected shrimp at 2% Aq and in the presence of PM (PM⁺). Aq significantly increased the expression of mucin-1, mucin-2, mucin-5AC, mucin-5B, and mucin-19 in both PM⁺ and PM[−] shrimp. Conclusively, organic acid in mixtures can protect farmed shrimp against WSSV infection and increase their survivability through a mediated gut health effect which includes resistance to oxidative stress and improved immunity.

Keywords: white spot syndrome; shrimp; natural antimicrobials; organic acids; AuraAqua

1. Introduction

Aquaculture has become one of the world's most important and most rapidly developing sectors of the global food industry, particularly in the Asia-Pacific region due to the demand in high quality protein [1]. The Pacific white shrimp (*Litopenaeus vannamei*)

represents over 70% of the world's crustacean production, is worth above USD 18 billion, and employs over a million people [1–3]. For instance, in 2021, over USD 33 billion was estimated in global shrimp exports, contributing greatly to global food security and economic development [4]. The economic implications of white spot syndrome virus (WSSV) are profound, shaping the research priorities in shrimp aquaculture, including vaccination, and the integration of herbal and probiotic immune enhancers to overcome these high-cost barriers. Moreover, because the global shrimp production now exceeds 5 million tons per year [5], as reported by the Food and Agriculture Organization of the United Nations (FAO), it is noteworthy to provide robust, economically viable solutions to mitigate and combat WSSV and to maintain the viability of this important industry [1].

WSSV infects a diverse range of crustacean hosts, including the commercially important shrimp species *L. vannamei* and *Penaeus monodon* ($\approx 90\%$ of the market), but it can also infect crabs as well crayfishes [6–9]. The virus targets tissues of both ectodermal and mesodermal origin, with a specific affinity for the hepatopancreas of shrimp, where the host exhibits clinical symptoms such as lethargy, a discoloured exoskeleton, and pronounced white spots on the carapace [7,9,10]. Of note, the hepatopancreas, a vital organ for shrimp immune function, serves as the primary battleground in the resolution against WSSV, mediating both metabolic and immune responses [7].

Several biologic compounds providing high efficacy against WSSV have been recently revealed, including baicalein from *Scutellaria baicalensis* (SBEE) and coumarin derivatives (P13) [5,11]. Baicalein, at concentrations of 100 μM , has demonstrated a 66.7% effectiveness in reducing WSSV genome copy numbers in infected shrimp larvae, while also upregulating immune genes like Toll-4, Dorsal, and AMPs. This dual mechanism restricts viral replication, statistically reduces viral loads in vivo, and remarkably blocks the horizontal transmission of WSSV between hosts by enhancing host immunity, making it a viable candidate for inclusion in feed additives [11]. Similarly, P13 [N-(4-methyl-2-oxo-2H-chromen-7-yl)acetamide], a synthesized coumarin derivative, at an effective concentration of $\text{EC}_{50} < 12 \text{ mg/L}$, has shown an antiviral response of over 90% in *L. vannamei* [5]. Following larvae infection with WSSV, treatment with P13 resulted in a notable viral inhibition and lower mortality rate of 45% compared with the control DMSO, which had a mortality rate of 80% at 120 hpi [5]. Plant-derived compounds like moringa (*Moringa oleifera*) leaf extract and olive (*Olea europaea*) leaf extract (OLE) have also gained importance for their immunomodulatory effects [12,13]. At the hepatopancreatic level, moringa leaf extracts at 0.5–1, including leaf powder at 25 g/kg, significantly enhanced the expression of immune genes such as alpha-2-macroglobulin ($\alpha 2\text{M}$), integrin β , LGBP, and peroxinectin (PX) [12]. Again, high doses of OLE improved both biochemical (total protein and glucose) and immunological parameters (OxH, SOD, PO activity, and clotting time), effectively supporting shrimp survival by up to 65% under viral challenges compared to the control [13]. Another promising compound, cuminaldehyde, achieved an 89% inhibition rate of WSSV replication at 40 $\mu\text{mol/L}$ after 72 hpi and demonstrated remarkable potential in limiting horizontal transmission [14].

AuraAqua (Aq), a mixture of organic acids and other biological compounds, has been previously identified as efficient in preventing parasitic and bacterial infections in crustaceans [15]. Unlike conventional antibiotics, these compounds exhibit minimal environmental residues, lower toxicity, and reduced potential for resistance development [16]. Moreover, their multifunctional properties—ranging from antiviral and antibacterial activities to immune modulation—could make them significant in addressing the multifaceted challenges posed by pathogens like WSSV [17]. With this study, we aimed to investigate if a mixture of multiple organic acids (AuraAqua) can also prevent WSSV infection in vitro and alleviate the negative impact of infection in vivo. Moreover, we aimed to describe the

biological mechanism by which the antimicrobial mixture exhibits its anti-viral effect and improves host immunity.

2. Materials and Methods

2.1. Virus, Antimicrobial Mixture and Shrimp Gut Primary Cell Isolation

White spot syndrome virus (WSSV) was propagated and isolated as previously described [18]. Post-larvae shrimps were purchased from a local hatchery in Bistrita, Romania and further cultivated in our research facilities as required in our experimental design. Ethical approval was not required for this study. After 48 h post-infection, the allantoic fluids were harvested using low-speed centrifugation and stored at $-70\text{ }^{\circ}\text{C}$. Virus preparations were performed as previously described [19]. In our study, we used AuraAqua, a mixture of maltodextrin (4%) and sodium chloride (0.5%). As additives, lactic acid (30%), citric acid (15%), and citrus extract (8%) were also included and the balance to 100% was made up with dH_2O . For primary cell preparations (SGPs), 5–10 *P. vannamei* gut tissue samples were harvested and prepared as previously described [20].

2.2. WSSV Quantification Using PCR

The virus quantification was performed as previously described [21]. Twenty milligrams of gut tissue from shrimp or infected SGP cells were homogenized in 500 μL of guanidine lysis buffer (50 mM Tris-HCl, 25 mM EDTA, 4 M guanidinium thiocyanate, 0.5% N-lauroylsarcosine, pH 8.0) at RT (room temperature). After centrifugation at $15,000\times g$ for 3 min, 20 μL of silica was added to the supernatant for DNA absorption. Then, the mixture was spun for 5 min and centrifuged at $15,000\times g$ for 30 s. The pellet was washed twice with 70% ethanol, resuspended in 20 μL dH_2O , and centrifuged at $15,000\times g$ for 2 min. WSSV-specific primers were used for PCR (forward primer 5'-tattgtctctctgacgtac-3' and reverse primer 5'-cacattcttcacgagctctac-3'). The parameters for PCR reactions were as follows: 5 min at $94\text{ }^{\circ}\text{C}$, 40 cycles at $94\text{ }^{\circ}\text{C}$ for 45 s and $68\text{ }^{\circ}\text{C}$ for 1 min, and extension at $68\text{ }^{\circ}\text{C}$ for 5 min. For quantitative analysis of viral DNA, the real-time PCR was conducted. The TaqMan probe was 5'-FAM-tgctgccgtctccaa-TAMRA-3'. DNA standards were prepared as previously described [18] in order to generate a standard curve for absolute quantitation. Viral DNA was isolated and amplified using the above-mentioned primers at similar conditions. For generation of the standard curve, ten-fold serial dilutions of the DNA were made over a range of 8 log units (10^9 – 10^2) (efficiency: 98.64%; R^2 : 0.99). The results of three independent experiments are shown as mean $\pm$ standard deviation (SD). *p* values of <0.05 were considered significant.

2.3. Cytotoxicity Assay (CC_{50} , EC_{50} , and SI)

The AuraAqua mixture was used to determine the EC_{50} and SI (selective index). The mixture was titrated from 1 to 1:128 CC_{50} and used for virus contact. After the addition of 10 μL MTT reagent (Sigma Aldrich, Gillingham, UK), the samples were incubated for 4 h at $37\text{ }^{\circ}\text{C}$. The EC_{50} concentrations were calculated against Aq concentrations. To find out the cytotoxic activity of Aq, the antimicrobial product was dissolved in water and added to the SGP medium at concentration of 2% to quantify the host cell effect. The SGP cells were grown in 0.001–0.2% Aq containing medium. The experiment was repeated three times and on three different occasions. The absorbance of each well was recorded at 620 nm in a microplate reader (Fluostar Omega, BMG Labtech, Belfast, UK) to calculate the percentage of cell survival.

2.4. In Vitro Antiviral Activity

For antiviral activity, SGP cells were exposed to Aq before infection or infected with Aq pre-exposed virus. Following supernatant removal, the cells were washed and infected. In the case when the antimicrobial mixture was used to pre-treat the virus, the incubation took place for 1 h at room temperature followed by SGP cell infection. The absorption period was analyzed by mixing the viruses individually with 0.1%, 0.5%, and 2% Aq followed by infection of cells. For replication measurement, the inoculum was removed and replaced with media containing 0.5% methylcellulose. The experiment was repeated three times and at three different occasions. Data were subjected to statistical analyses using the Graph Prism software package version 11.

2.5. In Vivo Shrimp Challenge Test

Firstly, the in vivo activity of AuraAqua against WSSV was performed to test its activity in preventing *Penaeus vannamei* post-larvae survival. Twenty-five shrimp post-larvae per replicate were plated in sterile Petri dishes and intramuscularly inoculated into *P. vannamei* (shrimp infectious dose with 50% endpoint) per mL. The antimicrobial mixture was applied at the time of infection in concentrations of 0% and 2% in 5 L flasks. Survival was determined by counting the larvae at 2 days after infection. The first controls included an un-infected group and in the absence of Aq. The second control included WSSV-challenged shrimp in the absence of Aq. The experiment was performed in triplicate. Secondly, we infected shrimp with removed and non-removed peritrophic membrane. The removal of the peritrophic membrane was performed as previously suggested [19]. Shrimps were inoculated orally with 50 µL of a 10^{-3} WSSV stock. A total of 10 DNA samples was used for analysis from each experimental group. As previously described [22], the resulting infected and un-infected shrimp gut tissue was also used to quantify using qPCR the relative expression levels of mucin-1 (F ggctcgaagtggcgatgatg; R cgatggctcaatggcgaagagg), mucin-2 (F tgccagccacgtctctcttg; R ccgcagccgaggcagtc), mucin-5AC (F agcaggactcaacgactacaacag; R gcgcgacgccgatgatgg), mucin-5B (F ctgacgcatacgtcagggttc; R tcgcgcgtctcatctctg), and mucin-19 (F gaagaggaggaagaggacgaggag; R ggaccaccaggcacaagaacatc). The β -actin gene was used as reference gene (F gccctgttcagccctcatt; R acggatgtccacgtcgact). The experiment was performed in triplicate. Quantification of LGBP gene expression using real-time RT-PCR was performed as previously described [23]. Briefly, the amplification was performed by using LGBP (β -1,3-glucan-binding protein) gene-specific primers (F ggtaaccagtacggaggaacga and R, tactcgacgtgggtcttctcga) from gut tissue cDNA, using an SYBR Green RT-PCR at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. As internal control, we used the actin gene (F-tgccgaactgctgaccaaga and R, ccggcttcagttcttacc). The experiment was repeated three times. The levels of oxygenase expression (HO-1) were detected as previously described [24] in the shrimp gut. The primers used for HO-1 amplification were qHO-1-F gcatggcagtgaccgagattga and qHO-1-R gtcgtgcttctctctcatc. The relative gene expression level for investigated genes was analyzed using the $2^{-\Delta\Delta C_t}$ method. The expression levels of LvECSIT were measured in gills at 2 days post-infection from each group (control, infected and infected + 2% Aq). The primers used were in this study were LvECSIT-F atgattcttatgaacgctt and LvECSIT-R aatttgggcatccagta [25]. β -actin was used as internal reference, and the PCR parameters were used as previously described [26]. The expression of carboxypeptidase B in the hepatopancreas and in the gut tissue of challenged shrimp and treated with 2% Aq was performed as previously described also using the primers CPB-real time-F gacatttcgtagaccatcacc and CPB-real time-R gaacttgccactatacagcgt [27].

2.6. Peritrophic Membrane (PM) Extraction and Isolation

P. vannamei ingesta free PMs were extracted as previously described [28]. Briefly, after 3 days of feed-free time, the PMs were suspended in 100 mL of 1% calcofluor (Sigma Aldrich, Gillingham, UK) containing protease inhibitors (Sigma, UK).

2.7. Measurements of H_2O_2 , SOD, and CAT

The effect of AuraAqua on superoxide dismutase (SOD) and catalase (CAT) in WSSV-infected SGP cells or in challenged shrimp was measured as previously described in 10 samples [20]. SOD activity was established by means of a commercially available SOD colorimetric activity kit (Thermo Fisher, Horsham, UK) and CAT by using a catalase activity kit (Abcam, Trumpington, UK, ab83464). The methods were performed as indicated in the instruction manuals. Inhibitors of NADPH activity, including diphenyleneiodonium chloride (DPI, Sigma; 15 μ M, 45 min preincubation and wash out) and bovine liver catalase (Sigma-Aldrich, Gillingham, UK; 300 U/mL), were used during the 24 h measuring interval. The SOD and CAT activity was measured in disrupted gut tissue using sonication for 60 s (4 $\times$) at 4 °C (in ice) in 1% saline solution and centrifuged at 2500 rpm at 4 °C for 5 min (Ultrawave DP200-00, Ultrawave Ltd., Cardiff, UK). Superoxide dismutase (SOD) and catalase (CAT) activity in WSSV-challenged shrimp was measured in the resulted supernatants. Hydrogen peroxide (H_2O_2) in the WSSV-infected SGP cells was measured using a PeroxiDetect™ Kit (Sigma-Aldrich, Gillingham, UK). All experiments were performed in triplicate.

2.8. IL 22 Expression Using qPCR and Western Blotting

To analyze the expression of IL-22 using qPCR, total RNA was extracted from infected SGP cells. The gut tissue was disrupted using sonication for 60 s (4 $\times$) at 4 °C (in ice) in 1% saline solution followed by centrifugation at 2500 rpm at 4 °C for 5 min (Ultrawave DP200-00, Ultrawave Ltd., Cardiff, UK). The RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Manchester, UK) and reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche, East Sussex, UK). The mRNA levels were determined using quantitative RT-PCR using QuantiNovaSYBR Green PCR Kit (Qiagen, Manchester, UK) on a LightCycler 96 (Roche, East Sussex, UK). For quantification, primers IL-22-qF ccgtactgttagcaacagtgcag and IL-22-qR tcacattcttgagagcaggattc were used [29]. The qPCR protocol included 1 $\times$ 95 °C for 30 s and 40 $\times$ 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. Fold changes of expression were calculated by comparing the relative expression levels of the experimental groups with those of corresponding control groups, as previously described, also including detection using Western blotting [30]. For Western blotting, the anti-IL-22 antibody (P211) was purchased from Thermo Fisher Scientific, Manchester, UK. Briefly, following extraction, the proteins were separated in 15% SDS-polyacrylamide gel, transferred to nitrocellulose membrane using a 25 mM Tris buffer. The membranes were blocked with 5% milk in 20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, at pH 8.0 (TBST buffer) overnight at 4 °C. The next day, the membrane was incubated with IL-22 primary antibody overnight at 4 °C. The membranes were extensively in TBST buffer followed by horseradish peroxidase-conjugated secondary antibody (Thermo Fisher, Horsham, UK) for 2 h at room temperature. The membranes were washed three times with TBST and the blots were detected by using enhanced chemiluminescence reagent (ECL) and exposed to photographic films (Kodak, Thermo Fischer Scientific, Manchester, UK). The experiment was repeated three times.

2.9. Statistical Analysis

GraphPad Prism 10 software was used to perform statistical analyses. A p value < 0.05 was considered statistically significant following estimations using the Student's t -test. An ANOVA was performed to analyze the percentage decrease between Aq concentrations compared to control using one-way ANOVA ($p < 0.0001$).

3. Results

3.1. The In Vitro Effect of Aq Against WSSV Replication and Ability to Infect SGP Cells

The direct effect of Aq on WSSV replication was first investigated in vitro. The 50% effective concentration (EC_{50}) of Aq was detected at $0.003 \mu\text{L/mL}$ (Table 1). Next, we aimed to demonstrate the effect of Aq on the WSSV replication by treating the SGP cells and the virus with Aq prior to infection, through their inclusion during adsorption or after adsorption. Infected SGP cells in the absence of Aq were used as a control (Figure 1). The pre-treatment of cells with Aq (0.1%, 0.5%, and 2%) led to a significant reduction in virus production compared to the control. Similar results were detected during viral replication, when Aq was included only in the cell media. Collectively, these results suggest that the anti-viral effect of AuraAqua is exerted through all phases of host cell infection. The concentration of 2% Aq was selected for further investigations.

Table 1. Cytotoxicity and efficiency of AuraAqua.

Virus	CC_{50} ($\mu\text{L/mL}$)	EC_{50} ($\mu\text{L/mL}$)	SI
WSSV	0.21	0.003	70

CC, cytotoxic concentration; EC, effective concentration; SI, selective index (CC_{50}/EC_{50}).

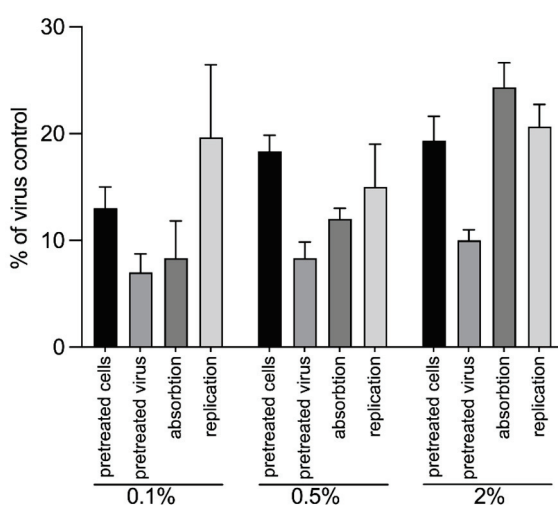


Figure 1. The anti-viral effect of AuraAqua against WSSV. AuraAqua (Aq) was added at the non-cytotoxic concentrations of 0.1%, 0.5%, and 2%. SGP cells were either pre-treated with Aq prior to WSSV infection, pre-treated with WSSV prior to infection, or Aq was included during the adsorption period (adsorption) or after penetration of the virus into cells (replication). One-way ANOVA ($p < 0.0001$) was used to analyze the data and significance of percentage decrease at the different concentrations.

The percent reduction in WSSV replication was calculated relative to the amount of virus produced in controls, and a concentration of 2% was used further in all assays. The presence of 2% Aq during the in vitro infection assay gradually decreased the WSSV replication levels until they reached significance ($p = 0.03$) at 2% Aq after 72 h (Figure 2A). Additionally, we found that, at 72 h post-infection, there was a significant increase in oxidative stress as measured by the amount of H_2O_2 released by the infected SGP cells

(Figure 2B). However, these levels were significantly reduced in the presence of 2% Aq ($p = 0.004$). Our results also showed that the addition of DPI during infection resulted in reduced levels of H_2O_2 being released due to their role in cellular NADPH inhibition. Both CAT (Figure 2C) and SOD (Figure 2D) activity were also decreased in the presence of 2% Aq, a consequence of decreased infection. Overall, these results show that Aq can prevent WSSV replication, cellular infection, and oxidation.

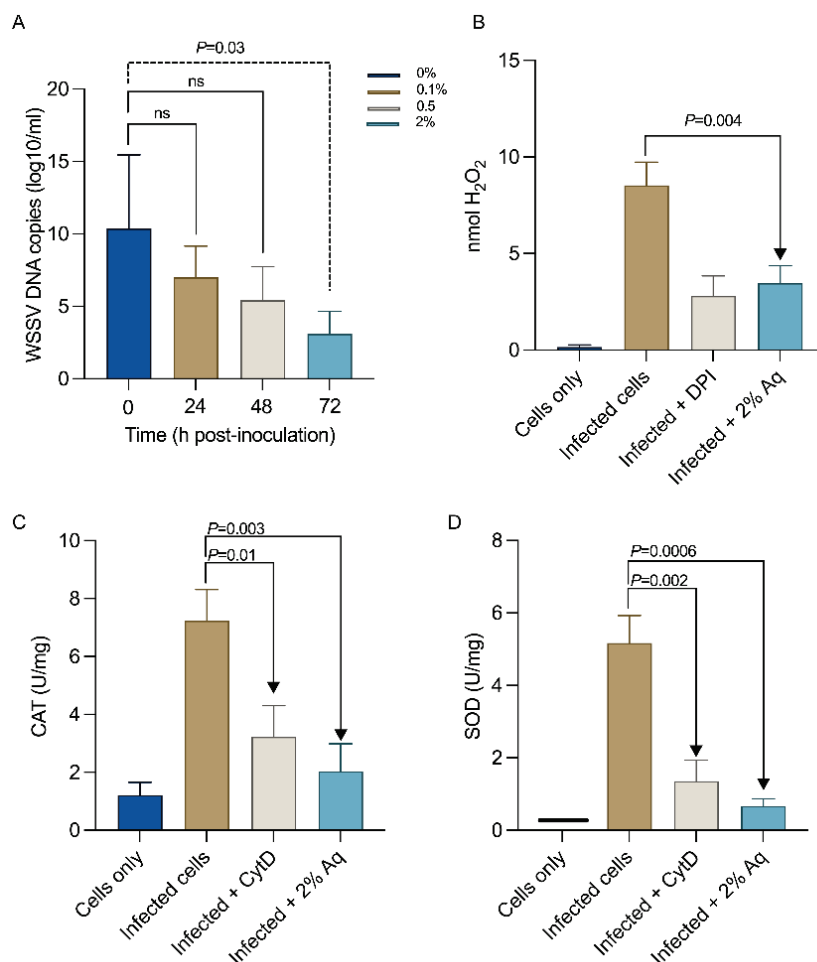


Figure 2. Time course of WSSV infection of SGP cells as assessed using qPCR (A) at 2% Aq. The WSSV DNA copies were measured at 27 °C at 0, 24, 48, and 72 h post-inoculation. H_2O_2 release (B), catalase (C), and superoxide dismutase (D). The results are presented as the mean of three independent experiments of the copy number of WSSV genomes in the WSSV-infected SGP cells incubated at 27 °C over time. The statistical significance was determined using Student *t*-test; *p* values are indicated on the graphs. (ns—not significant).

3.2. The Effect of 2% Aq in Preventing WSSV-Induced Mortality and Stimulating Mucin Production In Vivo and the Impact of PM

To estimate the efficiency of Aq, we performed challenge studies by infecting *P. vannamei* shrimps with WSSV (Figure 3). The mortality rates decreased from 96.66%, recorded in the infected shrimp (wild-type gut) and in the absence of Aq, to 7.33% in the presence of 2% Aq (Table 2) (the average mortality between the experiments is presented).

The decrease in mortality was proportional with the increase in Aq concentration. Moreover, as indicated in Figure 4E, a significant increase ($p < 0.05$) in mucin-1, mucin-2, mucin-5AC, mucin-5B, and mucin-19 genes was also detected. These results clearly indicate that the antimicrobial mixture has the potential to protect the shrimp cultures in vivo.

