

Research Article

Growth Performance, Immune Modulation, and Gene Expression Enhancement in *Labeo rohita* Through Integrated Probiotic *Bacillus licheniformis* and Phytobiotic *Cinnamomum verum* Strategies

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This study examines the dietary application of probiotic *Bacillus licheniformis* and phytobiotic *Cinnamomum verum* concerning growth, immunity, gene expression, and disease resistance in *Labeo rohita* fish. A controlled experiment was conducted over 12 weeks involving 800 fingerlings, which were divided into seven groups: one control group and three groups each receiving varying doses of *B. licheniformis* (D1 = 10^5 , D2 = 10^7 , and D3 = 10^9 CFU/g) and *C. verum* (D4 = 25, D5 = 50, and D6 = 75 mg/kg) individually, along with a group with no additions (control). Growth was measured in terms of weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER) alongside hematology and immunology. To evaluate resistance of the test organisms, pathogenic challenge with *Aeromonas hydrophila* was performed. The findings indicated that fish in the D3 group achieved the highest average final weight of 125.40 ± 0.1 g, a weight increment of 86.40 ± 0.53 g, and the most favorable FCR of 0.81 ± 0.01 . The D6 group followed, in contrast to the control group, which had a body weight of 95.20 ± 0.2 g, a WG of 56.73 ± 0.05 g, and a FCR of 1.03 ± 0.06 . The survival rates following the challenge were analyzed, revealing that group D3 exhibited the highest effectiveness ($p < 0.001$). The evaluation of immunological parameters in D3 indicated increased levels of total protein, immunoglobulin M (IgM), complement component 3 (C3), and lysozyme activity relative to D1 and D2. Analysis of gene expression revealed that interleukin- 1β (IL- 1β) and tumor necrosis factor α (TNF- α) were upregulated in the liver and head kidney tissues of fish in the D3 and D6 groups. *Bacillus licheniformis*, when administered at 10^9 CFU/g, enhances growth, feed utilization efficiency, immunity, and disease resistance in *L. rohita*. *Cinnamomum verum* at a dosage of 75 mg/kg improved the immune response. This study supports the consumption of probiotics and phytobiotics as substitutes for antibiotics in aquaculture.

Keywords: *Bacillus licheniformis*; *Cinnamomum verum*; gene expression assays; growth performance; hematological parameters

1. Introduction

Aquaculture is currently one of the fastest-growing sectors in global food production, actively seeking sustainable methods to enhance fish health, growth, and disease resistance, particularly in freshwater species like *Labeo rohita*. Nonetheless, concerns regarding the potential issues associated with antibiotic overuse have diminished the value of this practice. Resistant pathogens are difficult to control and can spread

to other animals and humans [1], diminishing the effectiveness of antibiotics for treating common infections. Furthermore, antibiotics in fish can lead to consumer health risks and environmental pollution, disrupting ecosystems [2].

Alternative strategies, including probiotics and phytobiotics, demonstrate effectiveness in captive fish, leading to increased interest in these approaches. Interventions appear effective in stimulating fish growth, pathogen resistance, and enhanced immune responses, thereby, aiding sustainable

aquaculture practices [3]. Among the various approaches, *Bacillus licheniformis*, a probiotic and *Cinnamomum verum* (true cinnamon), a phytobiotic have been reported due to their multidimensional application in aquaculture.

Probiotics, especially those derived from *Bacillus*, have the ability to improve the health of fish's gut microbiota and help them absorb nutrients and boost the immune response [4]. In particular, *B. licheniformis* has been on the rise as a spore-forming bacterium that improves growth performance and disease resistance in fish by maintaining a stable gut microflora and optimizing gut health. In a number of reports, *B. licheniformis* improved specific growth rate (SGR) and feed conversion ratio (FCR) of *L. rohita* by modulation of gut enzymes and boosting of immune responses [5]. Most of these effects are attributed to the ability of the said bacterium to synthesize embargoes against pathogenic microorganisms such as the fish pathogen *Aeromonas hydrophila* due to their production of bacteriocins [6].

Further, in aquaculture, probiotics such as *B. licheniformis* increase the production of short-chain fatty acids (SCFAs), which have been reported to be positively associated with gut health and nutritional absorption efficiency in aquatic animals [7].

The other important dimension of the usefulness of *B. licheniformis* in aquaculture is its ability to modulate the immune response. The bacterium is known to be able to increase the levels of immune-genes such as interleukin-1 β (IL-1 β) and tumor necrosis factor α (TNF- α) that helps inducing the immune response to pathogens in fish [8]. In addition, the probiotics can also enhance the secretion of lysozyme or immunoglobulin M (IgM) production which is part of the fish's immune system and hence, pacifying the fish against bacterial infections [9].

Phytobiotics are substances obtained from plant sources that are being accepted more and more as substitutes for chemicals in aquaculture. One such phytobiotic, *C. verum* is also referred to as true cinnamon and is reputed for its antimicrobial, antioxidant, and anti-inflammatory effect. More recent findings have shown that inclusion of *C. verum* in fish's diets contributes to better growth and immune response by improving gut health and reducing oxidative stress [10]. Some formulations having bioactive components of *C. verum* such as eugenol and cinnamaldehyde were effective against *A. hydrophila* a pathogenic bacterium for fish [11].

Furthermore, it has been noted that *C. verum* acts as an antioxidant by decreasing reactive oxygen species (ROS) in fishes which helps improve their general well-being [12]. This is common in aquaculture, more so when the rearing densities are high and needs to be done effectively to enhance the health of the fish. *C. verum* has more than just antioxidant activity, its application is also beneficial within the fish system on critical organs including the liver and kidneys which are always attacked by daily oxidative stress in such populations of fishes [13].

Even within probiotics and phytobiotics, there are successful application of each; however, in the direction of aquaculture, a number of studies have been performed on the fish for the combined action of these two groups of biologics.

Studies showed that dietary inclusion of single probiotic, *B. licheniformis*, or phytobiotic, *C. verum*, in fish gave sub optimal results when both were included in feed. The probiotic promotes the integrity and activity of the gut microbiome and the phytobiotic grant extra antimicrobial and antioxidant effect, making the immune system of the fish more comprehensive and effective.

In a research work with *L. rohita*, a mixture of *B. licheniformis* and *C. verum* demonstrate beneficial effects upon certain growth parameters such as weight gain (WG), SGR, and FCR as compared to control groups [14]. Further, the fish were not only able to overcome the challenge but also exhibited higher disease resistance to *A. hydrophila* infection, thus, emphasizing the role of the combined supplementation on disease resistance. Other combinations of probiotics and prebiotic, such as the use of synbiotics have also been attempted with positive response such as enhancement of digestive enzymes activity, feeding efficiency, and immune function [5].

Besides the interesting factors discussed above, the use of *B. licheniformis* and *C. verum* in aquaculture is associated with modulation of expression of immune gene which is of major interest in this study. It has been reported that supplementation of probiotics can lead to upregulation of pro-inflammatory cytokines producing genes such as IL-1 β and TNF- α which are important in the general initiation of the immune response [8]. Such upregulation is key in enabling fish to defend themselves against attacking pathogens especially during the beginning stages of infection.

Typically, phytobiotics such as *C. verum* have also been highlighted to influence gene expression through inhibition of pro-inflammatory cytokines thus averting (cutting the risk of in red) chronic inflammation and reducing the risk of tissue injury [11].

The interplay of probiotics and phytobiotics on gene expression also helps in achieving a proper level of immune response to infections in fish, avoiding unproductive over-reactions of inflammatory responses.

Combining *B. licheniformis* and *C. verum* into aquaculture diets is likely to be effective for improving the health, growth performance, and disease resistance of fish reared in a sustainable environment. The industry will move away from antibiotic treatment, and the use of probiotics and phytobiotics in conjunction will help further improve the natural resilience of *L. rohita* fish.

The study aims to investigate the comparative effects of *Bacillus licheniformis* and *C. verum* in terms of cost-effectiveness and their efficacy in the context of immunomodulation, fish health, and resistance to *A. hydrophila* in *L. rohita*. Additionally, it seeks to determine the optimal dosage that yields beneficial effects on the fish. We recommend conducting more studies on the applications of these additives and other strains of probiotics in aquaculture to establish optimal ratios that maximize benefits and maintain the sustainability of aquaculture practices.