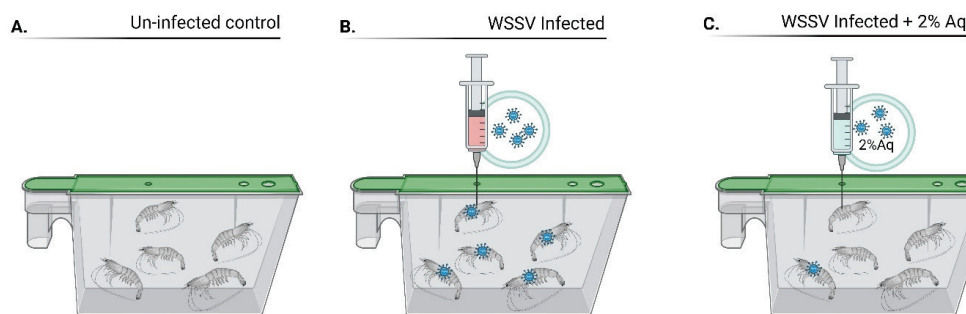


Figure 3. In vivo experimental design. The experiment included an un-infected and un-treated control (A); WSSV infected and un-treated (B); WSSV infected + 2% Aq (C). The experiment was performed for a period of 2 days and repeated three times. The experiment was also performed in the presence/absence of the peritrophic membrane (PM). Designed with Biorender.com.

Table 2. Mortality of *Penaeus vannamei* 2 days post-challenge after infection with WSSV.

AuraAqua Concentration (%)	% of Mortality
0	96.66
0.1	54
0.5	28
2	7.33

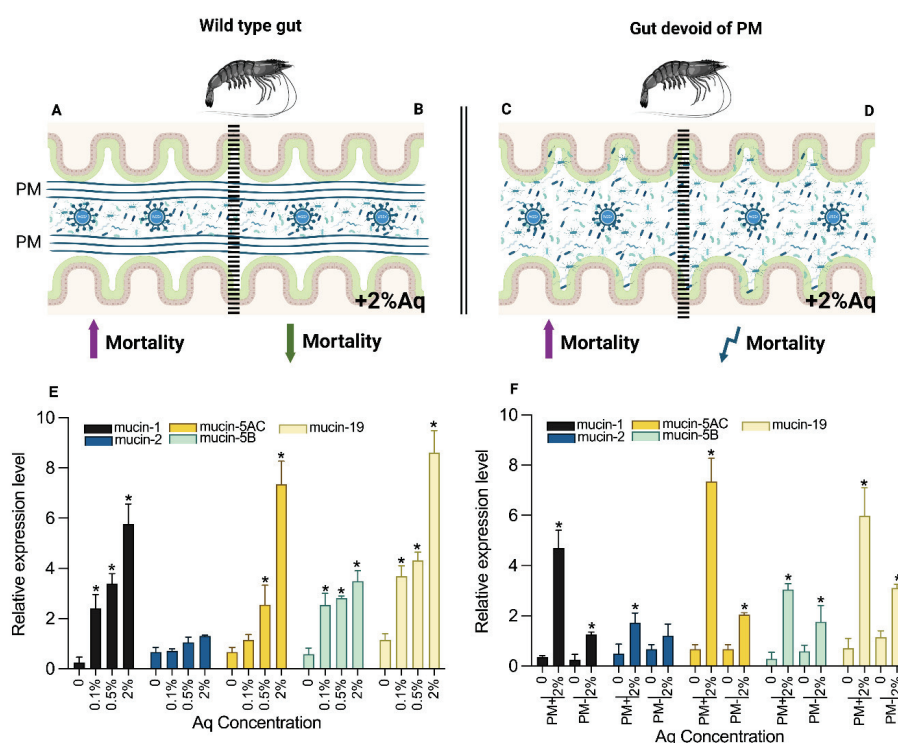


Figure 4. The impact of PM in mediating shrimp survival in the presence of Aq. Panels (A,B) (+2% Aq) indicate the experimental design in the infected wild-type shrimp. The design for the shrimp devoid of PM is presented in panels (C,D) (+2% Aq). Mucins gene expression levels in the intestines of wild-type *P. vannamei* at 2 days post-WSSV infection (E) and in the PM devoid gut (F). β -actin expression was used as reference. Vertical bars represent the mean $\pm$ SE (n = 3). Data indicated with asterisks (*) were significantly different ($p < 0.05$) among the two groups at the same time point. Panels (A,B) designed with Biorender.com.

Next, we aimed to explore if the peritrophic membrane (PM) has a role in mediating Aq efficacy. The data presented in Table 3 clearly indicate that in the absence of PM, the mortality rates decreased to only 27.8% compared to 9.34% in the presence of PM.

Table 3. Mortality of *Penaeus vannamei* 2 days post-challenge after infection with WSSV and in the presence/absence of PM.

AuraAqua Concentration (%)	% of Mortality (PM+)	% of Mortality (PM−)
0	100	97.4
0.1	63	85
0.5	18	67.3
2	9.34	27.8

This lower survival rate in the PM group clearly indicates that PM has a role in mediating the efficacy of Aq. In addition, the results presented in Figure 4F show that in the absence of PM (at 2% Aq), the gut responds by significantly increasing the expression of mucin-1, mucin-2, mucin-5AC, mucin-5B, and mucin-19 genes, and potentially contributes to lower WSSV infection rates in shrimp (Figure 4F). These results show that under the influence of Aq, the shrimp gut structures improve their resilience by increasing the expression of mucins with a direct impact on shrimp survivability during WSSV infection.

3.3. The Impact of Aq on the WSSV Levels in the Shrimp Gut and on CAT and SOD Expression

Our next goal was to measure the gastrointestinal levels of WSSV in the infected shrimp gut tissue. Clearly, as indicated in Figure 5A, the levels of WSS virus were significantly increased ($p < 0.05$) following infection of the un-treated group. However, in the presence of 2% Aq, the infection levels dropped significantly ($p < 0.05$). This reduction in virus load in the presence of 2% Aq was also associated with a significant reduction in SOD (Figure 5B) and CAT (Figure 5C) production in the infected shrimp. Firstly, these results confirmed the initial in vitro results indicating a decreased infection rate in the presence of 2% Aq. Secondly, the antioxidant role of CAT or SOD has become unnecessary in the presence of Aq, probably due to the decreased infection rate mediated via a separate mechanism.

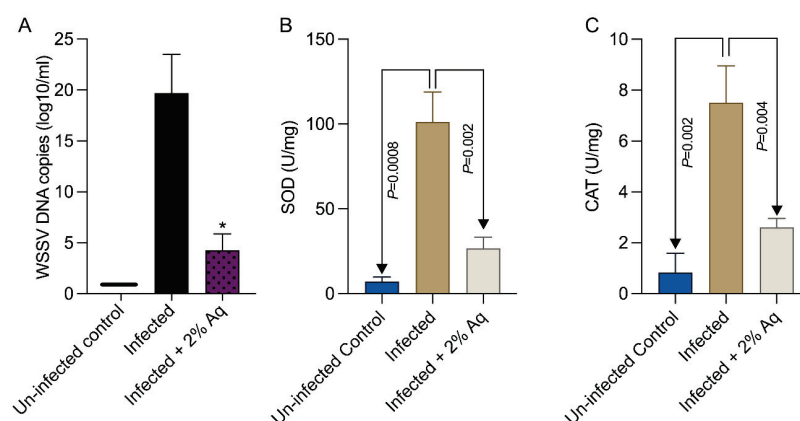


Figure 5. The WSSV (A), SOD (B), and CAT (C) levels in the shrimp gut tissue in the presence of 1% Aq. The results are presented as the mean of three independent experiments of the copy number of WSSV genomes in gut tissue at 2 days post-challenge. The statistical significance was determined using Student *t*-test; *p* values are indicated on the graphs. Data indicated with asterisks (*) were significantly different ($p < 0.05$) among the two groups at the same time point.

3.4. The Impact of Aq on the Expression of LGBP and HO-1 Proteins

To further explore the impact of Aq in mitigating WSSV-induced oxidative stress, we measured the expression of β -1,3-glucan-binding protein (LGBP) and the antioxidant protein HO-1 oxygenase in the gut tissue. The expression of the WSSV interaction protein, LGBP (Figure 6A), was significantly reduced ($p = 0.0003$), while the expression of the antioxidant protein, HO-1 (Figure 6B), exhibited a significant increase ($p = 0.001$). These results confirm that the reduction in WSSV infection was caused by Aq, firstly by blocking

the cellular entry mechanisms and secondly via an increased antioxidant mechanism to reduce the impact of infection-generated oxidative stress.

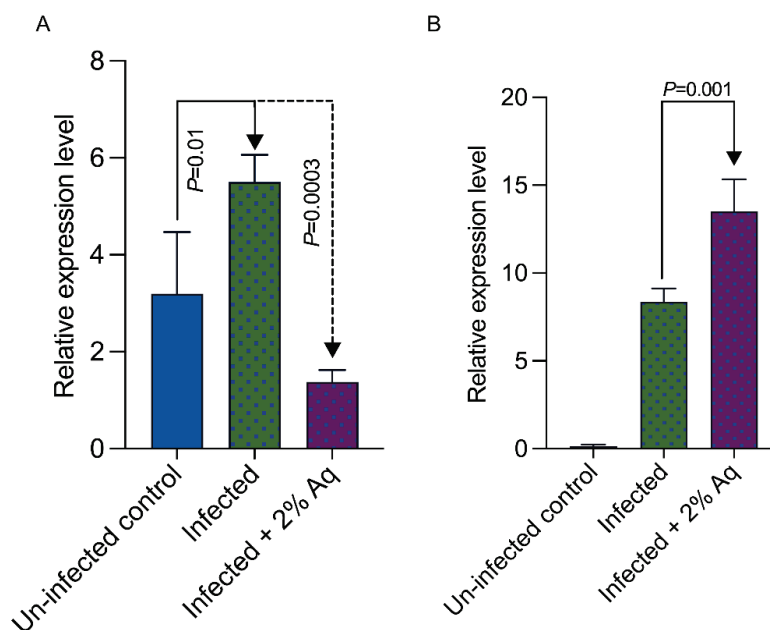


Figure 6. β -1,3-glucan-binding protein (LGBP) (A) and oxygenase (HO-1) (B) gene expression levels in the intestines of *P. vannamei* at 2 days post-WSSV infection. β -actin expression was used as reference. Vertical bars represent the mean $\pm$ SE (n = 3).

3.5. The Effect of Aq on the LvECSIT Expression in WSSV-Challenged Shrimp

Moreover, we aimed to further characterize the involvement of Aq in mediating the interaction between WSSV and the shrimp immune system. To gain an insight, we measured the expression levels of the evolutionarily conserved signaling intermediate in toll pathways (LvECSIT) in gills. As indicated in Figure 7, the expression of LvECSIT was significantly upregulated in infected shrimp ($p < 0.0001$), and this trend was reversed in the presence of Aq ($p < 0.0001$). These results clarify and confirm that Aq reduces the need for the host's innate immunity activation due to the reduction in WSSV infection rates.

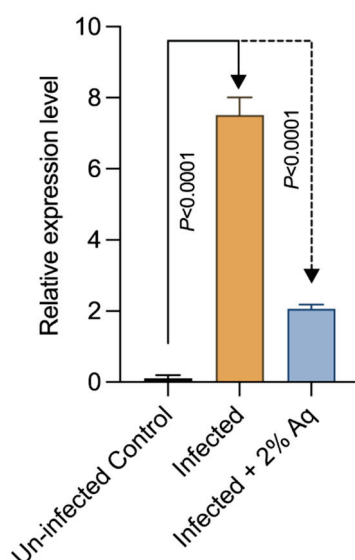


Figure 7. LvECSIT expression in the shrimp gills in the presence of 0.2% Aq at 2 days post-WSSV infection. β -actin expression was used as reference. Vertical bars represent the mean $\pm$ SE (n = 3). The statistical significance was determined using Student *t*-test; *p* values are indicated on the graphs.

3.6. The Impact of Aq on IL-22 Cytokine Production in WSSV-Infected Shrimp

To further prove the impact of Aq in mitigating the consequences of WSSV infection, we next measured the levels of IL-22 production, otherwise known as one of the responders to WSSV infection in shrimp. Our results (Figure 8A) confirmed the increase in IL-22 production in infected shrimp, followed by a significantly lower detection in the presence of 2% Aq. The decrease in gene expression was also confirmed when the amount of protein produced was also estimated using Western blotting (Figure 8B), indicating increased IL-22 levels in the infected shrimp and lower protein levels in the infected shrimp in the presence of 2% Aq.

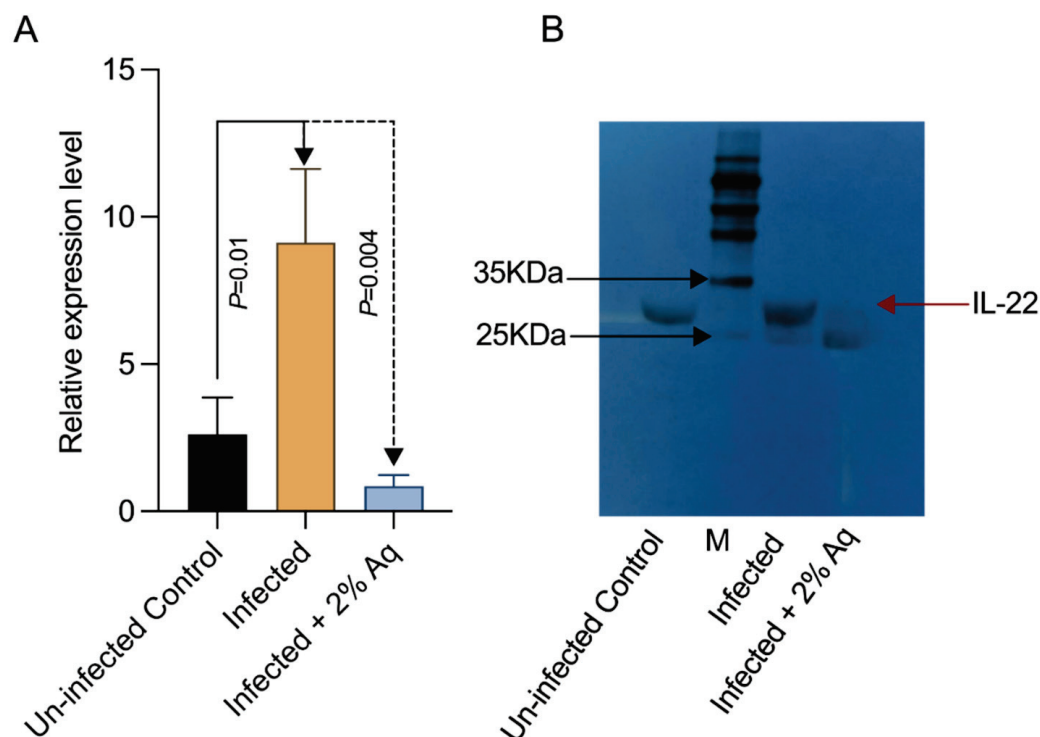


Figure 8. The shrimp Interleukin 22 (IL-22) relative expression levels as detected using qPCR at 2 days post-infection (A,B) Western blot analysis of the IL-22 protein expression levels of WSSV-challenged shrimp. The un-infected shrimp and un-treated shrimp were used as controls. Shrimp β -actin was used as loading control. M, protein marker. Statistically significant differences are indicated on the graph (A).

3.7. The Impact of 2% Aq on the Expression of Carboxypeptidase B in the Shrimp Gut

We used the expression of carboxypeptidase B to further prove the impact of Aq in reducing WSSV infections in shrimp. This proteolytic enzyme, which is usually downregulated in WSSV infected shrimp, exhibited a significantly recovered expression in both the hepatopancreas ($p = 0.009$) and in the gut tissue ($p = 0.001$) of WSSV-challenged shrimp (Figure 9). This impact on the carboxypeptidase B expression also suggests that Aq is involved in maintaining gut health and improve feed digestibility in shrimp due to its role in the degradation of proteins involved in the formation of peptides and hormones.

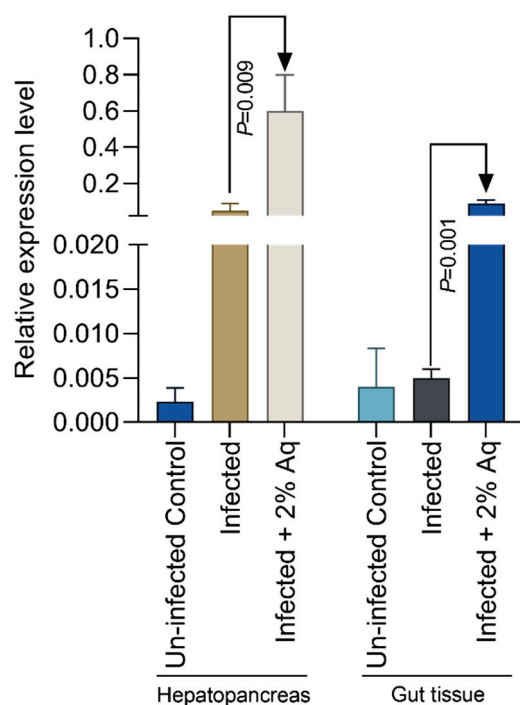


Figure 9. Carboxipeptidase B expression in the hepatopancreas and the gut tissue in challenged shrimp. The relative expression levels as detected using qPCR at 2 days post-infection in WSSV-challenged shrimp. The un-infected shrimp and un-treated shrimp were used as controls. Shrimp β -actin was used as loading control. M, protein marker. Statistically significant differences are indicated on the graph.

4. Discussion

White spot syndrome virus (WSSV) is responsible for significant economic losses in the shrimp industry by triggering the white spot disease (WSD) [31]. Finding and designing efficient on-farm interventions should focus on the virus only, since the host gut seems to be unaffected in WSSV-infected shrimp [32]. Mixtures of organic acids have been shown before to be effective in preventing viral infections both in vitro and in vivo in farmed animal models [17]. This type of intervention, other than vaccination, could be more effective in the long-term, given that recent data on WSSV outbreaks indicate that genetic variations can contribute to WSSV virulence and disease progression in shrimp [33].

Multiple host-entry pathways are employed by WSSV, for which the most common route is oral ingestion. VP28 is an integral viral envelope protein mediating host cell receptor interactions, entry, and systemic dissemination [9]. After infection, the process of viral hijacking of cellular machinery propagates, rendering metabolic reprogramming in immune cells as well as other target tissues [34]. Among these, glycolytic enzymes and the pentose phosphate pathway are necessary for providing the nucleotide precursors required for viral replication [34]. Additionally, these pathways supply nucleotide and lipid substrates that viruses utilize for biosynthesis [7]. Furthermore, lipid metabolism is upregulated to produce the components necessary for forming viral membranes, all providing substrates essential for viral replication and morphogenesis [7,35].

The peritrophic membrane (PM) provides protection to the gastrointestinal tissue and cells while simultaneously allowing the penetration of nutrients, water, and minerals into the host [36]. These characteristics give the PM an important role for both gut immunity and digestion in invertebrates [37]. Structurally, the PM in shrimp is known to include the lipopolysaccharide β -1,3-glucan-binding protein (LGBP) [28], which can act as a pathogen recognition protein and is involved in the activation of the shrimp immune

system. Critically for our study, this protein plays an important role in the interaction of WSSV with the host in shrimps [23]. The current results showed that when shrimp were devoid of PM and infected with WSSV, this resulted in higher mortality rates (27.8%) when compared to PM intact shrimp (9.33%) in the presence of 2% Aq. However, these reductions in mortality rates in both PM– and PM+ shrimp were only possible in the presence of Aq, since in the un-treated shrimp groups the mortality rates were over 97%. This reduction in mortality can also be explained by the increase in mucus production and a reduced expression of the LGBP gene, which might explain the attenuated infection in the presence of Aq. The inhibitory effects of natural products (plant extracts) against β -1,3-glucans and their biosynthesis pathways were previously described, including their chitin inhibitory effects, another component of PM [38]. It is very possible that the observed decrease in LGBP expression was a secondary effect caused by the reduced virus load levels. Moreover, Aq was responsible for the decline in host oxidative stress (both in vitro and in vivo), as indicated by the reduced H_2O_2 , CAT, and SOD levels in the presence of 2% Aq. Oxygenase, also identified in the PM (Heme oxygenase-1 HO-1), is usually induced to mitigate the damage caused by the WSSV-induced oxidative stress [24]. Our data clearly indicated that in WSSV-challenged shrimp, the gut HO-1 expression levels were significantly increased in the presence of 2% Aq.

The immune response in WSSV-infected shrimp is triggered via the LvECSIT, an evolutionarily conserved signaling intermediate in Toll pathways, which plays an important role in the innate immunity [39] and defense against WSSV [25]. In the presence of Aq, and at low infection levels, the expression of LvECSIT was detected at significantly low levels in gills. Interleukin-22 (IL-22), one of the cytokines upregulated in WSSV-infected shrimp [30], was detected in our study at very low levels both in gene and protein expression in the presence of Aq. Additionally, carboxypeptidase B, a proteolytic membrane involved in breaking peptide bonds at the amino-acid C terminus, has been marked as downregulated as a response to WSSV infection [40]. At low infection levels, in the presence of 2% Aq, this enzyme was significantly upregulated in the gut tissue ($p = 0.001$) and hepatopancreas ($p = 0.009$) of the challenged shrimp. These results suggest that Aq is involved in maintaining gut health in shrimp exposed to WSSV infection. The role of carboxypeptidase B in nutrition was also highlighted in other species, such as poultry, where this enzyme was found to be critical in the ileal digestion of proteins [41].

In addition to metabolic disruption, WSSV compromises the shrimp immune system by altering the expression of immune-related genes and metabolites. For instance, linoleic acid levels significantly increase post-infection, serving dual roles in antiviral immunity and viral replication inhibition [42]. WSSV also modulates lipid metabolism in haemocytes and the hepatopancreas, alternating between lipolysis and lipogenesis to meet its energy necessity and support virion assembly [35,42]. The innate immune system of shrimp plays a pivotal role in their defense versus WSSV as well as other pathogens. WSSV undermines the shrimp's immune system by reprogramming metabolic pathways and suppressing immune-related genes. The virus's rapid replication cycle (~24 h) and ability to modulate host immune responses make it exceptionally challenging to control [34]. Despite the absence of adaptive immunity in shrimp, innate immune mechanisms, such as AMPs and reactive oxygen species (ROS) production, melanization, phagocytosis, and apoptosis, provide limited defense [7,34]. Key enzymes, such as superoxide dismutase (SOD), catalase (CAT), and alkaline phosphatase (AKP), are integral to the shrimp's defense system [8]. SOD, for instance, mitigates oxidative stress by converting harmful superoxide radicals including ROS into hydrogen peroxide, which is subsequently broken down into water and oxygen by CAT [8]. Studies showed that shrimps fed with low concentrations of cup plant (*Silphium perfoliatum* L.) have demonstrated the ability to counter the deleterious effects of

WSSV and *Vibrio parahaemolyticus*, or even improve the resistance of shrimp by providing strong growth of beneficial intestinal bacteria and enhancing significantly the immune-related enzymes (SOD, CAT, AKP, LZM, α -AL and LIP) and modulated immune-related genes (*Crustin*, *Lysozyme*, *LGBP*, *LvLec*, *proPO* and *PEN-3 α*) from the hepatopancreas and intestine [43]. According to such observations, this not only improves the host's immune capacity, but also maintains the integrity of important tissues, reducing the susceptibility to WSSV.