Utilizing probiotics along with phytobiotics in aquaculture systems has beneficial implications for both productivity and fish health with fewer adverse environmental impacts. As the population continues to grow, the demand for fish will

TABLE 1: Ingredient composition (mg/kg) of the experimental diets (on dry matter basis).

Diet ingredients (g)	Experimental diets						
	Control	D1	D2	D3	D4	D5	D6
Soybean meal (g/kg)	350	350	350	350	350	350	350
Fish meal (g/kg)	220	220	220	220	220	220	220
Corn gluten (g/kg)	120	120	120	120	120	120	120
Rice polish (g/kg)	120	120	120	120	120	120	120
Vitamin premix (g/kg)	20	20	20	20	20	20	20
Mineral mixture (g/kg)	30	30	30	30	30	30	30
Fish oil (ml/kg)	10	10	10	10	10	10	10
Wheat flour (g/kg)	130	130	130	130	130	130	130
<i>Bacillus licheniformis</i> (CFU/g)	0	10 ⁵	10 ⁷	10 ⁹	0	0	0
<i>Cinnamomum verum</i> (mg/kg)	0	0	0	0	25	50	75
Total	1000	1000	1000	1000	1000	1000	1000
Proximate composition (%)							
Crude protein	35.27	35.46	35.25	35.45	35.34	35.34	35.33
Ether extract	15.59	15.49	15.35	15.47	15.59	15.57	15.47
Crude fiber	15.32	15.41	15.23	15.40	15.32	15.32	15.30
Total ash	12.45	13.03	12.86	11.98	12.98	12.98	12.98
Dry matter	92.12	92.04	92.14	92.35	92.12	92.12	92.12
Gross energy	341.32	342.01	340.55	342.28	341.6	341.3	340.9

Note: Vitamin premix (mg/IU per kg of premix): Vitamin A, 219,990,000 IU; Vitamin D, 41,990,000 IU; Vitamin E, 8350 IU; Vitamin K, 9100 mg; Vitamin B1, 1280 mg; Vitamin B2, 8000 mg; Vitamin B6, 6100 mg; Vitamin B12, 30,900 µg; Vitamin C, 10,600 mg; calcium pantothenate, 30,700 mg; nicotinic acid, 100,100 mg; folic acid, 2125 mg. Mineral premix (mg/IU per kg of premix): Ca, 155 g; P, 135 g; Mg, 55 g; Na, 45 g; Fe, 1000 mg; Zn, 3000 mg; Mn, 2000 mg; Cu, 600 mg; Co, 10 mg; I, 40, mg; Se, 3 mg.

rise, and these sustainable opportunities will play a crucial role in AQs meeting the global food security requirements.

2. Materials and Methods

2.1. Experimental Design and Fish Maintenance. The experiment spanned over a period of 12 weeks and was carried out with *L. rohita* fingerlings (mean weight: 10.53 ± 0.25 g). We obtained 800 fingerlings and randomly assigned them to seven treatment groups, each with three replicates and $n = 100$ per group. Each replicate was placed in a 300 L fiberglass tank, which had all water parameters maintained within controlled conditions with aeration, a temperature of $27 \pm 1^\circ\text{C}$, and dissolved oxygen levels of 5.8 ± 0.2 mg/L. Every day, we carried out a 30% water exchange to maintain good water quality. We maintained a photoperiod of 12 h of light and 12 h of darkness throughout the entire experiment. The experiment was preceded by a 2-week acclimatization period during which fish were just placed on a basal diet before the start of the trial.

2.2. Diet Preparation and Feeding Regime. We designed the test diets to be isoproteic (35% CP) and isocaloric (18.2 MJ/kg) to meet the nutrient requirements of *L. rohita* fry. The control diet was a basal phosphorus-free diet (control), and the test diets contained either *B. licheniformis* or *C. verum* in different proportions. We supplemented the formulation with purchased probiotic *B. licheniformis* colonies at three different levels: D1 = 105 CFU/g, D2 = 107 CFU/g, and D3 = 109 CFU/g. Similarly, we added the phyto probiotic *C. verum* at three different concentrations:

D4 = 25 mg/kg, D5 = 50 mg/kg, and D6 = 75 mg/kg mentioned in Table 1.

The powdered *C. verum* was acquired from an approved distributor and incorporated directly into the feed formulation. The diets were formulated by accurately combining the components, extruding the pellets with a noodle extruder, and subjecting them to a temperature of 45°C . The commercially available probiotic strain was uniformly sprayed over the feed using phosphate-buffered saline (PBS) media, as both PBS and NaCl media do not adversely affect bacterial viability or the palatability of the feed. The pellets were additionally coated with fish oil to minimize bacterial loss in water prior to ingestion. The trial involved testing the survival of probiotic bacteria in the diet by diluting the diet with saline and applying 0.1 mL of this dilution onto agar plates. The plates were maintained at 36°C for a duration of 24 h. Diets were employed to maintain the desired colony count.

2.3. Feeding Regime. The fish were fed in two equal portions at 09:00 and 16:00, amounting to 3% of their daily weight. Adjustments were made biweekly, informed by a comparable parameter measured for total biomass during the same interval.

2.4. Growth Performance Assessment. The evaluation of growth performance included WG, SGR, FCR, and protein efficiency ratio (PER). To evaluate the total biomass in each tank, biweekly weight measurements of each fish were conducted using a digital balance. WG was determined by calculating the difference between initial and final weights. The SGR was determined utilizing the formula:

TABLE 2: PCR Primers used in the study.

Gene	Gene primer sequence (5'→3')	Temperature (°C)	Gene bank ID
IL-1 β	F: ATCTTGAGAAATGTGATCGAAGAG R: GATACGTTTTTGTATCCTCAAGTGTGAAG	61.5	AM93255
TNF- α	F: CCAGGCTTTCACCTTCACG R: GCCATAGGAATCGGAGTAG [20]	61.1	FN543477
Reference gene β -actin	F: AGACCACCTTCAACTCCATCATG R: TCCGATCCAGACAGAGTATTACGCH	60.5	AY531753

Note: [21].

Abbreviations: IL-1 β , interleukin-1 β ; PCR, polymerase chain reaction; TNF- α , tumor necrosis factor α .

$$\text{SGR}(\%) = \frac{(\ln \text{Final weight} - \ln \text{initial weight})}{\text{Experimental days}} \times 100.$$

FCR was calculated as the ratio of the total feed consumed to the WG of the fish:

$$\text{FCR} = \frac{\text{Feed intake (g)}}{\text{WG (g)}}.$$

2.4.1. *PER*. *PER* was computed by dividing the WG by the protein intake.

2.5. Hematological and Immunological Parameters

2.5.1. *Sample Collection and Storage*. At the termination of the experiment, blood samples were collected from healthy fish that displayed no indications of infection. Five fish from each group were anesthetized with clove oil (50 mg clove oil L⁻¹) and blood was collected from the caudal vein using a sterile syringe [15]. Fifty percent (50%) of each blood sample was stored at 4°C in an aqueous heparin solution (anticoagulant) and later used for the immediate evaluation of hematological parameters. The residual blood was subjected to centrifugation at 1075 × *g* for 10 min at 4°C to isolate plasma. Furthermore, for the analysis of biochemical and immunological assays, the samples were stored at -80°C. The hematological parameters of bovine red blood cells (RBCs), white blood cells (WBCs), hemoglobin (Hb), and hematocrit (Hct) were assessed using standardized methods described in book [16]. The concentrations of total serum were conducted through Biuret's method [17]. Serum samples were analyzed to evaluate immunological parameters and biochemical markers, such as lysozyme activity, total protein levels, IgM, and complement component 3 (C3) concentrations, in accordance with the protocols outlined by Ullah et al. [18] and Anderson and Siwicki [19].