5. Conclusions

This study demonstrates the potential of a mixture of organic acids to attenuate WSSV infections in shrimp using *in vitro* and *in vivo* infection models. Our results showed that Aq exerts its anti-WSSV activity through multiple mechanisms, including the direct inhibition of viral replication across all phases of host cell infection, the modulation of oxidative stress responses, and the enhancement of innate immune pathways. *In vivo* experiments illustrated in Figure 10 further corroborate the protective effects of Aq in WSSV-challenged shrimp, reducing mortality rates significantly from 96.66% in un-treated shrimp to just 7.33% with 2% Aq.

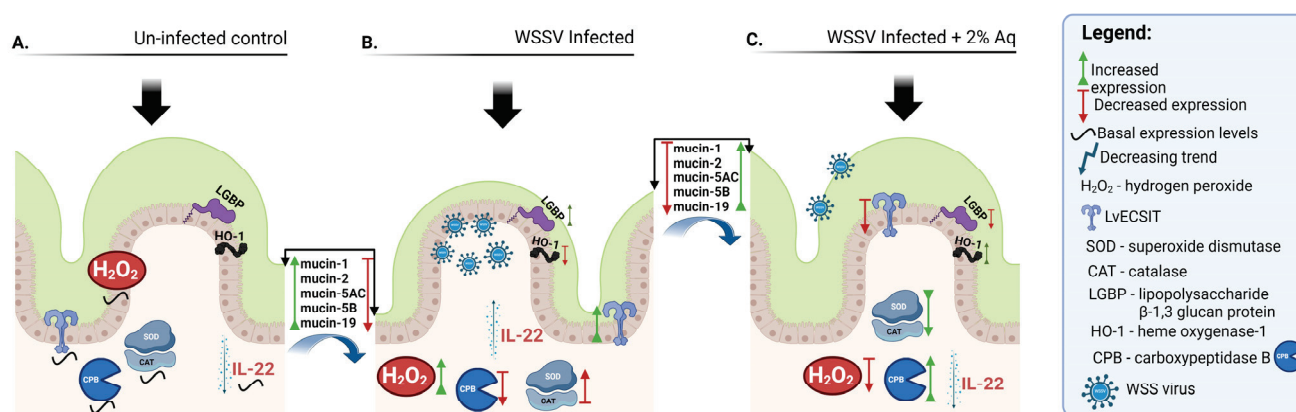


Figure 10. The molecular mechanisms of Aq and its role in preventing WSSV infection *in vivo* in a shrimp challenge model. The role of Aq in the un-infected and un-treated control (A), in WSSV infected shrimp (B), and in WSSV-infected shrimp in the presence of 2% Aq (C). Design created with Biorender.com.

The treatment-mediated reductions in H_2O_2 emphasize its antioxidant properties, which mitigate WSSV-induced cellular damage. Additionally, regulating immune responses by including the downregulation of IL-22 and LvECSIT in infected shrimp accentuates its role in tempering inflammatory and immune activation, while effectively controlling viral replication. The upregulation of CPB in gut and hepatopancreas tissue further underlines a resulting contribution to gut health and protein digestion, evidencing shrimp resilience against infection. By addressing WSSV infections via antiviral, immunomodulatory, and antioxidative mechanisms, such alternatives could offer a mitigating resolution to managing pathogens. Future research could further investigate the effect of water quality on the treatment efficiency and the long-term health effects. The resulting findings could pave the way to incorporate natural antimicrobials into sustainable aquaculture practices, the potential implications of which extend to other viral and bacterial diseases in farmed species.

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I.B.; supervision: I.C. and L.S.; writing—original draft and writing—review and editing: all authors contributed equally. All authors have read and agreed to the published version of the manuscript.

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Article

Moderate Dietary Cannabidiol Enhances Growth, Restructures Gut Microbiota, and Bolsters Environmental Stress Resilience in *Litopenaeus vannamei*

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Abstract

Intensive aquaculture induces severe environmental stress and disease susceptibility in Pacific white shrimp (*Litopenaeus vannamei*). Cannabidiol (CBD) offers significant potential as a bioactive stress-mitigating additive. This study evaluated the effects of dietary CBD supplementation (0, 10, 20, 40, and 80 mg/kg) on the growth, intestinal microecology, and stress tolerance of juvenile *L. vannamei* over an 8-week feeding trial, followed by a combined chronic ammonia and acute hypoxia challenge. Moderate CBD supplementation (10–40 mg/kg) significantly promoted growth, minimized feed conversion ratios, and enriched muscle eicosapentaenoic (EPA) and docosahexaenoic acids (DHA). Furthermore, CBD restructured the intestinal microbiota by suppressing opportunistic pathogens and enriching beneficial taxa. Under combined stress, moderate CBD prolonged the median lethal time (LT₅₀) by up-regulating hypoxia-inducible factor 1-alpha (*hif-1α*) and heat shock protein 70 (*hsp70*) transcription and boosting systemic antioxidant capacity to neutralize lipid peroxidation. Conversely, the highest dose (80 mg/kg) induced metabolic exhaustion and hepatopancreatic toxicity, evidenced by drastically elevated serum transaminases and diminished stress tolerance. Conclusively, dietary CBD exerts a classic biphasic effect in *L. vannamei*. Inclusion at 10–40 mg/kg safely promotes the best comprehensive effects on growth, immune homeostasis, and environmental resilience within the concentration range tested in this study, whereas excessive administration provokes severe metabolic burden, highlighting the critical need for strict dosage regulation.

Keywords: cannabidiol; *Litopenaeus vannamei*; intestinal microbiota; stress resilience; antioxidant capacity; growth; EPA; DHA

1. Introduction

The rapid expansion of the aquaculture sector is a direct response to the growing global demand for high-quality animal protein [1]. Among farmed species, the Pacific white

shrimp (*Litopenaeus vannamei*) has become the dominant contributor to global crustacean production, owing to its rapid developmental cycle, robust euryhaline traits, and capacity for high-density cultivation [2]. However, the shift towards hyper-intensive farming has introduced significant ecological and physiological challenges, such as water quality instability [3], ammonia nitrogen accumulation [4], and dissolved oxygen fluctuations [5]. These stressors collectively compromise the immune competence of shrimp, increasing their susceptibility to opportunistic pathogens and thereby threatening the profitability and sustainability of the industry [6,7]. Traditionally, the industry has relied upon broad-spectrum antibiotics and synthetic chemotherapeutants to manage these risks; however, the resulting emergence of antimicrobial resistance and stringent international regulations on chemical residues have necessitated the search for sustainable, bioactive alternatives [8–10].

Phytogenic compounds and nutraceuticals have emerged as promising candidates for improving the health and welfare of aquatic organisms [11,12]. Cannabidiol (CBD), a predominant non-psychoactive phytocannabinoid derived from the industrial hemp plant (*Cannabis sativa* L.), has recently gained significant scientific interest for its extensive range of therapeutic properties [13,14]. Unlike Δ^9 -tetrahydrocannabinol (THC), CBD is characterized by a high safety margin and does not exhibit addictive potential [15]. Extensive biomedical literature has firmly established its roles in modulating inflammation [16,17], neutralizing oxidative stress [18,19], and regulating immune responses in various mammalian models [20,21].

In recent years, the application of CBD in aquatic sciences has advanced from preliminary model organisms to commercially significant species [22]. Recent evidence demonstrates that waterborne administration of CBD can effectively mitigate transport-induced acute stress and behavioral anomalies in ornamental fishes [23]. Furthermore, dietary supplementation with CBD or cannabis derivatives has been documented to significantly enhance the welfare of Nile tilapia (*Oreochromis niloticus*) by ameliorating aggressive behavior, positively regulating hematological profiles, and boosting overall metabolic responses [22,24–26]. Similar physiological improvements have also been reported in marine teleosts, such as the large yellow croaker (*Larimichthys crocea*) [27]. Mechanistically, these benefits in finfish are frequently linked to the regulatory interaction between CBD and the endocannabinoid system or homologous neurological signaling networks [28].

For invertebrates such as *L. vannamei*, the polyphenol-like structure of CBD—particularly its rich hydroxyl groups—confers exceptional radical-scavenging capabilities [29,30]. This structural advantage highlights its unexplored potential to preserve physiological homeostasis and alleviate oxidative damage in shrimp subjected to environmental stressors. Beyond these direct biochemical benefits, CBD may also exert systemic protective effects via the host's gut-microbiome axis. The gastrointestinal tract serves as the primary organ for nutrient assimilation and the first line of defense against aquatic pathogens [31,32]. While mammalian models have indicated that CBD can interact with the gut microbiome to alleviate intestinal inflammation [33,34], its capacity to modulate the intestinal microecosystem in aquatic invertebrates remains entirely unknown.

The current study aimed to systematically evaluate the impact of dietary CBD supplementation on the growth trajectory, feed utilization, muscle nutritional profile, and intestinal microbiota architecture of *L. vannamei*. Furthermore, we subjected the shrimp to a combined chronic ammonia and acute hypoxia challenge to clarify the role of CBD in bolstering environmental stress tolerance. Crucially, by setting a wide range of inclusion gradients (0, 10, 20, 40 and 80 mg/kg diet, based on validated effective doses in aquaculture species [25–27]), this study also sought to elucidate the dose-dependent biphasic effects of CBD, aiming to identify the most effective dietary dosage, assess the physiological risks

associated with excessive administration, and provide empirical support for the application of CBD in sustainable intensive shrimp aquaculture.

2. Materials and Methods

2.1. Ethics Statement

The handling and culture of the animals used in this research study were carried out in compliance with the guidelines established by the Animal Ethics Committee of Shanghai Ocean University (Shanghai, China), following the approved protocol numbers SHOU-DW-2020-017 (Approved on 15 March 2020).

2.2. Feed Ingredients and Diets Formulation

Five isonitrogenous (42% crude protein) and isolipidic (6% crude lipid) experimental diets were formulated to meet the nutritional requirements of juvenile *L. vannamei*. Using fish meal, meat meal, soybean meal, peanut meal, and corn gluten meal as protein sources, and fish oil, soybean oil, and soybean lecithin as lipid sources, a basal diet was formulated. The functional additive used in this study was dried hemp (*C. sativa* L.) leaf powder, which contained approximately 5% active CBD and was kindly provided by the Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences (Changsha, China).

To achieve the target active CBD levels of 0, 10, 20, 40, and 80 mg/kg in the final diets (designated as Group A, B, C, D, and E, respectively), the hemp leaf powder was supplemented at exactly 0%, 0.02%, 0.04%, 0.08%, and 0.16% of the total diet, respectively. Corresponding reductions in α -cellulose content were made across all diets to maintain equal protein and lipid levels, ensuring the only variable among the five experimental diets was the inclusion level of CBD.

The CBD inclusion levels were set based on published effective doses in farmed fish [25–27], our core objective to clarify dose-dependent effects of CBD in shrimp, and species differences between crustaceans and teleosts, to ensure both the capture of significant biological effects and experimental safety. Due to the minimal inclusion levels of CBD ($\leq 0.16\%$ of diet), its contribution to the overall protein and lipid content was negligible, and the adjustments in cellulose effectively maintained isonitrogenous and isolipidic conditions across all diets.

All dry ingredients were ground through an 80-mesh sieve, weighed accurately according to the formulation, and thoroughly mixed in a stepwise manner. After adding fish oil, soybean lecithin, and an appropriate amount of water, the mixture was pelleted into 1.5 mm diameter feed pellets using a pelletizer. The pellets were immediately dried to a moisture content of 10% at low temperature, sealed in vacuum bags, and stored at $-20\text{ }^{\circ}\text{C}$ until use. The composition and nutritional levels of the basal diet are shown in Table 1.

Table 1. Formulation and proximate analysis of the experimental diets (per 100g dry diet).

Ingredients (%)	Diet No. (CBD Level mg/kg)				
	Diet A (0 mg/kg)	Diet B (10 mg/kg)	Diet C (20 mg/kg)	Diet D (40 mg/kg)	Diet E (80 mg/kg)
Protein mixture ^a	68	68	68	68	68
Wheat meal	13.8	13.8	13.8	13.8	13.8
α -starch	3	3	3	3	3
Cellulose	2.60	2.58	2.56	2.52	2.44
Fish oil	3	3	3	3	3

Table 1. Cont.

Ingredients (%)	Diet No. (CBD Level mg/kg)				
	Diet A (0 mg/kg)	Diet B (10 mg/kg)	Diet C (20 mg/kg)	Diet D (40 mg/kg)	Diet E (80 mg/kg)
Soybean oil	1.5	1.5	1.5	1.5	1.5
Soy lecithin meal	1	1	1	1	1
Mineral premix ^b	1.5	1.5	1.5	1.5	1.5
Vitamin premix ^c	1	1	1	1	1
Ca(H ₂ PO ₄) ₂ ·H ₂ O	2	2	2	2	2
Choline chloride	0.3	0.3	0.3	0.3	0.3
Squid paste	2	2	2	2	2
Methionine	0.2	0.2	0.2	0.2	0.2
Y ₂ O ₃	0.1	0.1	0.1	0.1	0.1
Hemp leaf powder (5% CBD)	0	0.02	0.04	0.08	0.16
Analyzed Composition (%)					
Crude protein	42.94	42.9	42.91	42.81	42.89
Crude lipid	6.46	6.49	6.48	6.48	6.47
Crude ash	9.31	9.37	9.34	9.35	9.38

^a The protein mixture consists of soybean meal (47.06%), fish meal (14.71%), peanut meal (11.76%), corn protein powder (11.76%), meat meal (5.88%), blood meal (4.41%), and brewer's yeast (4.41%). ^b Contained the following per kilogram of mineral premix: Ca 10.5 g, Fe 1.0 g, Co 0.8 g, Se 0.02 g, Mg 12 g, Mn 3.8 g, K 90 g, Cu 3.0 g, Zn 10 g. ^c Contained the following per kilogram of vitamin premix: VA8000000 IU, VD3 2000000 IU, VE 50 g, VK 10 g, Thiamine hydrochloride 5 g; riboflavin 15 g, nicotinamide 40 g, calcium D-pantothenate 25 g, pyridoxine 8 g, cyanocobalamin 0.02 g, folic acid 2 g, biotin 0.08 g, inositol 100 g.

2.3. Animals, Experimental Procedure, and Conditions

The feeding trial was conducted at the Binhai Aquaculture Base of Shanghai Ocean University. A total of 675 juvenile *L. vannamei* (purchased from Longyuan Seedling Company, Nantong, China) with uniform size, healthy constitution, and an average body weight of 0.52 ± 0.04 g were selected and randomly distributed into 15 indoor recirculating tanks (height: 117 cm, radius: 50 cm, spatially randomized), with 45 shrimp per tank. Each tank was covered with a net to prevent shrimp from jumping out. Shrimp were fed to satiation four times daily (at 07:35, 12:35, 17:35, and 22:35). Feed residues were collected 2 h after each feeding (this time point was set to ensure complete recovery of settled uneaten feed with minimal nutrient leaching into water) to monitor feeding activity, and daily feed rations were adjusted accordingly.

The 8-week feeding trial was conducted under controlled conditions: water temperature 27–29 °C, salinity 10‰, pH 7.8–8.5, with continuous aeration maintaining dissolved oxygen above 7 mg/L. The system operated under natural photoperiod with 20% water exchange every 3 days, while ammonia nitrogen was kept below 0.2 mg/L and water transparency was maintained at 20–40 cm throughout the experimental period.

At the end of the 8-week feeding trial, all samples for basal growth performance, physiological and biochemical analysis, and intestinal microbiota detection were collected first. The remaining healthy shrimp with uniform size in each replicate tank were fasted for 24 h and then used for the subsequent environmental stress challenge experiment.

2.4. Chronic Ammonia-Nitrite Stress

After the completion of basal sample collection, a 4-day combined ammonia and nitrite stress test was conducted. Healthy shrimp from each treatment were exposed to water containing 2 mg/L of nitrite-nitrogen (NO₂[−]-N) and 2 mg/L ammonia-nitrogen, maintained by adding reagent-grade NaNO₂ and NH₄Cl. Water quality was monitored twice daily to ensure stable stress concentrations.

2.5. Acute Hypoxia Stress and LDO₅₀/LT₅₀ Determination

Immediately following the 4-day chemical stress, an acute hypoxia challenge was performed to evaluate the resilience of the shrimp. Healthy shrimp with uniform size were randomly selected and fasted for 12 h prior to the hypoxia tolerance test. 10 shrimp from each replicate tank were transferred into a separate 20 L sealed bucket. Each replicate tank corresponded to 1 hypoxia challenge bucket, with 3 biological replicate buckets per dietary CBD treatment group, resulting in a total of 30 shrimp per treatment group for the hypoxia test.

Dissolved oxygen (DO) in the sealed buckets was allowed to decline naturally through shrimp respiration, and the DO concentration was continuously monitored using a portable dissolved oxygen meter. The time of death and the corresponding DO level for each individual shrimp were recorded precisely. Death was defined as the total cessation of pleopod movement and lack of response to mechanical stimulation. The median lethal dissolved oxygen (LDO₅₀) and the median lethal time (LT₅₀) for each treatment group were calculated using Probit regression analysis, which modeled the relationship between cumulative mortality and the DO concentration or logarithm of exposure time ($\log_{10} T$) for each of the 3 biological replicates per group, respectively, with the final group value presented as the mean of the 3 replicates.

The Kaplan–Meier cumulative survival curve for each dietary treatment group was plotted based on the exact death time of all 30 individual shrimp (merged from 3 biological replicate buckets) in the group.

2.6. Sample Collection and Biochemical Analyses

At the end of the 8-week feeding trial and prior to the initiation of the combined ammonia-nitrite and hypoxia stress challenge, fecal samples were collected 1.5 h post-feeding using siphoning during the final two weeks of the culture period [35]. This time point was selected as the excretion window for *L. vannamei*, ensuring feces with intact nutritional components for accurate apparent digestibility analysis. All collection operations adopted low-disturbance protocols: fecal collection was performed by gentle siphoning along the tank wall without touching shrimp, and all sampling was completed within 5 min per tank at a fixed time, which avoided acute stress responses of shrimp caused by human interference. Collected feces were transferred to 10 mL centrifuge tubes and stored at $-20\text{ }^{\circ}\text{C}$ for apparent digestibility analysis. Upon trial completion, shrimp were fasted for 24 h before harvest. Final body weight and survivability (SV) were recorded. In each tank, 12 intermolt shrimp were randomly selected and anesthetized (100 mgL^{-1} tricaine methane sulfonate; MS222; Sigma-Aldrich, St. Louis, MO, USA): three were used for whole-body composition analysis; the rest were used for hemolymph collection (withdrawn from the pericardial cavity using sterile 1 mL syringes). The hemolymph was then stored in 2 mL tubes at $-80\text{ }^{\circ}\text{C}$ for biochemical/enzymatic assays. Muscle tissues and intact hepatopancreas were dissected into 5 mL centrifuge tubes and preserved at $-80\text{ }^{\circ}\text{C}$ for digestive/antioxidant enzyme analysis. For gut microbiome analysis, three shrimp per recirculating tank were surface-sterilized with 75% alcohol, aseptically dissected, and the intestines were collected. After rinsing with sterile saline, the intestines were transferred to cryovials and stored at $-80\text{ }^{\circ}\text{C}$.

Proximate composition (crude protein, crude lipid, moisture, and ash contents) of experimental diets, shrimp body samples, and feces was determined according to the standard methods of the Association of Official Analytical Chemists [36]. Briefly, moisture content was measured by oven-drying at $105\text{ }^{\circ}\text{C}$ to a constant weight. Crude protein was analyzed by the Kjeldahl method using an automatic Kjeldahl analyzer (2300 Auto-analyzer, Foss Tecator, Höganäs, Sweden). Total lipids were extracted with chloroform-methanol (2:1,

v/v) containing 0.01% BHT following the methods of Cejas [37], then methylated with 14% BF_3 in methanol [38]. Ash content was determined by incinerating in a muffle furnace at 550 °C for six hours. Fecal Y_2O_3 content was quantified using ICP-MS (ICAPQc, SN03019R, ThermoFisher (Waltham, MA, USA)).

Fatty acids were extracted, and fatty acid methyl esters (FAMES) were prepared according to the ISO 5509 method [39]. Briefly, total lipids were first extracted from samples via Soxhlet extraction with petroleum ether at 60 °C for 6 h. The extracted lipids were then saponified with 0.5 mol/L potassium hydroxide-methanol solution at 60 °C for 20 min, followed by esterification with 14% boron trifluoride-methanol solution at 70 °C for 15 min to obtain FAMES, which were finally extracted with *n*-hexane and dehydrated with anhydrous sodium sulfate.

FAMES were subsequently analyzed using an Agilent 7890 capillary gas chromatograph (GLC) equipped with a flame ionization detector (FID; Agilent Technologies, Santa Clara, CA, USA). Separation was performed on a Supelco SP-2560 capillary column (30 m × 0.25 mm I.D., 0.5 µm film thickness; Supelco, Bellefonte, PA, USA). The chromatographic conditions were set as follows: injector temperature 210 °C, FID temperature 250 °C; carrier gas high-purity N_2 at a constant flow rate of 1.0 mL/min; split ratio 10:1; oven temperature programmed from 180 °C (held for 2 min) to 250 °C at a rate of 4 °C/min, and held at 250 °C for 10 min.

Qualitative and quantitative analyses of fatty acids were performed using a FAME Mix standard (Cat. No. 47885-U, Supelco, Bellefonte, PA, USA). Target fatty acids were identified by matching the retention time of sample peaks with those of the standard, and the relative content of each fatty acid was calculated by the peak area normalization method, expressed as a percentage of the total fatty acids.

Digestive enzymatic activities and biochemical assays were analyzed using the hepatopancreas from four randomly selected shrimp in total from the three replicate tanks of each dietary treatment. The hepatopancreas was homogenized with sterilized shrimp saline (0.85%, 1:9 *w/v*) and centrifuged (8000 rpm, 10 min, 4 °C). The supernatant was used for following analyses: protease activity was determined by Folin method (GB/T 23527.1-2023) [40]; lipase activity, amylase activity, Na^+ , K^+ -ATPase activity, total antioxidant capacity (T-AOC), malondialdehyde (MDA) content, and catalase (CAT) activity, glutathione peroxidase (GSH-Px) activity, and peroxidase (POD) activity were assayed using commercial kits (Nanjing Jiancheng Bioengineering Institute (Nanjing, China)); serum parameters including glucose (GLU), triglycerides (TG), alkaline phosphatase (ALP), acid phosphatase (ACP), lysozyme (LZM), alanine aminotransferase (ALT), and aspartate aminotransferase (AST), as well as serum T-AOC, CAT activity, and MDA content, were measured with corresponding kits following manufacturer's protocols (Nanjing Jiancheng Bioengineering Institute). To minimize subjective bias, the personnel involved in the outcome assessment and data analysis were blinded to the dietary group allocations.

2.7. RNA Isolation and qPCR Analysis

For gene expression analysis, 1 shrimp at the terminal moribund state was collected from each hypoxia challenge replicate bucket when the cumulative mortality of the bucket reached 100% (3 shrimp per dietary treatment group, corresponding to 3 independent biological replicates). The sampled shrimp was the last surviving individual in the bucket, with weak pleopod movement and minimal response to standardized mechanical stimulation, immediately before meeting the irreversible death criteria defined in Section 2.5. Hepatopancreas tissues were dissected immediately after sampling, snap-frozen in liquid nitrogen, and stored at −80 °C for subsequent RNA extraction.