2.6. *Gene Expression Analysis*. At the end trial, fish samples were collected to analyze tissue sample and gene expression. Six samples of liver and head kidney tissues from each group were collected and promptly frozen at -80°C. Gene expression analyses of specific tissues were conducted within 1 week of tissue collection to ensure sample integrity. Quantification of gene expression levels for IL-1 β and TNF- α was conducted using quantitative polymerase chain reaction

(qPCR). Total RNA was extracted from liver and head kidney tissues using TRIzol reagent and a commercial total RNA extraction kit. For each sample, 1 μ g of RNA was reverse transcribed to cDNA utilizing a standard cDNA synthesis kit (Cat#K1622, ThermoFisher Scientific, USA; Cat#G898, Applied Biological Materials, USA). The qPCR was performed in triplicate utilizing 1 μ L of cDNA template and SYBR green master mix (Cat#K0222, Thermo Fisher Scientific, USA) under defined conditions. Amplification and expression data were analyzed with LinRegPCR software, utilizing the Pfaffl method for quantification of expression. The following primer sequences were utilized (Table 2).

2.7. *Pathogenic Challenge with A. hydrophila*. Following the 12-week feeding trial, a pathogenic challenge using *A. hydrophila* was conducted to assess the disease resistance of the fish. This virulent strain was obtained from the Department of Microbiology at GCU Lahore, Pakistan. Fingerlings received an intraperitoneal injection of 0.1 mL of *A. hydrophila* suspension (1 × 10⁶ CFU/mL), while the control group was injected with an equivalent volume of sterile PBS. Mortality assessment was conducted over a 10-day period to observe any instances of mortality, and the relative percentage survival (RPS) was calculated using the following formula:

$$\text{RPS}(\%) = \left[1 - \frac{\text{Mortality in treatment group}}{\text{Mortality in control group}} \right] \times 100.$$

2.8. *Statistical Analysis*. The data was calculated as the three replicates average ($n = 3$) ± standard error of mean (SEM) and were statistically analyzed by one-way analysis of variance (ANOVA) by adjusting the significant level at $p < 0.05$. Tukey's multiple range test was used to determine differences among the groups. All statistical analyses were performed by using the statistical software SPSS (IBM Statistics version 25.0).

3. Results

3.1. *Growth Performance Metrics*. Table 3 summarizes the growth performance of *L. rohita* fingerlings that were fed diets supplemented with different doses of *B. licheniformis* and *C. verum* over a 12-week period. Group D3 exhibited the highest body-WG of 125.40 ± 0.10 g, WG of 86.40 ± 0.53 g, and SGR of 1.95 ± 0.02%/day, significantly surpassing the

TABLE 3: Growth performance metrics of fish fingerlings fed experimental diets.

Parameters	Experimental diets						ANOVA	
	Control	D1	D2	D3	D4	D5	D6	p-Value
IBW (g)	39.40 ± 0.52 ^a	39.20 ± 0.1 ^a	39.23 ± 0.25 ^a	39.20 ± 0.1 ^a	39.23 ± 0.11 ^a	39.20 ± 0.2 ^a	39.43 ± 0.28 ^a	0.001
FBW (g)	95.20 ± 0.2 ^d	105.23 ± 0.21 ^c	108.3 ± 0.1 ^{b,c}	125.40 ± 0.1 ^a	105.13 ± 0.231 ^c	112.33 ± 0.57 ^b	116.9 ± 1.62 ^b	0.001
WG (g)	56.73 ± 0.05 ^d	65.66 ± 0.41 ^c	69.20 ± 0.1 ^{b,c}	86.40 ± 0.53 ^a	66.03 ± 0.06 ^{b,c}	72.40 ± 0.36 ^b	77.17 ± 0.29 ^b	0.001
FCR	1.03 ± 0.06 ^e	0.99 ± 0.01 ^{c,d}	0.97 ± 0.017 ^c	0.81 ± 0.01 ^a	0.98 ± 0.01 ^{c,d}	0.94 ± 0.03 ^c	0.87 ± 0.01 ^b	0.001
SGR (%)	52.53 ± 0.25 ^e	61.87 ± 0.55 ^{d,e}	64.67 ± 0.65 ^c	81.56 ± 0.59 ^a	62.07 ± 0.40 ^d	69.06 ± 0.47 ^{b,c}	73.47 ± 0.32 ^b	0.001
PER	0.88 ± 0.01 ^e	1.03 ± 0.01 ^e	1.14 ± 0.01 ^{d,e}	1.22 ± 0.02 ^a	0.987 ± 0.01 ^{d,e}	1.063 ± 0.11 ^{c,d}	1.13 ± 0.06 ^{b,c}	0.001

Note: Values in the same row not sharing the same subscript are significantly different at $p < 0.05$.

Abbreviations: ANOVA, analysis of variance; FBW, final body weight; FCR, feed conversion ratio; IBW, initial body weight; PER, protein efficiency ratio; RER, relative efficiency ratio; SGR, specific growth rate; WG, weight gain.

TABLE 4: Hematological indices of fish fingerlings on varied experimental diets.

Hematological Parameters	Experimental diets						ANOVA	
	Control	D1	D2	D3	D4	D5	D6	p-Value
Hct (%)	26.03 ± 0.55 ^a	28.6 ± 0.88 ^a	33 ± 0.577 ^b	37.66 ± 0.88 ^a	27.33 ± 0.33 ^a	28.33 ± 0.33 ^a	32.6 ± 0.33 ^b	0.001
Hb (g/dL)	5.97 ± 0.44 ^{e,f}	7.83 ± 0.21 ^{b,c}	8.65 ± 0.07 ^b	9.83 ± 0.55 ^a	6.0 ± 0.43 ^e	7.0 ± 0.40 ^{c,d}	8.15 ± 0.02 ^{b,c}	0.001
MCHC (gdL ⁻¹)	24.7 ± 0.33 ^a	26.6 ± 0.26 ^{a,b}	28.1 ± 0.18 ^{b,c}	30.5 ± 0.29 ^a	26.8 ± 0.32 ^{a,b}	27.1 ± 0.86 ^{b,d}	29.5 ± 0.29 ^{c,e}	0.001
MCH (pg)	46.66 ± 1.20 ^a	42.33 ± 0.33 ^b	39.0 ± 0.57 ^b	34.3 ± 0.88 ^c	47.33 ± 1.20 ^a	42.3 ± 0.33 ^b	39 ± 0.577 ^b	0.001

Note: Values in the same row not sharing the same subscript are significantly different at $p < 0.05$.

Abbreviations: ANOVA, analysis of variance; Hb, hemoglobin concentration; Hct, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, MCH concentration.

TABLE 5: Immunological indices of fish fingerlings on varied experimental diets.

Immunological Parameters	Experimental diets						ANOVA	
	Control	D1	D2	D3	D4	D5	D6	p-Value
Total protein (g/dL)	35.03 ± 0.54 ^{d,e}	37.30 ± 0.30 ^b	38.6 ± 0.31 ^b	41.0 ± 0.57 ^a	35.3 ± 0.33 ^d	36.5 ± 0.28 ^d	38.9 ± 0.54 ^b	0.001
IgM (mg ml ⁻¹)	7.70 ± 0.11 ^d	8.97 ± 0.40 ^c	10.60 ± 0.25 ^b	11.6 ± 0.45 ^a	7.73 ± 0.03 ^d	9.07 ± 0.49 ^c	10.6 ± 0.27 ^b	0.001
C3 (mg ml ⁻¹)	55.4 ± 0.25 ^{a,c}	55.9 ± 0.37 ^c	57.03 ± 1.02 ^{a,b}	60.6 ± 0.98 ^a	55.4 ± 0.26 ^c	56.8 ± 0.52 ^c	57.0 ± 0.744 ^b	0.001
Lysozyme (μg ml ⁻¹)	7.80 ± 0.21 ^{d,e}	9.20 ± 0.21 ^d	10.3 ± 0.25 ^{b,c}	11.10 ± 0.57 ^a	7.87 ± 0.17 ^{d,e}	9.43 ± 0.27 ^d	10.2 ± 0.15 ^{b,c}	0.001

Note: Values in the same row not sharing the same subscript are significantly different at $p < 0.05$.

Abbreviations: ANOVA, analysis of variance; C3, complement component 3 levels; IgM, immunoglobulin M concentration.

control group, which recorded a final body weight of 95.20 ± 0.20 g, WG of 56.73 ± 0.05 g, and SGR of $1.56 \pm 0.01\%$ /day ($p < 0.05$). The FCR was optimal in group D3, recorded at 0.81 ± 0.01 , compared to the DMSO group, which had a value of 1.03 ± 0.06 . Conversely, under *C. verum*, it was noteworthy that fish in D6 (75 mg/kg) exhibited significant growth, while no growth was observed in the other groups relative to the control. Nonetheless, the performance in question remains inferior to that of the D3 group, as outlined in Table 3.