Total RNA was extracted from the hepatopancreas of size-matched shrimp using Total RNA Extraction Kits (Tiangen, Beijing, China). RNA integrity was verified by 1% agarose gel electrophoresis, and concentration/purity was determined via NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Subsequent cDNA synthesis employed PrimeScript™ RT reagent Kits (Takara Bio Inc., Kusatsu, Shiga, Japan). Gene-specific primer sequences for heat shock protein 70 (*hsp70*), cysteine-aspartic acid protease 3 (*caspase 3*), hexokinase (*hk*), hypoxia-inducible factor 1-alpha (*hif-1α*), and lactate dehydrogenase (*ldh*) are shown in Table 2. Quantitative PCR amplification followed Wang et al. [41], with relative gene expression calculated using the $2^{-\Delta\Delta C_t}$ method [42], with β -actin as the reference gene.

Table 2. Primers for qPCR analysis.

Gene Name		Nucleotide Sequence (5'-3')	Sources
<i>hsp70</i>	Forward	AGGAGACCGCTGAGGCTTAC	DQ813343.1
	Reverse	AGCACATTCAGACCCGAGAT	
<i>caspase 3</i>	Forward	AGTTAGTACAAACAGATTGGAGCG	KC660103.1
	Reverse	TTGTGGACAGACAGTATGAGGC	
<i>hif-1α</i>	Forward	GGAGTCTTTGAGAGAGAG	FJ807918 [43]
	Reverse	GCCTCCTTCCGTGATCTTC	
<i>hk</i>	Forward	AGTCGCAGCAACAGGAAGTT	EF102106.1 [44]
	Reverse	CGCTCTTCTGGCACATGATA	
<i>ldh</i>	Forward	TTGTTACTGCTGGTGCTCGT	XM_070135849.1
	Reverse	AGCGACACTCAGTTTCTGGG	
β -actin	Forward	CTTGTGTGCGACAATGGCTC	XM_027364954.1
	Reverse	TCGATGGGGTACTTGAGGGT	

2.8. DNA Extraction, Library Preparation, and High-Throughput Sequencing Data Analysis

Genomic DNA was extracted from intestinal samples using the EZNA® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's protocol. The hypervariable V3–V4 region of the bacterial 16S rRNA gene was amplified via PCR with extracted DNA as template. Amplification products were purified by electrophoresis on agarose gels and quantified using a Qubit® 4.0 Fluorometer (Thermo Fisher Scientific). Sequencing libraries were constructed with the NEXTFLEX® Rapid DNA-Seq Kit (Bioo Scientific (Austin, TX, USA)), involving adapter ligation, size selection with magnetic beads, and PCR enrichment. Final libraries were sequenced on the Illumina (San Diego, CA, USA) (NovaSeq 6000) platform. Raw sequence data have been uploaded to NCBI (PRJNA1434381).

Raw paired-end reads were quality-filtered and adapter-trimmed using fastp (v0.23.2). Overlapping reads were merged with FLASH (v1.2.11). Operational Taxonomic Units (OTUs) were clustered at 97% sequence similarity using USEARCH (v11.0). Sequences annotated as chloroplasts or mitochondria were removed. Taxonomic classification was performed against the SILVA (v138) 16S rRNA reference database using the RDP classifier (v2.13) with a confidence threshold of 0.8. Microbial community composition, including α -diversity indices and β -diversity metrics, was analyzed. Linear Discriminant Analysis Effect Size (LEfSe) was performed to identify the microbial biomarkers exhibiting significant differential abundance across groups (LDA score > 3.0). The differential abundance of specific bacterial taxa at the genus level was further validated using the Kruskal–Wallis H test. Functional profiles of bacterial communities were predicted using PICRUSt2 based on the Clusters of Orthologous Genes (COG) database.

2.9. Calculations

The performance indices were calculated as follows:

$$\text{Weight Gain Rate (WGR, \%)} = [(W_t - W_0)/W_0] \times 100$$

$$\text{Survivability (SV, \%)} = (N_t/N_0) \times 100$$

$$\text{Feed intake (FI, \%day)} = [W_f / ((W_t \times N_0/N_t + W_0)/2)] / t \times 100$$

$$\text{Feed Conversion Ratio (FCR)} = W_f / (W_t - W_0)$$

$$\text{Specific Growth Rate (SGR, \% d}^{-1}\text{)} = [\ln(W_t) - \ln(W_0)] / t \times 100$$

$$\text{Apparent Dry Matter Digestibility (ADDM, \%)} = [1 - (B_1/B_2)] \times 100$$

$$\text{Apparent Nutrient Digestibility (AND, \%)} = [1 - (A_1/A_2) \times (B_1/B_2)] \times 100$$

where W_t : Final mean body weight (g), W_0 : Initial mean body weight (g), W_f : Total dry matter intake (g), t : Experimental duration (days), N_t : Final number of shrimp per tank, N_0 : Initial number of shrimp per tank, A_1 : Nutrient concentration in feces (%), A_2 : Nutrient concentration in diet (%), B_1 : Y_2O_3 concentration in diet (%), B_2 : Y_2O_3 concentration in feces (%)

2.10. Statistical Analysis

All experimental results are expressed as means $\pm$ standard deviation (mean $\pm$ SD). For growth performance and survival rate, the experimental unit was the tank ($n = 3$ per treatment). For biochemical parameters, fatty acid composition, and enzyme activities, the experimental unit was the individual shrimp, with four shrimp randomly selected per treatment ($n = 4$ per treatment). Data normality and homogeneity of variances were verified prior to analysis. Statistical analyses were performed using SPSS 25.0 (IBM Corp., Armonk, NY, USA). Significant differences among treatment groups were determined by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test for post hoc comparisons when significant effects ($p < 0.05$) were detected. For microbiota data, non-parametric tests (e.g., Kruskal–Wallis) were applied where data did not meet parametric assumptions. Cumulative survival curves were generated using the Kaplan–Meier method in GraphPad Prism 9.0.

To systematically evaluate the potential internal relationships between macro-phenotypes and micro-physiological changes, Pearson correlation analysis was conducted among the core parameters of growth performance, digestive enzymes, antioxidant status, stress-related genes, and environmental stress resistance. The correlation coefficient (r) matrix and corresponding p -values were calculated and visualized using GraphPad Prism 9.0. Statistical significance for the correlation was defined as $p < 0.05$.

3. Results

3.1. Growth Performance, Feed Utilization, and Whole-Body Proximate Composition

The effects of dietary CBD on the growth performance, feed utilization, and survival of *L. vannamei* are presented in Table 3. The IBW showed no significant differences among the treatments ($p > 0.05$). At the end of the feeding trial, shrimp fed diets supplemented with varying levels of CBD exhibited significantly higher FBW, WGR, and SGR compared to the control group ($p < 0.05$). However, no significant differences in these growth parameters were observed among the different CBD-supplemented groups ($p > 0.05$).

Table 3. Effects of dietary CBD levels on growth performance and feed utilization of *L. vannamei*.

Parameters	Diet A (0 mg/kg)	Diet B (10 mg/kg)	Diet C (20 mg/kg)	Diet D (40 mg/kg)	Diet E (80 mg/kg)
IBW (g)	0.52 ± 0.04	0.52 ± 0.04	0.52 ± 0.04	0.52 ± 0.04	0.52 ± 0.04
FBW (g)	15.51 ± 0.53 ^b	17.09 ± 0.71 ^a	16.9 ± 0.63 ^a	16.71 ± 0.2 ^a	16.78 ± 0.29 ^a
WGR (%)	2883.24 ± 101.19 ^b	3185.76 ± 136.16 ^a	3150.31 ± 120.4 ^a	3113 ± 37.66 ^a	3127.37 ± 56.22 ^a
FI (%/day)	4.18 ± 0.05 ^b	4.28 ± 0.1 ^b	4.46 ± 0.2 ^{ab}	4.49 ± 0.18 ^{ab}	4.62 ± 0.2 ^a
SGR (%/day)	2.46 ± 0.02 ^b	2.53 ± 0.03 ^a	2.52 ± 0.03 ^a	2.51 ± 0.01 ^a	2.51 ± 0.01 ^a
FCR	1.9 ± 0.03 ^a	1.79 ± 0.02 ^b	1.76 ± 0.04 ^b	1.68 ± 0.01 ^c	1.86 ± 0.04 ^a
SV (%)	65.19 ± 3.39 ^c	70.37 ± 1.28 ^{bc}	74.81 ± 2.57 ^{ab}	80.74 ± 5.13 ^a	74.81 ± 4.63 ^c

Note: Data were expressed as means ± S.D. ($n = 3$ replicate tanks per treatment). Data in the same row sharing different superscript letters denote significant differences determined by Duncan's test ($p < 0.05$). Rows without superscript letters indicate no significant differences among treatments ($p > 0.05$). FBW: final body weight; FCR: feed conversion rate; FI: feed intake; IBW: initial body weight; SGR: specific growth rate; SV: survivability; WGR: weight gain rate.

Regarding feed utilization, the FCR exhibited a distinct U-shaped dose–response pattern. The FCR was significantly reduced in groups fed 10 to 40 mg/kg CBD compared to the control, reaching its lowest value of 1.68 in Group D ($p < 0.05$). However, when the CBD dosage increased to 80 mg/kg (Group E), the FCR deteriorated to 1.86, showing no significant difference from the control group ($p > 0.05$). Conversely, FI progressively increased, peaking significantly in Group E. Furthermore, the SV was significantly enhanced by moderate CBD supplementation, maximizing at 80.74% in Group D ($p < 0.05$). Notably, in the highest dosage group, the SV declined to 74.81%, which was not significantly different from the control group ($p > 0.05$).

The whole-body proximate composition of the shrimp fed the experimental diets is summarized in Table 4. Dietary CBD supplementation did not significantly affect the moisture, crude protein, and ash contents of the whole shrimp across all treatments ($p > 0.05$). Nevertheless, the total lipid content was significantly influenced by the dietary treatments. Compared to the control group (1.52%), the total lipid content remained relatively stable in the low-dose groups but significantly increased to its highest level (1.61%) in Group D (40 mg/kg) ($p < 0.05$). Interestingly, in the high-dose group (Group E, 80 mg/kg), the total lipid content significantly decreased to 1.48%, representing the lowest value among all experimental groups ($p < 0.05$).

Table 4. Proximate composition (% wet weight basis) of *L. vannamei* whole shrimp fed with experimental diets.

Parameters	Diet A (0 mg/kg)	Diet B (10 mg/kg)	Diet C (20 mg/kg)	Diet D (40 mg/kg)	Diet E (80 mg/kg)
Moisture	75.62 ± 0.37	75.71 ± 0.58	76.05 ± 0.61	76 ± 0.38	76.07 ± 0.48
Crude protein	18.88 ± 0.24	18.95 ± 0.15	18.67 ± 0.11	18.78 ± 0.09	18.78 ± 0.1
Total lipid	1.52 ± 0.03 ^b	1.53 ± 0.01 ^b	1.54 ± 0.05 ^b	1.61 ± 0.09 ^a	1.48 ± 0.1 ^c
Ash	2.65 ± 0.1	2.55 ± 0.08	2.64 ± 0.11	2.47 ± 0.09	2.59 ± 0.25

Note: Diets were supplemented with increasing levels of CBD (0, 10, 20, 40, and 80 mg/kg diet). Data were expressed as means ± S.D. ($n = 3$ replicate tanks per treatment). Data in the same row sharing different superscript letters denote significant differences determined by Duncan's test ($p < 0.05$). Rows without superscript letters indicate no significant differences among treatments ($p > 0.05$).

3.2. Muscle Fatty Acid Profile

We further analyzed the muscle fatty acid profile of *L. vannamei* fed with varying levels of CBD (Table 5). Overall, the total saturated fatty acid (Σ SFA) content remained highly

stable across all dietary treatments, showing no significant differences among the groups ($p > 0.05$).

Table 5. Effects of dietary CBD levels on the muscle fatty acid composition of *L. vannamei*.

Fatty Acids	Diet A (0 mg/kg)	Diet B (10 mg/kg)	Diet C (20 mg/kg)	Diet D (40 mg/kg)	Diet E (80 mg/kg)
C14:0	0.22 ± 0.03	0.21 ± 0.01	0.22 ± 0.02	0.23 ± 0	0.22 ± 0.02
C15:0	0.13 ± 0.04 ^b	0.14 ± 0.03 ^{ab}	0.15 ± 0.02 ^{ab}	0.17 ± 0.01 ^a	0.14 ± 0.02 ^{ab}
C16:0	15.2 ± 0.29	15.05 ± 0.22	15.1 ± 0.26	15.01 ± 0.2	15.21 ± 0.13
C17:0	0.82 ± 0.01 ^b	0.86 ± 0.02 ^a	0.81 ± 0.02 ^a	0.8 ± 0.03 ^b	0.64 ± 0.01 ^c
C18:0	9.54 ± 0.18	9.63 ± 0.15	9.59 ± 0.18	9.44 ± 0.27	9.59 ± 0.16
C19:0	1.34 ± 0.09 ^b	1.48 ± 0.1 ^{ab}	1.42 ± 0.1 ^{ab}	1.47 ± 0.11 ^{ab}	1.55 ± 0.06 ^a
C20:0	0.35 ± 0.02	0.32 ± 0.1	0.36 ± 0.03	0.37 ± 0.01	0.38 ± 0.01
ΣSFA	27.6 ± 0.25	27.69 ± 0.51	27.65 ± 0.2	27.48 ± 0.59	27.73 ± 0.28
C16:1n-7	1.13 ± 0.09 ^a	1.12 ± 0.03 ^a	1.12 ± 0.12 ^a	1.17 ± 0.06 ^a	0.96 ± 0.09 ^b
C17:1n-7	0.15 ± 0.05	0.17 ± 0.02	0.17 ± 0.03	0.18 ± 0.02	0.15 ± 0.07
C18:1n-9	20.13 ± 0.16 ^a	18.69 ± 0.35 ^d	19.18 ± 0.19 ^c	19.51 ± 0.05 ^b	19.56 ± 0.12 ^b
C20:1n-9	1.4 ± 0.01 ^a	1.3 ± 0.06 ^b	1.41 ± 0.02 ^a	1.41 ± 0.03 ^a	1.4 ± 0.03 ^a
C22:1n-9	0.38 ± 0.16 ^{ab}	0.33 ± 0.09 ^b	0.53 ± 0.13 ^a	0.41 ± 0.08 ^{ab}	0.42 ± 0.08 ^{ab}
ΣMUFA	23.2 ± 0.21 ^a	21.61 ± 0.27 ^c	22.42 ± 0.19 ^b	22.68 ± 0.06 ^b	22.48 ± 0.21 ^b
Σn-7MUFA	1.28 ± 0.13 ^{ab}	1.29 ± 0.04 ^{ab}	1.29 ± 0.10 ^{ab}	1.35 ± 0.08 ^a	1.11 ± 0.06 ^b
Σn-9MUFA	21.91 ± 0.15 ^a	20.32 ± 0.31 ^c	21.12 ± 0.13 ^b	21.33 ± 0.02 ^b	21.37 ± 0.20 ^b
C18:2n-6 (LA)	18.92 ± 0.18 ^a	16.78 ± 0.23 ^c	18.01 ± 0.24 ^b	19.02 ± 0.25 ^a	18.26 ± 0.15 ^b
C18:3n-3 (LNA)	1.36 ± 0.03 ^{ab}	1.21 ± 0.11 ^c	1.31 ± 0.03 ^{ab}	1.38 ± 0.05 ^a	1.28 ± 0.03 ^{bc}
C20:2n-6	2.08 ± 0.07 ^a	1.95 ± 0.03 ^b	2.1 ± 0.05 ^a	2.03 ± 0.01 ^{ab}	2.09 ± 0.08 ^a
C20:4n-6 (ARA)	1.96 ± 0.03 ^a	2.22 ± 0.06 ^b	1.94 ± 0.12 ^a	2.01 ± 0.08 ^a	2.02 ± 0.03 ^a
C20:5n-3 (EPA)	13.47 ± 0.29 ^{cd}	15.22 ± 0.16 ^a	13.77 ± 0.05 ^b	13.21 ± 0.18 ^d	13.72 ± 0.07 ^{bc}
C22:5n-3	0.67 ± 0.02 ^b	0.7 ± 0.03 ^{ab}	0.72 ± 0.03 ^a	0.71 ± 0.02 ^{ab}	0.69 ± 0.02 ^{ab}
C22:6n-3 (DHA)	10.74 ± 0.21 ^d	12.62 ± 0.16 ^a	12.08 ± 0.35 ^b	11.49 ± 0.21 ^c	11.73 ± 0.17 ^c
ΣPUFA	49.2 ± 0.32 ^c	50.7 ± 0.39 ^a	49.93 ± 0.33 ^b	49.84 ± 0.64 ^b	49.79 ± 0.2 ^{ab}
Σn-6PUFA	22.96 ± 0.2 ^b	20.95 ± 0.28 ^c	22.05 ± 0.21 ^b	23.06 ± 0.25 ^a	22.38 ± 0.22 ^b
Σn-3PUFA	26.24 ± 0.44 ^d	29.75 ± 0.26 ^a	27.88 ± 0.43 ^b	26.78 ± 0.39 ^c	27.41 ± 0.17 ^b
n-3/n-6PUFA	1.14 ± 0.03 ^d	1.42 ± 0.02 ^a	1.26 ± 0.03 ^b	1.16 ± 0.01 ^c	1.23 ± 0.02 ^b

Note: Data are expressed as mean ± SD ($n = 4$). Data in the same row sharing different superscript letters denote significant differences determined by Duncan's test ($p < 0.05$). Rows without superscript letters indicate no significant differences among treatments ($p > 0.05$). Only the main fatty acids are listed in the table, including: C14:0, C15:0, C16:0, C18:0, C19:0, C20:0, C16:1n-7, C17:1n-7, C18:1n-9, C20:1n-9, C22:1n-9, C18:2n-6 (LA), C18:3n-3 (LNA), C20:2n-6, C20:4n-6 (ARA), C20:5n-3 (EPA), C22:5n-3, and C22:6n-3 (DHA). ΣSFA: saturated fatty acids; ΣMUFA: monounsaturated fatty acids; ΣPUFA: polyunsaturated fatty acids.

Conversely, dietary CBD supplementation significantly altered the monounsaturated (ΣMUFA) and polyunsaturated fatty acid (ΣPUFA) profiles. The total ΣMUFA content was significantly reduced in all CBD-treated groups compared to the control group ($p < 0.05$). This reduction was primarily driven by a significant decrease in oleic acid (C18:1), which dropped from 20.13% in the control group to its lowest level of 18.69% in Group B (10 mg/kg) ($p < 0.05$).

Most notably, CBD supplementation significantly promoted the accumulation of ΣPUFAs in the shrimp muscle, with the most pronounced effect observed at the lowest inclusion level. The total ΣPUFA content reached a maximum of 50.70% in Group B ($p < 0.05$). This overall increase was heavily attributed to the significant enrichment of n-3 PUFAs. Specifically, the levels of eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) peaked significantly in Group B at 15.22% and 12.62%, respectively, both of which were significantly higher than those in the control group (13.47% and 10.74%) ($p < 0.05$). Although the contents of n-3 PUFA, EPA, and DHA slightly declined as the

CBD dosage further increased from 20 to 80 mg/kg (Groups C, D, and E), they generally remained elevated compared to the control. Meanwhile, the total n-6 PUFA content showed slight fluctuations among the treatments, largely reflecting the variations in linoleic acid (LA, C18:2n-6), which was significantly minimized in Group B.

3.3. Hepatopancreatic Digestive Enzyme Activities and Apparent Digestibility

The effects of dietary CBD supplementation on hepatopancreatic digestive enzyme activities and the apparent digestibility of nutrients in *L. vannamei* are presented in Table 6. Regarding the digestive enzymes, dietary CBD significantly stimulated the secretion and activity of key metabolic enzymes compared to the control ($p < 0.05$). Specifically, the hepatopancreatic protease activity was significantly elevated in all CBD-supplemented groups compared to Group A, with no significant differences observed among the varying CBD dosages ($p > 0.05$). Amylase activity exhibited a dose-dependent increase, peaking significantly in Group D (40 mg/kg) ($p < 0.05$). Interestingly, the activities of lipase and Na^+ , K^+ -ATPase followed an inverted U-shaped trend. Both enzymes reached their maximum activities in Group C (20 mg/kg), which were significantly higher than those of the control group ($p < 0.05$). However, as the CBD dosage further increased to 80 mg/kg, the activities of lipase and Na^+ , K^+ -ATPase declined to levels that showed no significant difference from the control group ($p > 0.05$).

Table 6. Effects of dietary CBD levels on hepatopancreatic digestive enzyme activities and apparent digestibility of nutrients in *L. vannamei*.

Parameters	Diet A (0 mg/kg)	Diet B (10 mg/kg)	Diet C (20 mg/kg)	Diet D (40 mg/kg)	Diet E (80 mg/kg)
Digestive Enzymatic Activity					
Amylase/(U/gprot)	0.98 ± 0.04^d	1.01 ± 0.02^{cd}	1.03 ± 0.01^{bc}	1.16 ± 0.03^a	1.06 ± 0.02^b
Protease/(U/gprot)	2841.73 ± 34.97^b	2965.67 ± 30.64^a	2931.04 ± 37.35^a	2930.44 ± 44.15^a	2924.97 ± 24.09^a
Lipase/(U/gprot)	2.19 ± 0.49^c	4.22 ± 1.29^{ab}	5.03 ± 1.04^a	3.22 ± 0.65^{bc}	2.85 ± 0.57^c
Na^+ , K^+ -ATPase/(U/mgprot)	23.57 ± 0.49^b	24.27 ± 0.74^b	27.15 ± 1.53^a	23.88 ± 0.65^b	23.74 ± 1.01^b
Apparent Digestibility					
Dry matter	81.53 ± 1.77^b	81.71 ± 1.33^b	84.83 ± 1.48^a	82.5 ± 0.84^{ab}	81.53 ± 2.53^b
Crude protein	86.96 ± 1.25^b	87.03 ± 0.94^b	89.31 ± 1.04^a	88.07 ± 0.57^{ab}	87.14 ± 1.76^b
Crude lipid	89.84 ± 0.98^b	92.45 ± 0.55^a	92.93 ± 0.69^a	90.5 ± 0.45^b	89.08 ± 1.5^b

Note: Data are expressed as mean $\pm$ SD ($n = 4$). Data in the same row sharing different superscript letters denote significant differences determined by Duncan's test ($p < 0.05$).

Consistent with the trends observed in hepatopancreatic enzymatic activities, the apparent digestibility of nutrients was notably enhanced by moderate CBD inclusion. The apparent digestibility of dry matter and crude protein reached their peak values in Group C (20 mg/kg) at 84.83% and 89.31%, respectively, both of which were significantly higher than those in the control group ($p < 0.05$). Similarly, the apparent digestibility of crude lipid was significantly improved in both Group B (10 mg/kg) and Group C (20 mg/kg) compared to Group A ($p < 0.05$). Consistent with the enzymatic decline at high dosages, when the CBD supplementation reached 80 mg/kg (Group E), the apparent digestibility parameters for dry matter, crude protein, and crude lipid all decreased, showing no significant differences from the control group ($p > 0.05$).

3.4. Serum Biochemical Parameters

The activities of serum transaminases, which serve as indicators of hepatopancreatic health, were significantly affected by dietary CBD (Table 7). The ALT activity remained stable across the control and low-to-moderate dosage groups ($p > 0.05$). However, ALT activity exhibited a sharp and significant increase in the high-dose group (Group E, 80 mg/kg),

reaching more than double the activity level of the control group ($p < 0.05$). Conversely, AST activity was significantly reduced only in Group B (10 mg/kg) ($p < 0.05$), while no significant differences were observed among the other treatments ($p > 0.05$).

Table 7. Effects of dietary CBD levels on serum biochemical parameters of *L. vannamei*.

Parameters	Diet A (0 mg/kg)	Diet B (10 mg/kg)	Diet C (20 mg/kg)	Diet D (40 mg/kg)	Diet E (80 mg/kg)
ALT/(U/L)	1.69 ± 1.09 ^b	2.09 ± 1.31 ^b	1.88 ± 0.53 ^b	1.61 ± 0.38 ^b	3.72 ± 0.46 ^a
AST/(U/L)	32.69 ± 2.55 ^a	20.93 ± 2.46 ^b	29.46 ± 1.18 ^a	29.33 ± 1.86 ^a	30.31 ± 2.02 ^a
LZM/(U/mL)	164.06 ± 23.59 ^b	220.31 ± 38.65 ^{ab}	206.24 ± 15.31 ^{ab}	224.99 ± 26.52 ^a	178.12 ± 24.21 ^{ab}
ALP/(U_KA/100 mL)	8.38 ± 0.35 ^c	9.48 ± 0.51 ^b	10.94 ± 0.94 ^a	10.84 ± 0.49 ^a	9.23 ± 0.91 ^{bc}
ACP/(U_KA/100 mL)	13.49 ± 0.37 ^b	12.93 ± 0.27 ^b	14.85 ± 0.55 ^a	14.94 ± 0.56 ^a	13.32 ± 0.29 ^b
T-AOC/(mM)	0.34 ± 0.02 ^c	0.43 ± 0.01 ^b	0.49 ± 0.01 ^a	0.35 ± 0.01 ^c	0.3 ± 0.01 ^d
CAT/(U/mL)	1.71 ± 0.23 ^b	1.87 ± 0.38 ^{ab}	1.82 ± 0.11 ^b	2.21 ± 0.19 ^a	1.75 ± 0.21 ^b
MDA/(mmol/L)	4.62 ± 0.57 ^a	3.94 ± 0.25 ^b	3.71 ± 0.62 ^b	3.86 ± 0.38 ^b	4.85 ± 0.25 ^a

Note: Data are expressed as mean ± SD ($n = 4$). Data in the same row sharing different superscript letters denote significant differences determined by Duncan's test ($p < 0.05$).

Dietary CBD supplementation generally enhanced serum non-specific immune responses at moderate dosages. The LZM activity showed an increasing trend with CBD supplementation, reaching its highest level in Group D (40 mg/kg), which was significantly higher than that of the control group ($p < 0.05$). Similarly, the activities of ALP and ACP were significantly elevated in groups fed 20 and 40 mg/kg CBD compared to the control group ($p < 0.05$). When the CBD dosage increased to 80 mg/kg, the activities of LZM, ALP, and ACP declined, showing no significant differences from the control group ($p > 0.05$).

The antioxidant capacity and lipid peroxidation levels exhibited a clear dose-dependent response to CBD. The T-AOC was significantly enhanced in Groups B and C, peaking at 0.49 mM in Group C (20 mg/kg) ($p < 0.05$). However, T-AOC significantly dropped below the control level in Group E ($p < 0.05$). CAT activity peaked significantly in Group D (40 mg/kg) ($p < 0.05$) and returned to baseline levels in Group E. Consequently, the concentration of MDA, a primary marker of oxidative stress and lipid peroxidation, was significantly reduced in Groups B, C, and D compared to the control ($p < 0.05$). Notably, in the 80 mg/kg group, the MDA content rebounded to 4.85 mmol/L, which was the highest among all groups and not significantly different from the control ($p > 0.05$).

3.5. Hepatopancreatic Antioxidant Parameters

The effects of dietary CBD levels on the hepatopancreatic antioxidant capacity of *L. vannamei* are detailed in Table 8. Overall, dietary CBD supplementation significantly enhanced the antioxidant defense system in the hepatopancreas compared to the control ($p < 0.05$).

Table 8. Effects of dietary CBD levels on hepatopancreatic antioxidant-related parameters in *L. vannamei*.

Parameters	Diet A (0 mg/kg)	Diet B (10 mg/kg)	Diet C (20 mg/kg)	Diet D (40 mg/kg)	Diet E (80 mg/kg)
T-AOC/(mmol/gprot)	1.41 ± 0.06 ^c	2.04 ± 0.14 ^a	1.71 ± 0.09 ^b	1.71 ± 0.08 ^b	1.58 ± 0.1 ^b
CAT/(U/mgprot)	3.5 ± 0.46 ^b	4.37 ± 0.57 ^a	4.34 ± 0.36 ^a	4.59 ± 0.65 ^a	4.26 ± 0.57 ^{ab}
GSH-Px/(U/mgprot)	1637.75 ± 41.77 ^c	1853.55 ± 78.09 ^a	1731.24 ± 38.79 ^b	1739.48 ± 43.71 ^b	1674.25 ± 53.04 ^{bc}
POD/(U/mgprot)	21.58 ± 3.17 ^b	31.06 ± 4.37 ^a	28.66 ± 3.36 ^a	33.51 ± 3.54 ^a	27.83 ± 2.99 ^{ab}
MDA/(nmol/mgprot)	26.7 ± 0.73 ^a	23.15 ± 0.62 ^b	22.38 ± 0.86 ^b	23.12 ± 1.04 ^b	22.81 ± 0.23 ^b

Note: Data are expressed as mean ± SD ($n = 4$). Data in the same row sharing different superscript letters denote significant differences determined by Duncan's test ($p < 0.05$).

The T-AOC and the activity of GSH-Px exhibited similar dose–response patterns, both reaching their maximum values in Group B (10 mg/kg), which were significantly higher than those in all other treatments ($p < 0.05$). Although their activities decreased as the CBD inclusion level further increased (Groups C, D, and E), T-AOC remained significantly elevated compared to the control group ($p < 0.05$). However, the GSH-Px activity in the highest dosage group (Group E, 80 mg/kg) declined to a level that showed no significant difference from the control group ($p > 0.05$).

Similarly, the activities of CAT and POD were significantly up-regulated in the low- to moderate-dose groups (Groups B, C, and D) compared to Group A ($p < 0.05$), with both enzymes recording their peak activities in Group D (40 mg/kg). Consistent with the other antioxidant markers, when the CBD dosage increased to 80 mg/kg (Group E), the activities of CAT and POD diminished, exhibiting no significant differences from the control group ($p > 0.05$).

Consequent to the up-regulation of antioxidant enzymes, the concentration of MDA, a primary biomarker of lipid peroxidation and oxidative damage, was significantly reduced by CBD supplementation. The hepatopancreatic MDA content in all CBD-treated groups (Groups B to E) was significantly lower than that in the control group ($p < 0.05$).

3.6. Modulation of Intestinal Microbiota Composition by Dietary CBD

3.6.1. Sequencing Quality and Alpha Diversity

To evaluate the impact of dietary CBD on the intestinal microecosystem of *L. vannamei*, high-throughput 16S rRNA gene sequencing was performed. The sequencing depth and species richness were first validated. As illustrated by the rarefaction curves (Figure 1B), all samples tended to reach a plateau, and the coverage index for all groups was greater than 99.9%, indicating that the sequencing depth was sufficient to capture most of the microbial diversity within the shrimp intestine. Community richness was evaluated using the Ace and Chao indices, while diversity was assessed by the Shannon and Simpson indices (Figure 1A). Regarding alpha diversity, the Simpson index showed a trend of decreasing first and then increasing with the elevation of dietary CBD supplementation. The lowest Simpson index was observed in Group C, which was significantly different from Groups A, D, and E ($p < 0.05$), but showed no significant difference compared to Group B ($p > 0.05$). Conversely, Group E recorded the highest value, which was significantly higher than those of Groups B and C ($p < 0.05$). No significant differences were observed in the other indices ($p > 0.05$).

3.6.2. Beta Diversity and Community Structure

To evaluate the beta diversity and visualize the structural variations across different treatments, Principal Coordinates Analysis (PCoA) was performed at both the phylum and genus levels (Figure 1C). At the phylum level, the first two principal coordinates (PC1 and PC2) accounted for 50.94% and 22.27% of the total structural variation, respectively. The scatter plot revealed that Group E (80 mg/kg CBD) was distinctly separated from all other groups, indicating a unique and profound shift in the microbial community architecture at this dosage. The control group (Group A) showed no spatial intersection with Groups B and E but exhibited a partial overlap with Groups C and D. Data points for Groups B, C, and D overlapped with each other, suggesting a relatively high degree of structural similarity among these moderate-dose treatments. At the genus level (PC1: 40.16%, PC2: 22.21%), Group E remained clearly isolated. Group A displayed no overlap with Groups C and E. Notably, while Groups C and D did not overlap directly, they were tightly clustered in close spatial proximity. Group B appeared as a transitional cluster, overlapping simultaneously with Groups A, C, and D. Together, these spatial distributions indicate that the highest

CBD dosage (Group E, 80 mg/kg) establishes a microbial community profile structurally distinct from both the control and the lower-dosage groups.

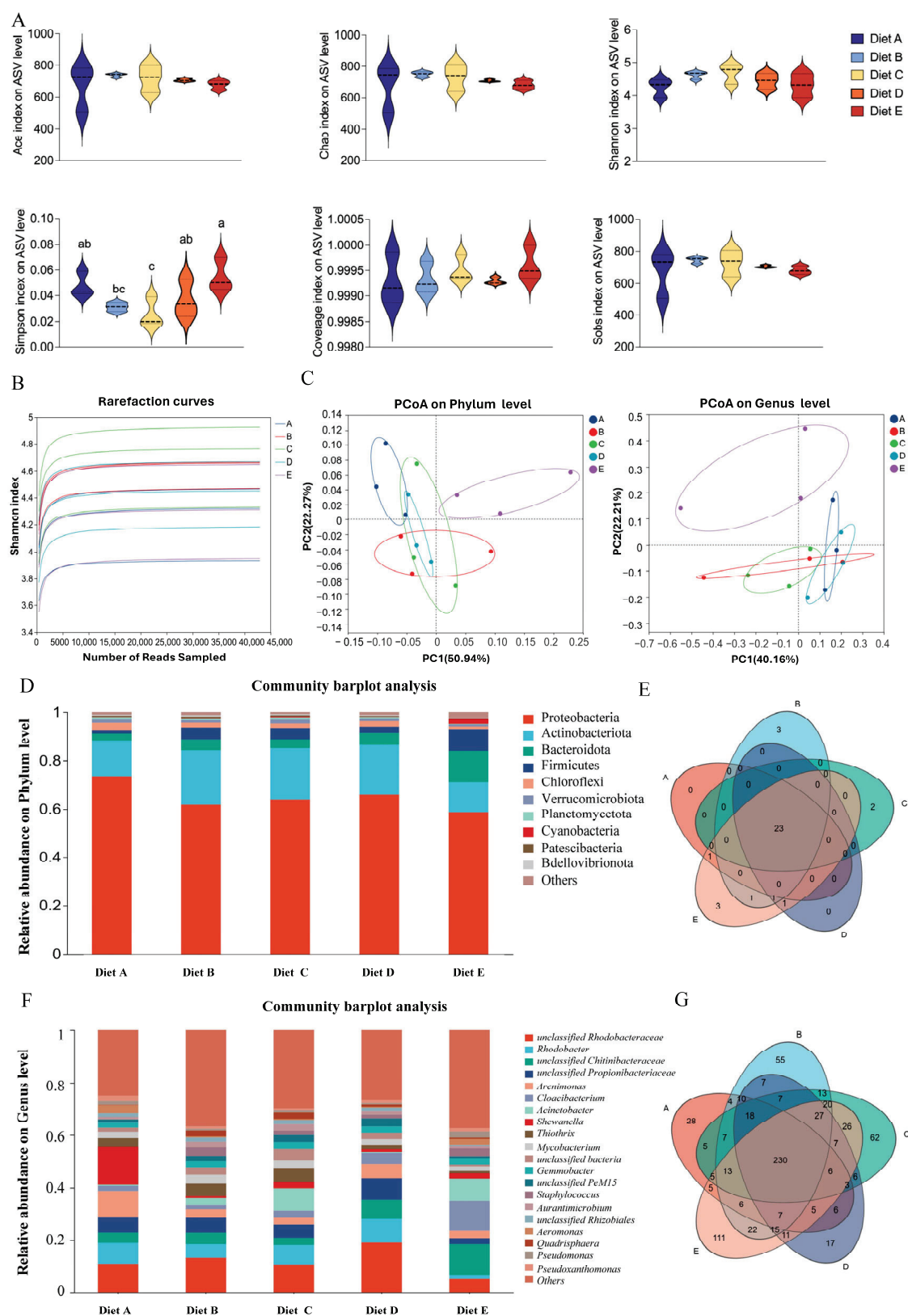


Figure 1. Effects of dietary cannabidiol (CBD) supplementation on the diversity and taxonomic composition of the intestinal microbiota in *L. vannamei*. (A) Alpha diversity indices of the intestinal

microbiota, including Ace, Chao, Shannon, and Simpson indices. Different lowercase letters above the violin plots indicate statistically significant differences among dietary treatments ($p < 0.05$). (B) Rarefaction curves validating the sequencing depth and species richness across all samples. (C) Principal Coordinates Analysis (PCoA) plots illustrating beta diversity and structural variations of the microbial communities at the phylum (left) and genus (right) levels. (D) Community barplot analysis showing the relative abundance of dominant bacterial taxa at the phylum level. (E) Venn diagram displaying the shared and unique bacterial phyla among the five experimental groups. (F) Community barplot analysis detailing the relative abundance of dominant bacterial taxa at the genus level. (G) Venn diagram illustrating the shared and unique operational taxonomic units (OTUs) at the genus level. Groups A, B, C, D, and E correspond to dietary CBD inclusion levels of 0, 10, 20, 40, and 80 mg/kg, respectively.

3.6.3. Taxonomic Composition Profiles

At the phylum level (Figure 1D), the dominant bacterial phyla across all groups were *Proteobacteria*, *Actinobacteriota*, *Bacteroidota*, and *Firmicutes*. The Venn diagram (Figure 1E) showed a highly conserved core microbiome, sharing numerous phyla across all groups, with only a few unique phyla appearing in specific treatments. At the genus level (Figure 1F), the community was predominantly composed of *unclassified_Rhodobacteraceae*, *Rhodobacter*, *unclassified_Chitinibacteraceae*, and *unclassified_Propionibacteriaceae*. The genus-level Venn diagram (Figure 1G) revealed a shared core of 230 operational taxonomic units (OTUs) among all five groups, demonstrating that CBD treatments recruited specific, unique bacterial members to the intestinal environment while maintaining the core community.

3.7. Differential Taxa and Functional Profiling of the Microbiome

3.7.1. Hierarchical Clustering and Abundance Patterns

To further identify specific microbial shifts, a community heatmap at the genus level (Figure 2A) was generated to provide hierarchical clustering of the top abundant genera. The color gradients visually confirmed that CBD treatments induced distinct abundance patterns compared to the control group. Specifically, Group A exhibited a relative enrichment of taxa such as *Shewanella*, *Arenimonas*, and the opportunistic pathogen *Aeromonas*. In contrast, the low- to moderate-dose CBD treatments (Groups B, C, and D) effectively suppressed these taxa. Concurrently, these moderate dosage groups displayed a distinct enrichment of core microflora, e.g., *unclassified_Rhodobacteraceae* and *Staphylococcus*. Notably, the high-dose group (Group E, 80 mg/kg) displayed a completely divergent clustering profile. Within this group, the previously enriched *Rhodobacter* and *Rhodobacteraceae* were relatively depleted, replaced by a profound surge in *norank_f__Chitinibacteraceae* and *Cloacibacterium*. Furthermore, potentially pathogenic genera like *Pseudomonas* and *Aeromonas* showed a rebounding trend compared to the moderate-dose groups. These visual trends corroborate that while moderate CBD supplementation positively modulates the intestinal microbiota, the highest dosage group (80 mg/kg) triggers a drastic structural reconstruction in the current study.

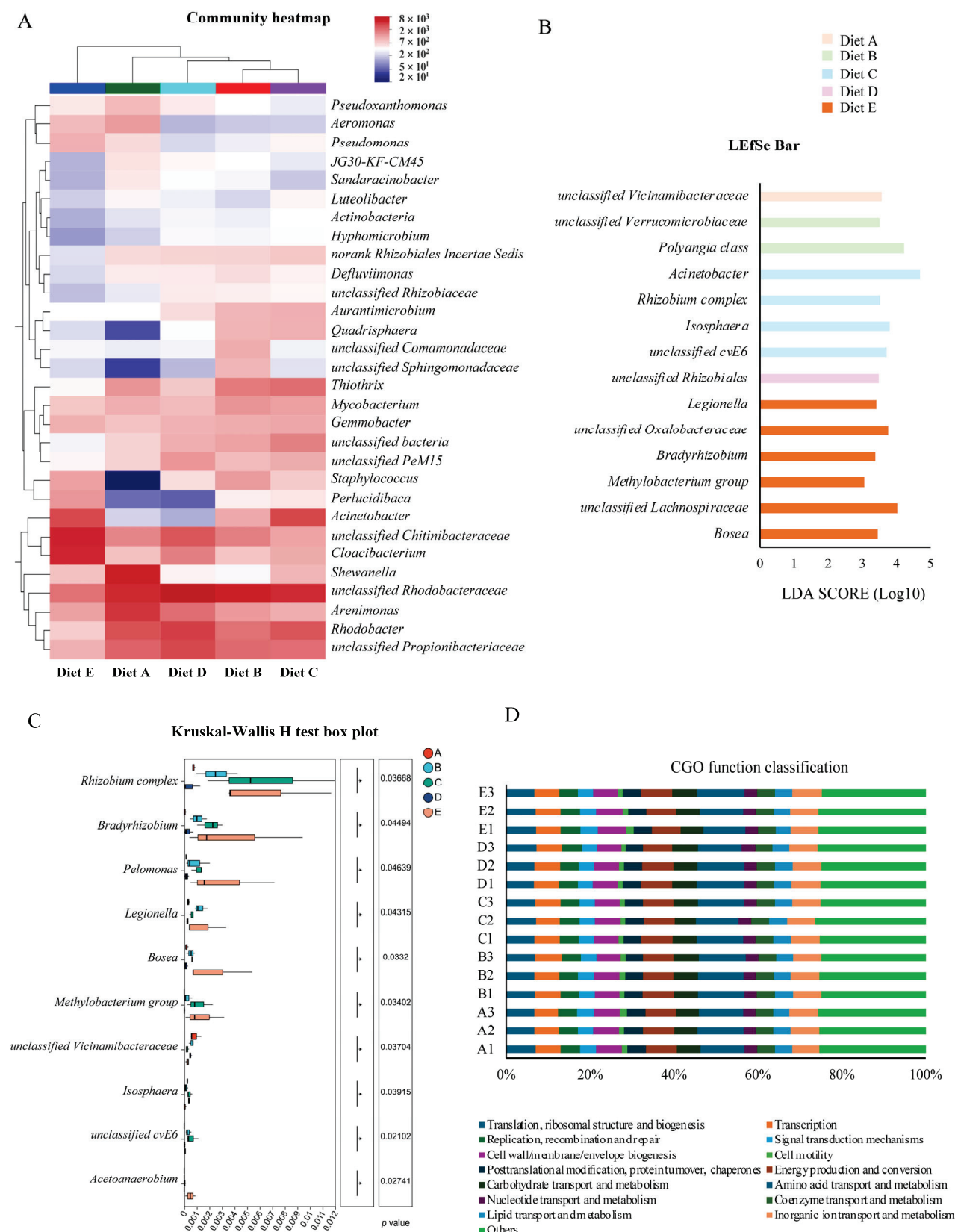


Figure 2. Differential abundance and functional prediction of the intestinal microbiota in *L. vannamei* across different dietary CBD treatments. **(A)** Community heatmap and hierarchical clustering of the top abundant bacterial genera. Color gradients indicate the relative abundance of specific microbial taxa. **(B)** Linear discriminant analysis Effect Size (LEfSe) identifying significantly enriched microbial

biomarkers across the experimental groups (logarithmic LDA score threshold > 3.0 , $p < 0.05$). (C) Differential abundance analysis of specific key bacterial genera evaluated using the Kruskal–Wallis H test (*, $p < 0.05$). (D) Predicted functional profiles of the intestinal microbiome based on the Clusters of Orthologous Genes (COG) database. Groups A, B, C, D, and E correspond to dietary CBD inclusion levels of 0, 10, 20, 40, and 80 mg/kg, respectively.

3.7.2. Identification of Specific Microbial Biomarkers

To identify the specific bacterial taxa driving the structural differences, Linear Discriminant Analysis Effect Size (LEfSe) was performed. A total of 14 discriminative features were identified as significantly enriched biomarkers based on a logarithmic LDA score threshold of > 3.0 and a significance level of $p < 0.05$ (Figure 2B). Overall, dietary CBD significantly altered the microbial biomarkers in a dose-associated manner. In the low- to moderate-dose groups (Groups B, C, and D), specific taxa such as *Polyangia* class, *Acinetobacter* (the highest overall LDA score of 4.69 in Group C), and *unclassified_Rhizobiales* were significantly enriched. Notably, Group E (80 mg/kg CBD) recruited the highest number of specific biomarkers (six taxa). Within this high-dose group, *unclassified_Lachnospiraceae* (LDA = 4.02) and *unclassified_Oxalobacteraceae* (LDA = 3.76) emerged as prominently enriched clades, alongside *Bosea* and *Bradyrhizobium*. These results demonstrate that the high dosage of CBD exerts a strong selective pressure on the microecosystem, leading to an over-representation of specific microbial lineages.

3.7.3. Differential Abundance of Key Genera

To further validate the taxonomic shifts, a differential abundance analysis of key genera was conducted using the Kruskal–Wallis H test (Figure 2C). The results demonstrated that dietary CBD significantly altered the relative abundances of ten specific bacterial genera ($p < 0.05$). Specifically, the control group exhibited the highest abundance of *unclassified_Vicinamibacteraceae*. In the moderate-dose CBD groups, specific taxa such as *unclassified_cvE6* and *Isosphaera* were significantly enriched. Notably, the high-dose treatment (Group E, 80 mg/kg) exerted the most profound effect, triggering the maximum accumulation of multiple distinct genera, predominantly including *Bradyrhizobium*, *Pelomonas*, and *Bosea*. These findings objectively confirm that the 80 mg/kg CBD dosage imposes a strong environmental selection, leading to a targeted expansion of specific bacterial clades.

3.7.4. Functional Prediction of the Microbiome

To evaluate the potential metabolic and ecological functions, the Clusters of Orthologous Genes (COG) functional classification was performed (Figure 2D). Across all experimental groups, the predicted functional profiles were consistently enriched in basic metabolic and cellular processes. Excluding the uncharacterized “Others” category, the most abundant functional classifications were “Amino acid transport and metabolism” (10.0–11.3%), followed by “Energy production and conversion” (6.8–7.4%), “Translation, ribosomal structure and biogenesis” (6.6–7.2%), and “Inorganic ion transport and metabolism” (6.5–7.0%). Statistical analysis revealed no significant differences in the relative abundances of these COG functional categories among the five dietary treatments ($p > 0.05$). These results indicate that despite the structural and taxonomic shifts induced by CBD, the core functional homeostasis and overall metabolic stability of the intestinal microecosystem in *L. vannamei* were well preserved.

3.8. Median Lethal Dissolved Oxygen and Median Lethal Time (LT50) After Combined Stress

To evaluate the effect of dietary CBD on the environmental stress resistance of *L. vannamei*, the shrimp were subjected to a 4-day chronic stress of ammonia and nitrite (2 mg/L)

and followed by an acute hypoxia challenge test. The median lethal dissolved oxygen (LDO₅₀) and median lethal time (LT₅₀) were determined under a combined stress challenge.

The results for LDO₅₀ are detailed in Figure 3. There were no statistically significant differences in the LDO₅₀ values among the five groups ($p > 0.05$). Across all treatments, mortality began to occur at DO levels between 0.51 and 0.72 mg/L, and the calculated LDO₅₀ hovered consistently around 0.57 mg/L.

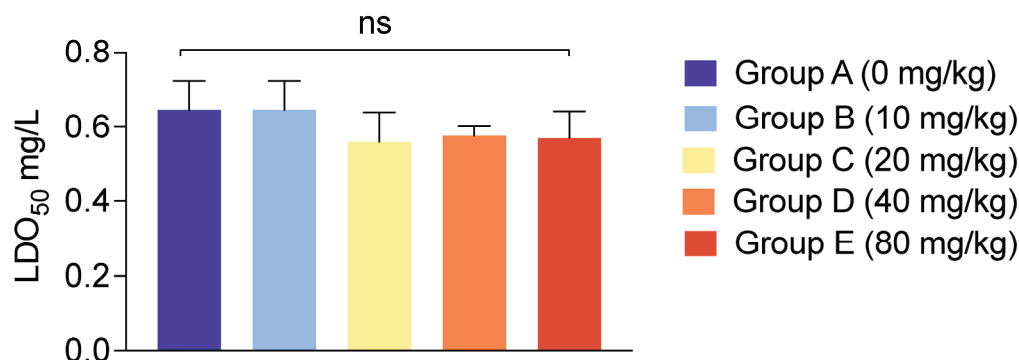


Figure 3. Effects of dietary CBD supplementation on the median lethal dissolved oxygen (LDO₅₀) of *L. vannamei* under combined environmental stress. Shrimp were subjected to a 4-day chronic ammonia and nitrite stress followed by an acute hypoxia challenge. LDO₅₀ values are presented as mean $\pm$ SD of 3 independent biological replicate buckets per treatment group. Groups A, B, C, D, and E correspond to CBD inclusion levels of 0, 10, 20, 40, and 80 mg/kg, respectively. ns, no statistically significant differences were observed among the dietary treatments determined by Duncan's test ($p > 0.05$).

In terms of LT₅₀ (Figure 4A), the control group recorded the lowest average LT₅₀ (131.94 ± 4.15 min). With the inclusion of dietary CBD at low to moderate levels, the LT₅₀ values were significantly prolonged. Specifically, shrimp in Groups B, C, and D exhibited significantly higher LT₅₀ values than those in the control group ($p < 0.05$), reaching a maximum of 170.63 ± 2.33 min in Group B. There were no significant differences in the LT₅₀ values among these three moderate dosage groups ($p > 0.05$). However, as the CBD supplementation level further increased to 80 mg/kg (Group E), the LT₅₀ value declined to 148.82 ± 9.21 min, which showed no significant statistical difference from either the control group or the other CBD-treated groups ($p > 0.05$). The cumulative survival curves further illustrate this trend, showing a clear rightward shift in the survival trajectories of the CBD groups (Figure 4B).

3.9. qPCR Analysis of Stress-Related Genes in the Hepatopancreas

To evaluate the molecular response of the hepatopancreas to the combined stress challenge, the relative mRNA expression levels of key stress, hypoxia, and metabolism-related genes were quantified (Figure 5). The relative expression of *hsp70* exhibited a significant dose-dependent response. Its expression peaked in Group B (10 mg/kg), which was significantly higher than both the control group and the highest dosage group ($p < 0.05$). Conversely, Group E recorded the lowest *hsp70* expression level among all treatments. Similarly, the expression of *hif-1 α* was significantly elevated. Groups B (10 mg/kg) and D (40 mg/kg) showed significantly higher *hif-1 α* expression compared to the control (m), while Groups C and E exhibited intermediate levels with no significant difference from the control. No significant differences in the expression levels of *ldh*, *caspase3*, and *hk* genes among any of the experimental groups ($p > 0.05$) were observed.

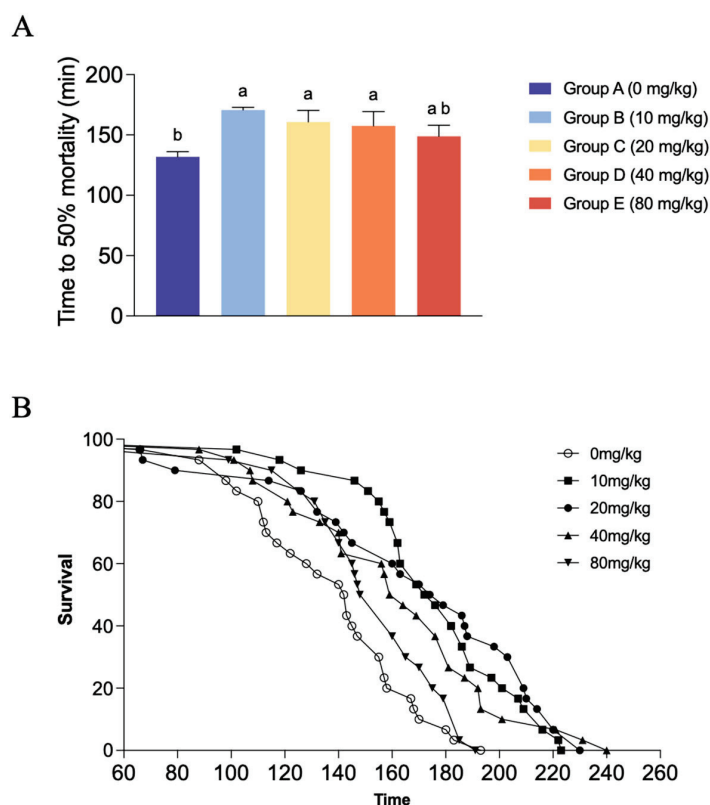


Figure 4. Effects of dietary CBD supplementation on the hypoxia tolerance of *L. vannamei* under combined environmental stress. **(A)** Median lethal time (LT₅₀) across different dietary treatments during the acute hypoxia challenge. LT₅₀ values are presented as mean $\pm$ SD of 3 independent biological replicate buckets per treatment group. Different lowercase letters indicate statistically significant differences among the groups ($p < 0.05$). Groups A, B, C, D, and E correspond to CBD inclusion levels of 0, 10, 20, 40, and 80 mg/kg, respectively. **(B)** Cumulative survival curves generated using the Kaplan–Meier method based on the death time of all 30 individual shrimp merged from 3 biological replicate buckets per treatment group, illustrating the rightward shift in survival trajectories for the CBD-supplemented groups.

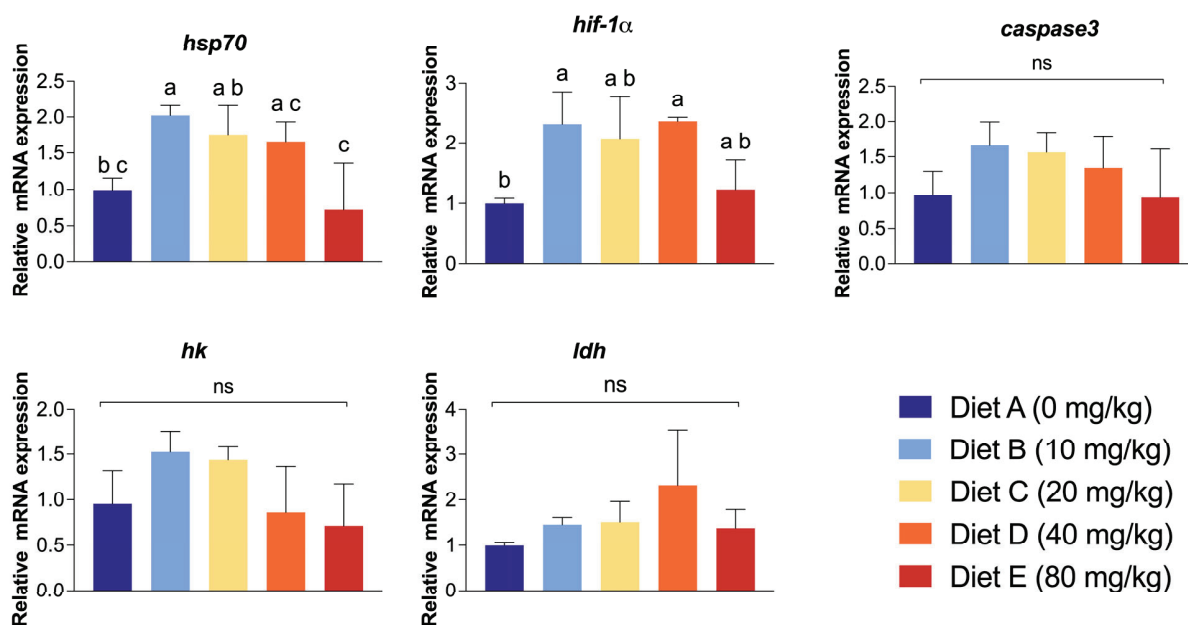


Figure 5. Relative mRNA expression levels of key stress, hypoxia, and metabolism-related genes in the hepatopancreas of *L. vannamei*. The quantified genes include heat shock protein 70 (*hsp70*),

hypoxia-inducible factor 1-alpha (*hif-1α*), cysteine-aspartic acid protease 3 (*caspase3*), hexokinase (*hk*), and lactate dehydrogenase (*ldh*). Data are presented as mean ± SD ($n = 3$ per treatment). Different lowercase letters above the error bars denote statistically significant differences among the dietary treatments ($p < 0.05$), while “ns” indicates no significant difference. Groups A, B, C, D, and E correspond to CBD inclusion levels of 0, 10, 20, 40, and 80 mg/kg, respectively.

3.10. Correlation Analysis Among Growth, Physiology, and Stress Resistance

To elucidate the potential internal relationships between macroscopic phenotypes and physiological shifts, a Pearson correlation analysis was performed (Figure 6). The correlation matrix revealed robust, statistically significant interactions integrating feed utilization, antioxidant capacity, and ultimate stress endurance. Notably, the LT_{50} under combined stress exhibited strong positive correlations with the T-AOC ($r = 0.842$), *hif-1α* expression ($r = 0.786$), and protease activity ($r = 0.748$, $p < 0.05$). Conversely, LT_{50} was significantly and negatively correlated with the FCR, the oxidative damage marker MDA, and the hepatopancreatic injury indicator AST ($p < 0.05$). Furthermore, FCR was profoundly and negatively correlated with amylase activity ($r = -0.764$) and protective molecules like POD and *hsp70*, while positively correlating with MDA ($p < 0.05$). Within the physiological parameters, T-AOC showed highly synergistic positive correlations with GSH-Px and *hif-1α*, and strong negative correlations with AST and MDA ($p < 0.05$).

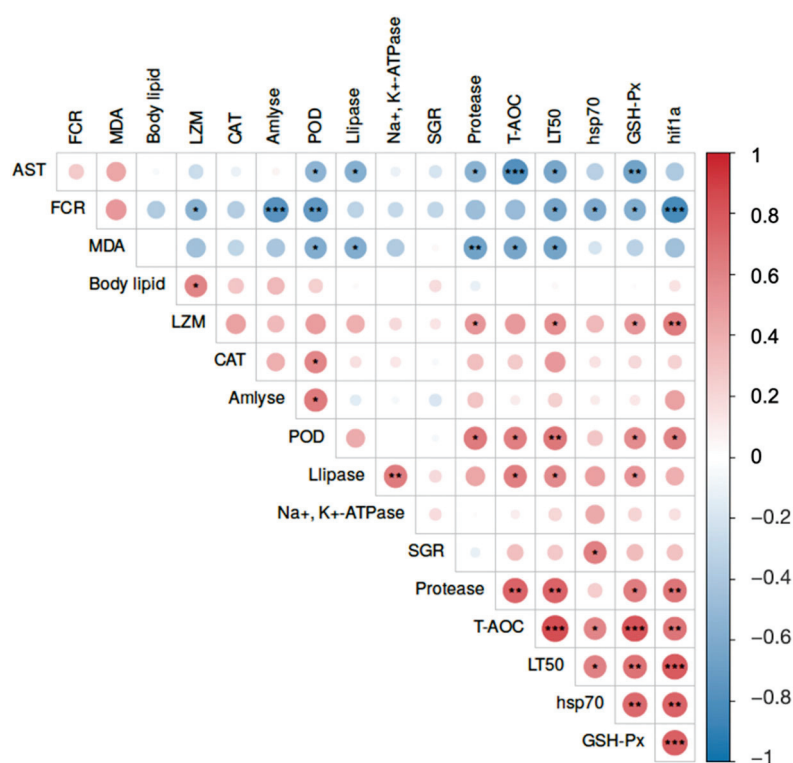


Figure 6. Pearson correlation matrix integrating macroscopic phenotypes and micro-physiological parameters in *L. vannamei*. The heatmap elucidates the potential internal relationships among core parameters of feed utilization (FCR), digestive capacity (protease, amylase), antioxidant status (T-AOC, GSH-Px, POD, MDA), hepatopancreatic health (AST), stress-related gene expression (*hif-1α*, *hsp70*), and ultimate environmental stress endurance (LT_{50}). Color gradients represent the correlation coefficient (r), with positive correlations and negative correlations distinctly color-coded. Statistical significance for the correlation is defined as *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, respectively.

4. Discussion

Given the physiological challenges imposed by intensive farming practices, the exploration of bioactive phytochemicals to enhance shrimp health and productivity has become increasingly relevant. In this context, the present study evaluated the effects of CBD on *L. vannamei*, revealing dose-dependent responses in growth, immunity, and intestinal microecology.

4.1. Moderate CBD Improves Feed Efficiency and Muscle Fatty Acid Composition

Our findings demonstrate that moderate CBD supplementation effectively promotes somatic growth and minimizes the FCR in *L. vannamei*. These growth-promoting effects align closely with recent findings in various teleost models. For instance, dietary supplementation with cannabis extracts significantly improved the feed conversion and growth performance of Nile tilapia (*O. niloticus*) [22], while CBD administration enhanced appetite and somatic growth in juvenile large yellow croaker (*L. crocea*) fed high-lipid diets [27]. Rather than merely stimulating feed intake, our correlation matrix (Figure 6) highlights that the enhanced feed efficiency is fundamentally driven by the robust secretagogue effect of CBD on the hepatopancreas. By positively modulating the digestive enzymatic cascade—particularly amylase and protease activities—CBD facilitates the breakdown and apparent digestibility of dietary macromolecules. This is further corroborated by recent mammalian evidence showing that CBD profoundly regulates intestinal lipid absorption and metabolic transport [45].

Interestingly, CBD also exhibited a highly specific, bidirectional modulation of lipid metabolism. While the best somatic growth was observed at 40 mg/kg within the concentration range tested in this study, the lowest inclusion level (10 mg/kg) preferentially directed lipid fluxes toward the biosynthesis of HUFAs, maximizing the deposition of EPA and DHA in the muscle. This phenomenon is powerfully supported by recent biomedical research. For instance, Bielawiec et al. (2023) demonstrated that CBD administration significantly ameliorates muscular lipid profiles by inhibiting de novo lipogenesis and specifically altering the desaturation and elongation ratios of fatty acids in skeletal muscle [46]. Furthermore, CBD is well-documented to reduce intracellular lipid accumulation and prevent hepatosteatosis [47]. Taken together, these mechanistic insights suggest that low-dose CBD may act as a potent elicitor for endogenous lipid-modifying enzymes (i.e., elongases or desaturases) in crustaceans, diverting energy from simple fat storage toward the synthesis of essential structural lipids. This metabolic shift not only improves the physiological quality of the shrimp but also significantly elevates its nutritional value for human consumption.

4.2. CBD Enhances Environmental Stress Tolerance via Antioxidant and Hif-1 α Pathways

Intensive shrimp farming is frequently compromised by cyclic hypoxia and ammonia accumulation, which provoke severe oxidative stress and tissue damage. The present study reveals that CBD significantly fortifies the environmental resilience of shrimp, effectively extending their survival window (LT₅₀) under combined lethal stressors. Mechanistically, this survival advantage is deeply rooted in CBD's intrinsic biochemical properties. The rich hydroxyl groups within the polyphenol-like structure of CBD confer exceptional free-radical scavenging capabilities [29,30]. Consequently, dietary CBD effectively neutralized ROS overproduction during hypoxia, preventing lipid peroxidation, as evidenced by suppressed MDA levels, and sparing endogenous antioxidant enzymes, such as GSH-Px and CAT, from rapid depletion. This robust systemic stress mitigation mirrors observations in teleosts, where CBD administration significantly attenuated cortisol levels and mitigated both acute and chronic stress responses in Nile tilapia [25,26].

Furthermore, our correlation network delineates a critical molecular pathway mediating this enhanced hypoxia tolerance: the up-regulation of the *hif-1 α* signaling cascade. Strikingly, this phenotypic observation is strongly corroborated by a recent mammalian model study, which demonstrated that CBD administration effectively prevented antioxidant depletion, reduced lipid peroxidation, and notably stabilized HIF-1 α protein levels during global hypoxia [48]. By sensitizing the *hif-1 α* pathway prior to the acute hypoxic event, CBD enables the hepatopancreas of the shrimp to proactively assemble a robust antioxidant shield and heat shock protein chaperones [49]. This proactive defense mechanism minimizes structural damage to vital organs and sustains cellular energy production under extreme environmental duress.

4.3. CBD Modulates Intestinal Microbiota and Suppresses Opportunistic Pathogens

The gastrointestinal tract is not only the primary site for nutrient assimilation but also the foremost immunological barrier in crustaceans [50–52]. Our 16S rRNA sequencing revealed that moderate CBD supplementation exerted a targeted ecological selection pressure on the intestinal microbiota, positively restructuring the core community. Notably, the relative abundances of *Aeromonas* and *Pseudomonas*—classic opportunistic pathogens in aquaculture—were substantially suppressed in the low- to moderate-dose CBD groups [53]. This antimicrobial potential of CBD is highly consistent with recent *in silico* assessments, which identified that CBD exhibits potent inhibitory binding affinities against crucial structural proteins of severe aquatic pathogens [28].

Beyond direct molecular interactions, emerging biomedical research highlights CBD's profound capacity to indirectly eliminate pathogens and protect the gut barrier via the host-microbiome axis. For instance, cannabinoid interventions have been shown to remodel the gut microecosystem by diminishing inflammation-associated opportunistic taxa (e.g., *Desulfovibrionaceae*) and promoting the intestinal secretion of antimicrobial peptides [34]. Furthermore, even when exposed to high pathogenic loads or bacterial endotoxins like lipopolysaccharides, CBD effectively antagonizes LPS-induced reactive enteric gliosis, intense intestinal inflammation, and epithelial apoptosis, thereby preserving the structural integrity of the mucosal barrier [34].

Concurrently, CBD-enriched core beneficial taxa, such as *Rhodobacteraceae*, are well documented for their roles in aquatic biogeochemical cycling and competitive pathogen exclusion [54,55]. This positive structural modulation of the microbiome likely alleviates the local inflammatory burden on the intestinal epithelium. Consequently, the host can reallocate energy resources to bolster systemic non-specific immunity, completely elucidating the significant elevation in serum lysozyme and phosphatase activities observed in our study.

Interestingly, the enhancement of baseline LZM activity by moderate CBD supplementation highlights its role as a proactive immune elicitor. In our study, CBD pre-conditioned the non-specific immune system of healthy shrimp prior to environmental challenges. This immunomodulatory behavior of CBD appears to be highly context-dependent. For instance, in a recent teleost model subjected to severe chronic stress, CBD exerted a stress-mitigating effect by reducing the abnormally spiked LZM levels back to physiological baselines [56]. Together with our findings, this dynamic contrast suggests that CBD functions not merely as a unidirectional immune stimulant, but rather as a profound homeostatic regulator, which bolsters immune readiness under normal conditions while preventing immune overexhaustion during severe stress.

4.4. Excessive CBD Supplementation Induces Metabolic Burden and Hepatopancreatic Toxicity

A critical objective of pharmacological and nutritional studies is identifying the safety threshold of novel additives. Our study revealed a classic biphasic dose–response effect of CBD in *L. vannamei*. While doses of 10–40 mg/kg conferred comprehensive physiological benefits within the range tested in this study, the highest dosage (80 mg/kg) aggressively compromised the host’s performance, leading to a deterioration in FCR and a diminished LT₅₀ advantage.

This macroscopic decline was strictly mirrored by microscopic toxicological indicators, illuminating a severe metabolic burden. At 80 mg/kg, massive energy resources were seemingly diverted from somatic growth to the detoxification of excessive xenobiotics, drastically depleting whole-body lipid stores. More alarmingly, the serum ALT activity in the high-dose group surged to more than double that of the control, providing definitive evidence of increased hepatopancreatic membrane permeability and substantial tissue damage. Crucially, this finding aligns with recent toxicological warnings in mammalian models and clinical safety updates, where the administration of high-dose CBD has been explicitly linked to hepatotoxicity and drastic elevations in liver transaminases [57,58]. Furthermore, heavy or overdose applications of phytocannabinoids are increasingly recognized to provoke adverse systemic effects, overwhelming the host’s detoxification capacity and inducing severe metabolic exhaustion [59]. While low doses of CBD (10 and 20 mg/kg) efficiently enhance animal welfare and physiological homeostasis in Nile tilapia [25], the exhaustion of protective mechanisms at 80 mg/kg was evident in our study, characterized by the severe down-regulation of *hsp70* transcription and rebounding MDA levels. Collectively, these findings caution that while CBD is a highly promising functional additive, its dietary inclusion must be strictly regulated (recommended below 40 mg/kg based on the results of the present study) to prevent metabolic exhaustion and hepatopancreatic toxicity in intensive shrimp culture.

Despite the comprehensive physiological benefits observed, several limitations of the present study should be acknowledged. First, the 8-week feeding trial primarily focused on the juvenile stage of *L. vannamei*. Long-term studies encompassing the entire grow-out phase are required to fully evaluate the lifelong impacts and potential bioaccumulation of CBD. Second, the laboratory-controlled acute stress models may not completely replicate the complex, multifactorial stressors encountered in actual open-pond aquaculture systems, suggesting that large-scale field trials are necessary before commercial translation.

5. Conclusions

The present study establishes CBD as a highly promising functional feed additive for *L. vannamei*, characterized by a distinct biphasic dose–response. Moderate dietary CBD supplementation (10–40 mg/kg) significantly improves feed efficiency, enriches EPA and DHA in the muscle, and actively restructures the intestinal microecosystem by suppressing opportunistic pathogens within the concentration range tested in this study. Crucially, CBD supplementation at 10–40 mg/kg up-regulates *hif-1α* and *hsp70* pathways and amplifies systemic antioxidant networks, thereby dramatically extending survival under severe ammonia and hypoxia stress. However, excessive CBD inclusion (80 mg/kg) overwhelms the host’s detoxification capacity, resulting in lipid depletion, severe hepatopancreatic tissue damage, and compromised environmental resilience. Therefore, careful dosage regulation is imperative. Dietary CBD at the effective levels identified in this study (up to 40 mg/kg) represents a safe, sustainable, and powerful nutritional strategy to enhance growth, immune homeostasis, and stress endurance in intensive crustacean aquaculture.

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Abbreviations

The following abbreviations are used in this manuscript:

ACP	Acid phosphatase
ADDM	Apparent Dry Matter Digestibility
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AND	Apparent Nutrient Digestibility
ANOVA	Analysis of variance
ARA	Arachidonic acid
AST	Aspartate aminotransferase
<i>caspase 3</i>	Cysteine-aspartic acid protease 3
CAT	Catalase
CBD	Cannabidiol
COG	Clusters of Orthologous Genes
DHA	Docosahexaenoic acid
DO	Dissolved oxygen
EPA	Eicosapentaenoic acid
FBW	Final body weight
FCR	Feed Conversion Ratio
FI	Feed intake
GLU	Glucose
GSH-Px	Glutathione peroxidase
<i>hif-1α</i>	Hypoxia-inducible factor 1-alpha
<i>hk</i>	Hexokinase
<i>hsp70</i>	Heat shock protein 70
HUFA	Highly unsaturated fatty acids
IBW	Initial body weight
LA	Linoleic acid
LDA	Linear discriminant analysis
LDO50	Median lethal dissolved oxygen

ldh	Lactate dehydrogenase
LEfSe	Linear Discriminant Analysis Effect Size
LNA	Linolenic acid
LPS	Lipopolysaccharides
LT50	Median lethal time
LZM	Lysozyme
MDA	Malondialdehyde
OTUs	Operational Taxonomic Units
PC1/PC2	Principal coordinates
PCoA	Principal Coordinates Analysis
POD	Peroxidase
ROS	Reactive oxygen species
SD	Standard deviation
SGR	Specific Growth Rate
SV	Survivability
T-AOC	Total antioxidant capacity
TG	Triglycerides
THC	Δ^9 -tetrahydrocannabinol
WGR	Weight Gain Rate
Σ MUFA	Monounsaturated fatty acids
Σ PUFA	Polyunsaturated fatty acids
Σ SFA	Saturated fatty acids

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Article

Antioxidant Capacity, Lipid Oxidation, and Quality Traits of Slow- and Fast-Growing Meagre (*Argyrosomus regius*) Fillets During Cold Storage

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Abstract: Meagre (*Argyrosomus regius*) is an important species in aquaculture, with size and flesh quality playing key roles in its production and marketability. This study aimed to examine the relationship between growth and flesh quality parameters, including fatty acid content (FA), total antioxidant capacity (TOAC), superoxide dismutase (SOD), lipid oxidation (LO), muscle cellularity (MC), and filleting yield (FY) during cold storage. Fish from the same hatchery raised under identical conditions showed size variation after 12 months. Fish below 600 g were classified as slow growing (SG), while those above 1000 g were classified as fast-growing (FG). The results showed that FG fish had higher body weight, moisture, and FY but exhibited lower levels of fat and polyunsaturated fatty acids (PUFA). SG fish had higher TOAC and SOD activity, which significantly declined during cold storage in both groups but remained higher in SG fish. Despite the higher lipid content in SG fish, no significant differences in malondialdehyde (MDA) levels, an indicator of LO, were observed between the two groups, suggesting that the elevated antioxidant defenses in SG fish mitigated lipid peroxidation. This study underscores intrinsic antioxidants' potential to preserve lipid quality of fish fillets during cold storage.

Keywords: intrinsic antioxidant activity; polyunsaturated fatty acids; lipid oxidation

1. Introduction

Consumers are increasingly discerning in their seafood choices, prioritizing options that offer both product sensory and nutritional value. The global demand for fish species rich in high quality protein and ω -3 fatty acids has surged, reflecting a growing health consciousness among consumers [1]. Meagre (*Argyrosomus regius*) has gained considerable attention in the aquaculture sector due to its favorable farming attributes, including rapid growth and adaptability as well as its desirable sensory attributes. These attributes, closely related to consumer acceptance, make meagre a sought-after species. Renowned for its high protein content,

low fat levels, and essential nutrients, such as ω -3 polyunsaturated fatty acids (PUFAs), meagre appeals to health-conscious consumers [2,3]. At the same time, the impressive aquaculture performance of meagre, marked by rapid growth rates, short production cycles, and broad market acceptance, solidifies its position in Mediterranean aquaculture [4,5].

The market size of aquacultured meagre typically starts at approximately 500 g. However, larger sizes are also frequently available, particularly those exceeding 1 kg, all of which are obtained after 12 months' time, catering to the growing demand for premium-quality fish in diverse culinary markets. The availability of a broad weight range contributes to the versatility and economic viability of meagre aquaculture, enabling it to meet market demands across different sectors from local fresh fish markets to high-end restaurants [1–3]. A notable challenge within several aquaculture fish species is the significant variability in individual growth rates, hindering production efficiency maximization. Even within the same fish farm, growth rates can vary significantly due to genetic and environmental factors as well as resource competition. Such a variability affects the composition of the fish's muscle tissue, raising questions for producers and consumers alike regarding potential flesh quality differences between fish of different sizes [6,7].

From a fish farm management perspective, growth variability leads to differences in harvested fish size and lipid quality, necessitating labor-intensive size grading of farmed fish. After size grading, fish from a common pond or floating cage must be sorted into different ponds or floating cages according to their size, resulting in increased demand for resources to ensure uniform product quality and marketability [6–8]. In terms of consumers' perspective, several studies suggest that fish proximate composition may vary with size and age [9,10]. Growth rate variability may also affect fillet proximate composition, with faster-growing fish potentially accumulating more lipids or showing differences in muscle fiber size and density [11], important parameters for texture and water holding capacity of fish flesh. Understanding these differences is crucial for various aquaculture aspects, including selective breeding, feed formulation, and product quality management, to enhance production efficiency, product consistency, and sustainability. Furthermore, variations in lipid quality and quantity among individual fish, i.e., fast growing vs. slow growing, may affect post-harvest lipid oxidation, off-flavor development, and fillet shelf life [12–15]. This, in turn, impacts the nutritional value and overall product quality.

Lipid oxidation significantly impacts the quality and shelf life of fish fillets, especially those rich in omega-3 polyunsaturated fatty acids, which are highly susceptible to oxidative degradation. Lipid oxidation alongside muscle cellularity—defined as the size and number of muscle fibers (myofibers) within a myotome—play a pivotal role in determining the oxidative stability of fish fillets. Larger myofibers, for instance, have a greater capacity to retain water, directly impacting product drip loss and the extent of oxidative changes post-harvest [15]. Unlike mammals, fish muscle lacks complex connective tissue and relies on segmented blocks (myotomes) for structure. This structural simplicity increases susceptibility to postmortem stiffening (rigor mortis), drip loss, and oxidation, ultimately compromising product texture and oxidative stability [16,17]. The interplay between lipid and protein oxidation further accelerates quality degradation, as reactive lipid oxidation products, such as aldehydes, can react with proteins, amplifying oxidative damage in muscle tissue [18], while the interplay between oxidation processes and other key parameters, such as proximate composition, muscle growth, and muscle cellularity, further highlights the complexity of fish flesh quality. Growth rate is inherently linked to lipid content and fatty acid composition, as faster growth often correlates with higher intramuscular fat deposition, which may increase susceptibility to oxidation if not managed appropriately [16]. Similarly, proximate composition—including protein, fat, moisture, and ash content—not only provides insights into the nutritional value of the fish but also influences the oxidative stability of the fillets [13,19]. Higher fat content,

particularly polyunsaturated fatty acids (PUFAs), enhances the nutritional value of the fish but also increases the risk of lipid oxidation, necessitating strategies to improve oxidative stability [1,12] and lipid quality of fish fillets [1,19–21]. Lipid oxidation of muscle cells represents a key challenge in the preservation of biological membranes, where it can propagate oxidative reactions and compromise structural integrity. These alterations weaken lipid–lipid and lipid–protein interactions, compromising membrane integrity of muscle cells. In the absence of antioxidants, lipid oxidation can accelerate, contributing to post-harvest quality losses in fish fillets. Intrinsic antioxidants present in fish fillets, collectively measured as total antioxidant capacity (TOAC), play a critical role in delaying lipid oxidation. TOAC encompasses both enzymatic antioxidants, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), and non-enzymatic antioxidants, including vitamins C and E. These antioxidants act synergistically to neutralize reactive oxygen species (ROS) and interrupt the chain reactions that propagate oxidative damage. Nevertheless, the protective effects of TOAC diminish over time, antioxidant molecules are gradually depleted, while the activity of several enzymes, including antioxidant enzymes, declines during prolonged storage [22–24].

Although rapid growth is often considered a desirable trait in aquaculture, the nutritional quality of the resulting fillets, particularly regarding lipid composition and omega-3 fatty acid content, remains underexplored. In some aquaculture fish species, growth and lipid quality are associated; it can be hypothesized that growth rate significantly influences lipid quality, omega-3 fatty acid composition, and oxidative stability in meagre fillets, with potential implications for their shelf life during cold storage. For example, it is reasonable to hypothesize that differences in growth rate may result in differences in lipid content and muscle cellularity with consequences for lipid oxidation during cold storage [11,16]. Therefore, differences in growth rate may not only affect lipid content and muscle structure but also increase the risk of lipid oxidation, making the role of intrinsic antioxidants critical, as they can be expected to at least partially mitigate lipid oxidation initially.

The aim of this study was to investigate the potential relationship between growth rate and muscle composition in farmed meagre, focusing on fatty acid content, intrinsic antioxidant capacity, oxidative stability, as well as other important fish quality parameters during cold storage in relation to prevention of oxidative damage, particularly within the context of lipid quality preservation, ensuring both stability and enhanced shelf life in aquaculture products.

2. Materials and Methods

2.1. Fish Samples and Somatometric Traits

Fish were obtained in June 2022 from a commercial fish farm in northwestern Greece, originating from the same batch and grown under identical conditions in floating cages. All fish in this study were 12 months of age, ensuring uniformity in terms of age. They were fed a commercial diet (48% protein, 16% fat, 3% fiber, 8.3% ash). Despite weekly monitoring and adjusted feeding rates, high variability in body weight after 12 months highlighted the need for refined growth management. To address this, size grading was implemented, categorizing fish into “small” and “large”, and fish were subsequently placed in different floating cages. For the needs of our research, subsamples of small and large fish were further categorized as slow growing (SG) and fast growing (FG); fish weighing 450–650 g were classified as SG, while 1000–1200 g were defined as the FG category. This approach ensured the “small” group included fish in the lower half of the size distribution, and the “large” group captured those above the median.

Samples from two cages per growth group were selected, and random subsamples ($n = 20$) were analyzed for body length, weight, testis, viscera, and liver weight, providing a comparative assessment of slow-growing (SG) and fast-growing (FG) fish. A visual comparison of the slow-growing (SG) and fast-growing (FG) fish can be seen in Figure 1.



Figure 1. Representative samples of slow-growing (SG) and fast-growing (FG) meagre used in the present study.

The sampling was conducted during the summer, a period that typically coincides with the peak of gonadal development in older fish, when reproductive organs are more pronounced and easier to differentiate between males and females. In fact, sexual maturation in *Argyrosomus regius* generally occurs at weights well above 1 kg between the second and third year of life, with studies indicating that 50% of males reach maturity at approximately 2.7 years of age [25].

However, due to the young age of the specimens in this study (1+ year age group), the gonads were barely visible. Macroscopic examination classified them as stage I of gonadal development; this stage is characterized by thread-shaped testes that range in color from white to rose or brown, appearing either translucent or opaque. These testes have a small but discernible width and thickness and are far smaller than sexually mature fish [23,24]. To avoid any sex-related growth differences [25–27], only male fish were used; all specimens were screened for the presence of male gonads, as identifying female gonads at this stage proved challenging. At this stage (immature), the lobes of the ovary are thin and dark red, while the gonadal lobes of the testes are thin with a creamy white appearance and relatively easy to identify by macroscopic examination.

Physiological and quality indices determined in SG and FG samples included gonadosomatic index ($GSI = \text{gonad weight (g)} \times \text{body weight}^{-1} (\text{g}) \times 100$); hepatosomatic index ($HSI = \text{liver weight (g)} \times \text{body weight}^{-1} (\text{g}) \times 100$); viscerosomatic index ($VSI = \text{visceral weight (g)} \times \text{body weight}^{-1} (\text{g}) \times 100$); and perivisceral fat (PVF) = perivisceral fat weight (g) $\times$ body weight^{−1} (g) $\times$ 100.

Samples of white epaxial muscle tissue were collected; the left side of the fillets was used for histological analysis, and the right side was used for proximate composition, fatty acid profile (FA), total antioxidant capacity (TAOC), oxidative stability (lipid oxidation), and muscle cellularity analysis.

2.2. Proximate Composition and Fatty Acid Content

For proximate composition analysis, the moisture, protein, lipid, and ash contents of deep white muscle tissue obtained from the upper epaxial anatomical region of the skeletal musculature were determined at the first day of sampling according to AOAC (2005) [28] using samples of 3 pools of 5 fish per group. Similarly, the analysis of FAs was performed once in 3 pools of 5 fish per growing group. Lipids were extracted using a 2:1 chloroform and ethanol solvent ratio and converted to their corresponding fatty acid methyl esters (FAMES) directly from freeze-dried muscle tissue under acidic conditions. FAs were methyl-esterified using a 12% boron trifluoride in methanol solution (BF₃-MeOH). The resulting methyl esters were extracted with hexane. The methyl ester analysis was performed by using a Chromatec-Crystal Model 9000 (Yoshkar-Ola, Russia) chromatograph equipped with a flame ionization detector and an Agilent HP-88 (88-cyanopropyl)-aryl-polysiloxane) column

(100 m × 0.25 mm i.d., 0.2 µm film). A sample of 1 µL was injected with a 1:20 split ratio at 250 °C. Oven temperature was programmed to increase from 120 °C to 180 °C in 10 min (6 °C/min), then kept at 180 °C for 10 min, increased to 220 °C in 5 min (8 °C/min), kept at 220 °C for another 5 min, increased to 240 °C in 5 min (4 °C/min), and finally kept at 240 °C for 4 min. The detector was set at 280 °C. Helium was used as the carrier gas at a constant flow of 2 mL/min. The identification and quantification of the fatty acid methyl esters were performed based on the Supelco 37 fatty acids methyl ester standard purchased from Sigma Aldrich (CRM47885). Methyl ester of behenic acid was added as the internal standard.

2.3. Total Antioxidant Capacity, SOD Activity, and Lipid Oxidation

The process was conducted repeatedly over a 10-day period using cold-stored (2 °C) fish fillets. At each time point, muscle tissue samples were collected and analyzed to track changes in total antioxidant capacity (TAOC), superoxide dismutase (SOD) activity, and lipid oxidation. All assays were performed in duplicate at 20 °C.

Total antioxidant capacity (TAOC) was determined by preparing muscle tissue samples according to the manufacturer's instructions for the Antioxidant Assay Kit (CS0790, Sigma-Aldrich, Germany), as modified by Fernando et al. [29] for fish tissues. Muscle tissue samples from three pools of three fish per group were diluted (1:10 *v/v*) in Tris-HCl buffer, homogenized, and centrifuged at 12,000 × *g* for 5 min. The resulting supernatant was collected and stored at −80 °C until analysis. The TAOC was measured spectrophotometrically using a Beckman DU-50 series spectrophotometer at a wavelength of 405 nm. The assay is based on the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) by a ferryl myoglobin radical, where the degree of oxidation is inversely proportional to the antioxidant activity. Trolox was used as a standard, and TAOC values were expressed as mM Trolox/mg.

For the SOD assays, white muscle tissue samples were excised from three pools of five fish each and immediately placed on ice. The tissue from each pool was minced and homogenized separately in ice-cold 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA. Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed as described by Antonopoulou et al. [30]. The assay was performed at 20 °C in a medium containing 800 µL of 50 mM phosphate buffer (pH 7.4), 55 µL of a 100/50 mM EDTA/Mn solution, 40 µL of 7.5 mM NADH solution, and 100 µL of 10 mM β-mercaptoethanol solution. The decrease in NADH absorbance at 340 nm was monitored, and the extinction coefficient for NADH ($\epsilon = 6.22 \text{ mM}^{-1}$) was used to calculate the rate of NADH oxidation. One unit of SOD activity was defined as the amount of tissue extract required to reduce the NADH oxidation rate of the control by 50%, and the activity was expressed as units per milligram of protein.

Lipid oxidation was measured using a previously described protocol [15] based on malondialdehyde (MDA) and determined spectrophotometrically using the thiobarbituric acid (TBA) method ($n = 3$ pools of 5 fish each per group). The concentration of TBA reactive substances (TBARS) was expressed as mg MDA Kg^{−1} of tissue wet weight.

2.4. Muscle Cellularity

In order to evaluate muscle cellularity, samples from the white epaxial muscle of the post-anal caudal area were collected (5 fish per group) and processed for routine histological processing. The samples were fixed in 10% phosphate-buffered formaldehyde saline, subsequently dehydrated in a graded series of ethanol solutions, and embedded in paraffin. Transverse sections were obtained (6 µm thick) and stained with H&E stains. The cross-sectional area (CSA) of at least 150 individual myofibers from each fish was traced using a computerized image analysis system (Seescan plc, Cambridge, UK). The density of myofibers in each sample of a cross-sectional area was estimated by using the ratio of the cross-sectional area divided by the mean myofiber size [31].

2.5. Chemicals

Histological consumables (4% formaldehyde, histology grade paraffin wax, and xylene) were purchased from Biognost Ltd., Zagreb, Croatia. All other chemicals and reagents used were ACS grade and were purchased from Sigma-Aldrich: boric acid; boron trifluoride-methanol solution; copper (II) sulfate pentahydrate; hydrochloric acid; methanol; n-hexane; phosphotungstic acid; potassium hydroxide; potassium sulfate; sodium chloride; sodium hydroxide; sulfuric acid; TBA; and trichloroacetic acid.

2.6. Statistical Analysis

For the statistical analysis, the data were first tested for normality using the Shapiro–Wilk test. The mean values of the variables between groups were compared using a *t*-test (for body traits and muscle quality indices) or one-way ANOVA (for TOAC, SOD, MDA, and muscle size classes). When ANOVA revealed significant differences, post hoc comparisons were performed using Tukey’s honest significant difference (HSD) test to identify specific group differences. All statistical analyses were performed using IBM SPSS Statistics (v. 22). The significance level was set at $\alpha = 0.05$.

3. Results and Discussion

3.1. Body Traits and Proximate Composition of Muscle Tissue

The analysis of body traits in slow-growing (SG) and fast-growing (FG) meagre revealed significant differences (Table 1). While both groups had similar viscerosomatic (VSI) and hepatosomatic (HSI) values, the FG fish displayed a significantly higher body weight (BW) and gonadosomatic (GSI) index value but lower perivisceral fat (PVF) ($p < 0.05$). Filleting yield (FY) was higher ($p < 0.05$) in FG fish (Table 1).

Table 1. Mean ($\pm$ sd) values of body traits and muscle tissue proximate composition of slow-growing (SG) and fast-growing (FG) (12 months old) meagre.

Body Traits and Muscle Tissue Proximate Composition	SG	FG	<i>p</i> -Value
Body weight (BW, g)	459.09 (21.01)	1148.8 (215.32)	*
Gonadosomatic (GSI, %)	0.03 (0.04)	0.12 (0.05)	*
Hepatosomatic (HSI, %)	1.2 (0.24)	1.22 (0.25)	Nsd
Viscerosomatic (VSI, %)	4.69 (2.01)	4.78 (0.57)	Nsd
Perivisceral fat (PVF, %)	2.08 (0.25)	0.54 (0.33)	*
Filleting yield (FY, %)	31.53 (3.16)	33.46(3.53)	*
Moisture (%)	78.29 (0.78)	80.40(1.40)	*
Proteins (%)	19.0 (0.44)	18.92(0.61)	Nsd
Lipids (%)	0.78 (0.05)	0.27(0.04)	*
Ash (%)	1.10 (0.02)	1.02(0.04)	Nsd

An asterisk * indicates significant differences $p < 0.05$; Nsd, non-significant difference between FG and SG fish.

The lipid and moisture contents differed significantly between groups. FG fish had significantly higher moisture (80.40 vs. 78.29%, Table 1) and significantly lower lipid contents than SG (0.27 vs. 0.78%, $p < 0.05$, Table 1). Protein and ash contents were similar ($p > 0.05$) between groups, averaging around 19% and 1% (Table 1).

Analysis of body traits and proximate composition indicated that slow-growing and fast-growing meagre exhibited distinct growth and metabolic characteristics. FG fish showed lower fat deposition and higher reproductive investment ($p < 0.05$), as indicated by the GSI%. These differences could have implications on flesh quality. FG fish, for example, exhibited less perivisceral and muscle fat ($p < 0.05$) compared to SG fish. These factors may affect the product sensory attributes and consumer appeal. The present values of moisture and fillet yield are similar to those of Grigorakis et al. [30], who reported a filleting yield (38.03%) and

moisture (80.40%) for reared meagre. With respect to fat content, the results of the present study are lower than those of Grigorakis et al. [30] (1.06%) and Giogios et al. [1] (1.09% and 0.73% for small and large reared meagre, respectively). Differences in fat content between the two studies may be attributed to the fact that, in the present study, fat determination was carried out on deep white muscle, which contains substantially less fat compared to red muscle tissue. Dorsal muscle samples typically contain a mixture of predominantly white muscle fibers but also some red muscle fibers, which are known to have higher fat content [31]. This mixture could introduce variability, making it more challenging to obtain consistent results. Focusing on deep white muscle ensures that the fat content analysis is more reliable, as this tissue is the most abundant and uniform in terms of composition.

In a similar study on the flesh quality of farmed meagre, Saavedra et al. [32] reported pronounced differences in fat content between farmed meagre of different weights. In smaller fish (800 g), the fat content was 1.3%, roughly half of that for 1500 g (2.5%) and 2500 g (2.9%) fish. In contrast, with regard to physiological indices, Martelli et al. [33] reported similar gonadosomatic (0.15), hepatosomatic (1.20), and viscerosomatic (4.78) index values for large meagre harvested in May but substantially higher fillet yields (50.64%) compared to those of the present study.

3.2. Fatty Acid (FA) Analysis

The analysis of FAs revealed significant differences between meagre growing groups. FG meagre had significantly higher contents in palmitic ($p = 0.013$), stearic ($p = 0.016$), palmitoleic ($p = 0.000$), dihomog- γ -linolenic ($p = 0.002$), and eicosatrienoic acids ($p = 0.014$) (Table 2). However, SG meagre had a similar content in eicosapentaenoic (EPA, $p > 0.05$) but a significantly higher content in docosahexaenoic acid (DHA) compared to FG meagre ($p = 0.033$). The most abundant fatty acids in both FG and SG were oleic, palmitic, docosahexaenoic, linoleic, and nervonic acids. A similar trend regarding the abundance of fatty acids was also recorded by Grigorakis et al. [30], Giogios et al. [1], and Saavedra et al. [32].

Table 2. Mean ($\pm$ sd) fatty acid concentration (%) in muscle tissue of slow-growing (SG) and fast-growing (FG) (12 months old) meagre.

Fatty Acid	Chemical Type	SG	FG	<i>p</i> -Value
Myristic acid	C14:0	2.91 (0.75)	3.63 (1.03)	Nsd
Palmitic acid	C16:0	12.1 (1.54)	16.54 (0.92)	*
Stearic acid	C18:0	4.19 (1.26)	7.62 (0.79)	*
Palmitoleic acid	C16:1 (ω -7)	0.21 (0.19)	3.89 (0.20)	***
cis-11-octadecenoic acid	C18:1 (ω -7)	0.28 (0.25)	0.63 (0.55)	Nsd
Oleic acid	C18:1 (ω -9)	19.57 (1.48)	18.88 (2.54)	Nsd
Linoleic acid (LA)	C18:2 (ω -6)	9.83 (1.18)	8.27 (2.10)	Nsd
Linolenic acid (LNA)	C18:3 (ω -3)	0.38 (0.11)	0.97 (0.88)	Nsd
Eicosadienoic acid	C20:2 (ω -6)	0.25 (0.21)	0.82 (0.71)	Nsd
Dihomo- γ -linolenic acid (DGLH)	C20:3 (ω -6)	0.19 (0.18)	2.24 (0.46)	**
Eicosatrienoic acid	C20:3 (ω -3)	2.66 (0.52)	4.37 (0.48)	*
Arachidonic acid	C20:4 (ω -6)	3.43 (0.88)	3.71 (1.77)	Nsd
Eicosapentaenoic acid (EPA)	C20:5 (ω -3)	7.75 (8.20)	3.69 (0.45)	Nsd
Docosahexaenoic acid (DHA)	C22:6 (ω -3)	13.90 (3.31)	7.47 (1.11)	*
Nervonic acid	C24:1 (ω -9)	7.81 (0.79)	7.25 (1.24)	Nsd

An asterisk * indicates significant differences between groups: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Nsd, non-significant differences.

FG meagre had significantly lower contents in omega-3 (ω -3) FAs (15.52 vs. 24.30, $p = 0.001$, Table 3), PUFA (31.35 vs. 38.38, $p = 0.016$), and ω -3/ ω -6 ratio value (1.07 vs. 1.78, $p = 0.021$). However, FG meagre content in SFA was significantly higher compared to SG meagre (27.79 vs. 19.21, $p = 0.006$). MUFA and ω -6 fatty acid contents were similar in

both FG and SG groups. In a study on the essential fatty acid nutritional needs of juvenile meagre, Phalzgraff et al. [34] reported ω -3 (17.09%), ω -6 (13.25%), ω -3/ ω -6 (1.29; Σ MUFA (43.55%), and Σ PUFA (33.73%) values in meagre flesh, which are in general agreement with those of the present study for SG and FG meagre with the exception of Σ MUFA content, which was considerably higher. Likewise, Saavedra et al. [32] reported ω -3 (15.6–19.5%), ω -6 (15.4–18.0%), ω -3/ ω -6 (0.9–1.1), Σ MUFA (28.2–35.0%), and Σ PUFA (33.5–38.0%) for farmed meagre of different weights.

Table 3. Mean ($\pm$ sd) concentration (%) of lipid quality indices in white muscle of slow-growing (SG) and fast-growing (FG) (12 months old) meagre.

Lipid Quality Indices	SG	FG	<i>p</i> -Value
ω -3	24.30 (1.45)	15.52 (1.26)	**
ω -6	13.70 (1.39)	15.04 (3.00)	nsd
ω -3/ ω -6	1.78 (0.14)	1.07 (0.30)	*
SFA	19.21 (2.66)	27.79 (0.95)	**
Σ MUFA	27.88 (1.72)	29.86 (1.37)	nsd
Σ PUFA	38.38 (31.53)	31.53 (1.22)	*

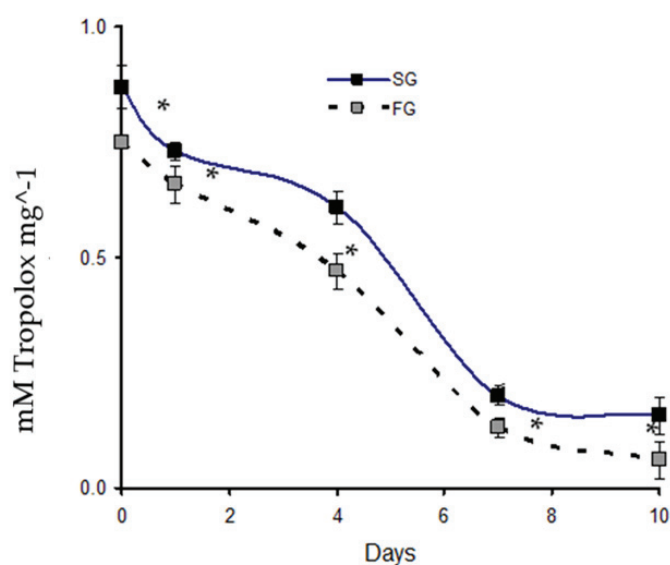
SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids. Asterisk indicates significant differences: * $p < 0.05$, ** $p < 0.01$. nsd: non-significant differences.

Although fast growth and high filleting yield are primary goals in aquaculture, the present study revealed intriguing findings regarding the lipid content of slow-growing (SG) meagre. Despite the justified emphasis on rapid growth in aquaculture, the fatty acid analysis demonstrated that SG meagre exhibited a higher lipid content, which could be considered a desirable trait in terms of consumers [14]. Interestingly, the fatty acid profile of the SG fish revealed a higher content of docosahexaenoic acid (DHA), a key omega-3 polyunsaturated fatty acid known for its significant health benefits. DHA plays a vital role in supporting cardiovascular health, brain function, and development, making it a valuable nutrient for human consumption [17,19,35]. Moreover, the ratio of omega-3 (n -3) to omega-6 (n -6) fatty acids was significantly higher in SG meagre, reflecting the potential benefits of consuming this fish species. A higher n -3/ n -6 ratio is often considered advantageous in terms of reducing inflammation and promoting overall health [17,19,36], further underscoring the value of SG meagre as a healthier option for human diets.

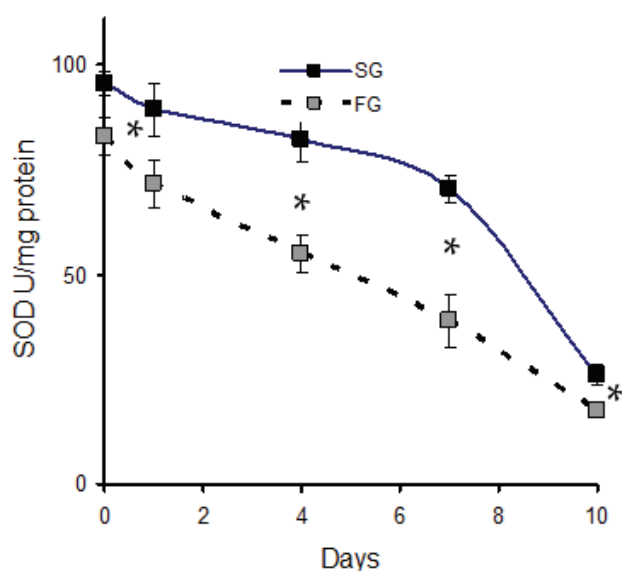
The results of the present work suggest that better growth does not always coincide with better lipid quality, challenging the common notion that faster-growing fish are always nutritionally superior. Fish metabolism and growth dynamics are crucial in shaping the fatty acid composition, as they directly influence how lipids are synthesized, stored, and utilized within the fish body [37–39]. Metabolic processes regulate the conversion of dietary fats into specific fatty acids, affecting the balance of ω -3 and ω -6 fatty acids, which in turn impacts the nutritional quality of the fish [40,41]. The observed fatty acid profile of both fast-growing (FG) and SG meagre indicates that SG meagre may offer enhanced nutritional value, particularly for human consumption. The higher ω -3 content along with the increased ω -3/ ω -6 ratio align with dietary recommendations aimed at promoting cardiovascular health and overall well-being. Additionally, the significant increase in polyunsaturated fatty acids in SG meagre strengthens the case for its potential as a healthier alternative. Fish growth dynamics should be carefully managed in aquaculture, not only to optimize production efficiency but also to enhance the nutritional quality of the fish, particularly in terms of essential fatty acids. By understanding the interplay between growth rate, lipid accumulation, and fatty acid composition, aquaculture producers can make more informed decisions that cater to both market demands and the health needs of consumers.

3.3. Total Antioxidant Capacity (TOAC), SOD Activity, and Lipid Oxidation

TAOC reflects the cumulative effect of all antioxidants, including enzymes like SOD and GPx, as well as non-enzymatic antioxidants, such as vitamins C and E. Figure 2a–c illustrate the postmortem changes in TOAC (Figure 2a), SOD activity (Figure 2b), and lipid oxidation (Figure 2c) of SG and FG meagre fillets. There was a significant effect of growth rate on TOAC, ANOVA, $F(1,4) = 27.51$, $p = 0.006$, $n^2 = 0.87$. Post hoc comparisons using the Bonferroni correction revealed that the SG group exhibited significantly higher TOAC than the FG group across all sampling days ($p < 0.05$). Similarly, SOD activity was consistently higher in SG fillets compared to FG fillets across all sampling days ($p < 0.05$). Both TOAC and SOD levels decreased significantly during cold storage, with reductions of approximately 80% observed up to day 10 of storage.



(a)



(b)

Figure 2. Cont.

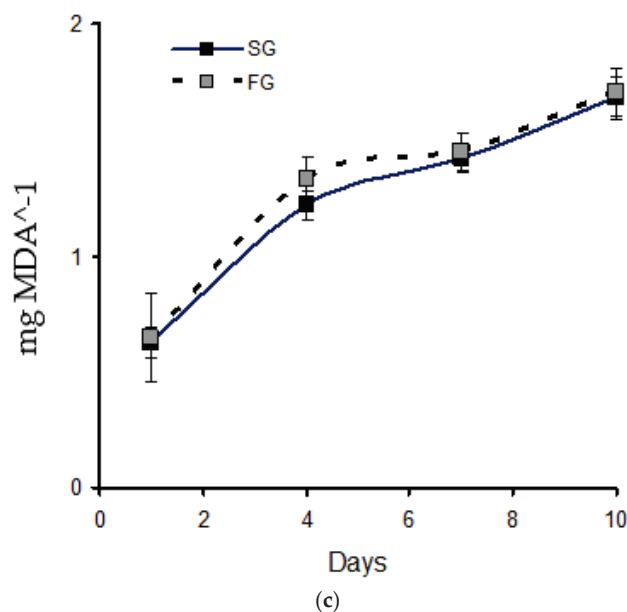


Figure 2. (a) TAOC (mM Trolox mg^{-1} of muscle tissue) of meagre fillets during storage at 2 °C from slow-growing (SG, grey squares, dotted line) and fast-growing (FG, black squares, solid line) meagre. Significant differences were observed between FG and SG groups on all sampling days between day 0 and day 10 ($p < 0.05$), Significant differences between FG and SG groups on specific sampling days between day 0 and day 10, as determined by Tukey's HSD test, are marked with an asterisk (*). (b) SOD activity (units/mg protein) of meagre fillets during storage at 2 °C from slow-growing (SG, grey squares, dotted line) and fast-growing (FG, black squares, solid line) meagre. Significant differences were observed between FG and SG groups on all sampling days between day 0 and day 10 ($p < 0.05$), as determined by Tukey's HSD test. (c) Mean ($\pm$ SD) lipid oxidation values (mg MDA kg^{-1}) of meagre fillets during storage at 2 °C from slow-growing (SG, dotted line) and fast-growing (FG, solid line) meagre. Non-significant differences were found between FG and SG groups for any sampling day ($p > 0.05$).

In contrast to the decline in TOAC and SOD activity, malondialdehyde (MDA) levels increased in fillets from both SG and FG groups during storage at 2 °C, indicating an increase in lipid oxidation. However, unlike TOAC and SOD, which differed significantly between the two groups, no significant differences in lipid oxidation levels were observed between SG and FG fillets on any sampling day ($p > 0.05$).

The reduction in total antioxidant capacity (TOAC) observed in postmortem SG and FG meagre fish fillets during cold storage is likely due to the combined effects of antioxidant consumption in neutralizing lipid oxidation products and oxidative damage to antioxidant molecules [20,21].

MDA levels of both fillet groups remained below the threshold value of 2.0 mg MDA.kg^{-1} during the ten-day storage period, indicating minimal lipid oxidation and satisfactory freshness [42,43]. There was a gradual increase, reaching MDA values above 1.5 mg MDA kg^{-1} by day 10 (Figure 2c) yet below the limit of 2.0 mg MDA kg^{-1} , signaling the onset of rancidity. This finding contrasts with the initial expectation that the higher lipid content in slow-growing (SG) fish, particularly the elevated levels of polyunsaturated fatty acids (PUFAs)—which are more susceptible to oxidation than saturated fatty acids (SFAs)—would lead to more rapid lipid oxidation. However, no significant differences in lipid oxidation were observed between SG and fast-growing (FG) fillets, which suggests that factors beyond lipid content and PUFA composition, such as intrinsic antioxidant levels, may have played a key role in mitigating oxidation. The MDA values observed in this study are comparable to those reported by Matulik et al. [13], who found oxidized tissue MDA levels in meagre ranging between 0.01 and 0.04 $\mu\text{mol g}^{-1}$ (or 0.72 to 2.88 mg MDA.kg^{-1}).

The differences in TAOC between SG and FG fish may be attributed to differences in their metabolism. Fast-growing fish generally exhibit higher metabolic rates to sustain rapid growth, which can increase the production of reactive oxygen species (ROS) and elevate oxidative stress [28,44–47]. Although FG fish may upregulate antioxidant production to counteract this stress, these antioxidants are likely more rapidly depleted in the process of neutralizing ROS [48–50]. This could at least partially explain the lower TAOC exhibited by the FG compared to SG fish, whose lower metabolic rates are expected to produce fewer ROS, probably allowing them to retain more antioxidants [49,50]. Furthermore, growth-related genes, which influence antioxidant enzyme activity, may be differently expressed in SG and FG fish, leading to varied antioxidant responses [49,51].

Meagre is known to exhibit a strong antioxidant capacity under a range of environmental and dietary regimes, particularly through the expression of key antioxidant enzymes, such as catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx) [52]. Furthermore, elevated TAOC levels are also beneficial postmortem, as antioxidant activity can slow down protein and lipid oxidation [53,54], particularly in PUFAs, which are highly susceptible to oxidative degradation.

Antioxidants can delay lipid oxidation, reducing rancidity and extending shelf life during storage. Although high TAOC and SOD values indicate a strong ability to neutralize ROS and delay lipid oxidation, their activity and, consequently, their protective effect diminished over time [20,21,54].

3.4. White Muscle Fiber Cellularity

Muscle cellularity was evaluated by measuring myofiber cross-sectional area (MCSA), fiber density (MFD), and size distribution of myofibers on histological sections of muscle tissue. MCSA, MFD, and size distribution values of myofibers were similar between the SG and FG groups (t -test ($p > 0.05$), Table 4, Figures 3 and 4).

Table 4. Mean ($\pm$ sd) values of myofiber cross-sectional area (MCSA) and density (MFD) in meagre white muscle.

Myofiber Characteristics	SG	FG	<i>p</i> -Value
MCSA (μm^2)	1516.42 ($\pm$ 63.7) [0.0015 mm ²]	1667.44 ($\pm$ 448.9) [0.0017 mm ²] [0.0017 mm ²]	nsd
MFD (number/mm ²)	510.48($\pm$ 131.8)	449.68 ($\pm$ 106.8)	nsd

Numbers in brackets indicate the MCSA expressed in mm². nsd: non-significant differences.

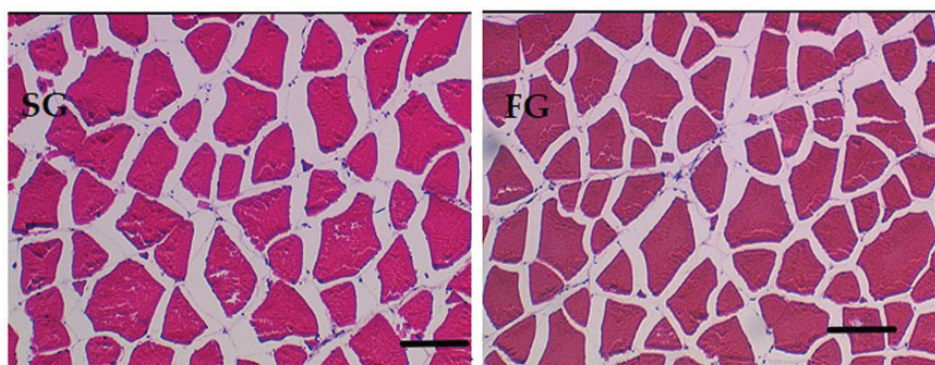


Figure 3. Hematoxylin and eosin (H&E) stained transverse cross-sections of white epaxial muscle tissue of slow-growing (SG) and fast-growing (FG) reared meagre displaying the arrangement and size mosaic of muscle fibers. Bar scale: 100 μm .

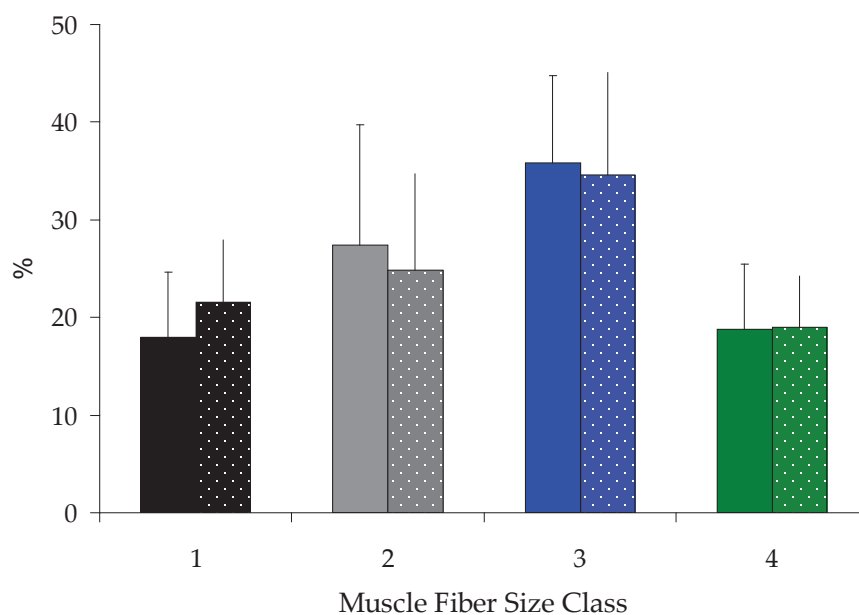


Figure 4. Frequency distribution (mean $\pm$ sd) of white muscle fibers cross-sectional area in the SG (solid bars) and FG (dotted bars) groups. Size classes: 1 (20–350 μm^2); 2 (350–650 μm^2); 3 (650–950 μm^2); and 4 (>950 μm^2). No significant differences were found between the two groups across any size class (ANOVA, $p > 0.05$).

Present average fiber areas and fiber densities are in agreement with those of Saavedra [32], who reported fiber areas ranging from 0.0014 to 0.0018 mm^2 for meagre with a range in body weight from 800 to 2500 g. Respective average fiber densities ranged from 431 to 297 fibers mm^2 ^{−1}.

Muscle fiber characteristics may influence lipid oxidation by affecting the surface area exposed to moisture loss and oxygen within the muscle tissue, which, in turn, may influence texture and lipid oxidation during storage. Larger muscle cells (hypertrophy) can retain more moisture, resulting in less drip loss during processing and storage, which affects product textural properties [54,55]. As both groups exhibited similar muscle fiber size and density, other parameters known to affect lipid oxidation, such as differences in the fatty acid profile and intrinsic antioxidant capacity (TOAC) in SG meagre, may have contributed to the observed differences in lipid oxidation between the two groups.

Our results on muscle fiber size and density suggest that both hyperplasia and hypertrophy contributed to the observed growth differences between SG and FG meagre. This indicates that, despite differences in growth rates, SG and FG fillets shared similar muscle fiber characteristics, which may have contributed to the comparable rates of lipid oxidation despite their differing fatty acid content. Previous studies have shown that, during cold storage, muscle structure changes slowly, as observed in sea bream [15], sea bass [56], and Atlantic Herring [57].

The parameters investigated in this study—muscle cellularity, lipid profile, and oxidative stability—are crucial indices of fillet quality and are related to texture, nutritional value, freshness, and shelf life. Fillet lipid content and fatty acid profiles have been frequently reported to vary with season, size, feeding regime, and environmental conditions, all of which can, in turn, affect key parameters of fillet quality [19,20].

Our findings suggest that growth dynamics, particularly rapid growth in FG fish, may compromise the quantity and quality of fatty acids—especially EPA (eicosapentaenoic acid), DHA (docosahexaenoic acid), and other polyunsaturated fatty acids (PUFAs)—making these fillets more vulnerable to oxidative degradation during cold storage. Despite the higher lipid content in SG fish, no significant differences in MDA levels were observed

between SG and FG fillets during cold storage. This may be explained by the qualitative differences in lipid profiles between the two groups combined with the antioxidant defenses shown by the higher total antioxidant capacity (TOAC) and superoxide dismutase (SOD) activity in SG fillets. The elevated TOAC in SG fillets likely played a key role in mitigating lipid oxidation, effectively counteracting the susceptibility of their PUFAs to oxidative degradation. Although TOAC and SOD levels declined over time in both groups, their higher initial levels in SG fillets may have provided greater oxidative stability, resulting in similar lipid oxidation rates between SG and FG fillets. In this context, the antioxidant capacity of fish fillets becomes a critical factor in preserving lipid stability. While SG fillets exhibited higher TOAC and SOD levels, these levels declined gradually over time in both groups, reflecting the depletion of antioxidant reserves in neutralizing reactive oxygen species. This decline was associated with reduced oxidative stability, as evidenced by increasing MDA levels during cold storage in both groups.

4. Conclusions

This study highlights the critical role of intrinsic antioxidants in preserving lipid quality and mitigating lipid oxidation in meagre (*Argyrosomus regius*) fillets during cold storage. The findings suggest that, despite the higher lipid content and polyunsaturated fatty acids (PUFAs) in slow-growing (SG) fish, no significant differences in lipid oxidation were observed between SG and fast-growing (FG) fish. This contrasts with initial expectations that the higher lipid content, particularly the elevated PUFAs in SG fish, would lead to more rapid lipid oxidation. Instead, the elevated total antioxidant capacity (TOAC) and superoxide dismutase (SOD) activity in SG fish likely played a key role in mitigating oxidative damage, preserving lipid stability during storage.

While no differences were found in muscle cellularity between the two groups, it is important to acknowledge the potential influence of sex-related variations on lipid metabolism, muscle structure, and post-harvest quality. Since only male fish were used in this study, future research that includes both males and females may provide further insights into the role of hormonal and metabolic differences in shaping flesh quality and antioxidant responses.

The use of 1-year-old meagre in this study is justified as fish of this age typically reach marketable sizes of 500–600 g, making them suitable for commercialization. This aligns with industry practices of size grading and allows for optimal resource utilization and revenue generation. However, further studies are needed to examine how growth dynamics, including size and age, affect not only lipid oxidation and antioxidant defense but also the broader economic and nutritional viability of meagre aquaculture.

In conclusion, this study underscores the importance of managing growth dynamics and antioxidant capacity in aquaculture to improve both the shelf life and nutritional quality of meagre fillets, especially in terms of ω -3 fatty acids. Selective breeding or dietary interventions aimed at enhancing antioxidant levels and optimizing lipid composition could contribute to improving the overall quality and sustainability of meagre farming.

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Data Availability Statement: Dataset available on request from the authors.

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