3.2. Hematological Parameters. Significant alterations were observed in the hematological indices of the subjects following the administration of dietary supplements, particularly in RBC count, WBC count, Hb concentration, and Hct (Table 4). The D3 group exhibited the highest RBC count ($3.80 \pm 0.05 \times 10^6$ cells/mm³) and Hb concentration (9.83 ± 0.55 g/dL), significantly surpassing the other groups ($p < 0.05$). Conversely, the control group recorded the lowest values

(RBC: $2.98 \pm 0.03 \times 10^6$ cells/mm³ and Hb: 5.97 ± 0.44 g/dL). As mentioned in Table 4, fish in the D6 group demonstrated enhancements in hematological parameters compared to the control group, specifically in WBC levels ($7.85 \pm 0.08 \times 10^3$ cells/mm³) and Hct ($37.66\% \pm 0.88\%$).

3.3. Immunological Parameters. The intervention positively influenced several immunological markers, including serum lysozyme enzyme activity, IgM, and C3. One hundred twenty fish receiving the D3 diet exhibited the highest mean lysozyme activity of 11.10 ± 0.57 μg/mL, which was significantly ($p < 0.05$) greater than that of the control group, which had a mean of 7.80 ± 0.21 μg/mL. The IgM levels were elevated in the D3 group relative to the control group (11.6 ± 0.45 and 7.70 ± 0.11 mg/dL, respectively), while the D6 group indicated the second-highest IgM titer at 10.6 ± 0.27 mg/dL. The complement C3 levels were consistent with the tissue cultures for the D3 group, which recorded a level of 60.6 ± 0.98 mg/mL. Details are outlined in Table 5, meanwhile

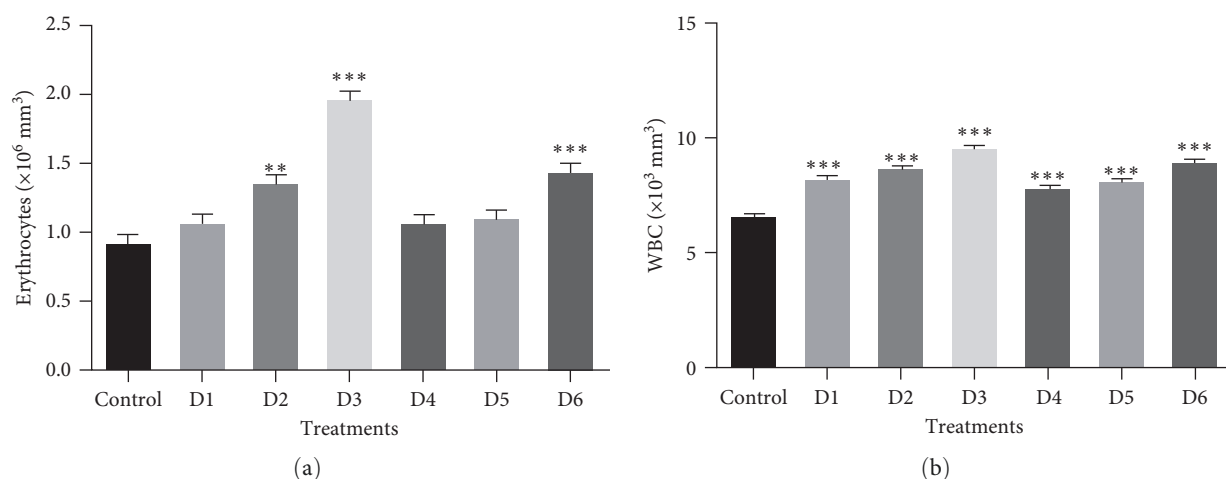


FIGURE 1: Comparative hematological profiles (a) RBC and (b) WBC count of fish fingerlings on varied experimental diets. * p -value < 0.05 (5% significance level), ** p -value < 0.01 (1% significance level), *** p -value < 0.001 (0.1% significance level). All groups showed significant improvements, with statistically significant results except Control. RBC, red blood cell; WBC, white blood cell.

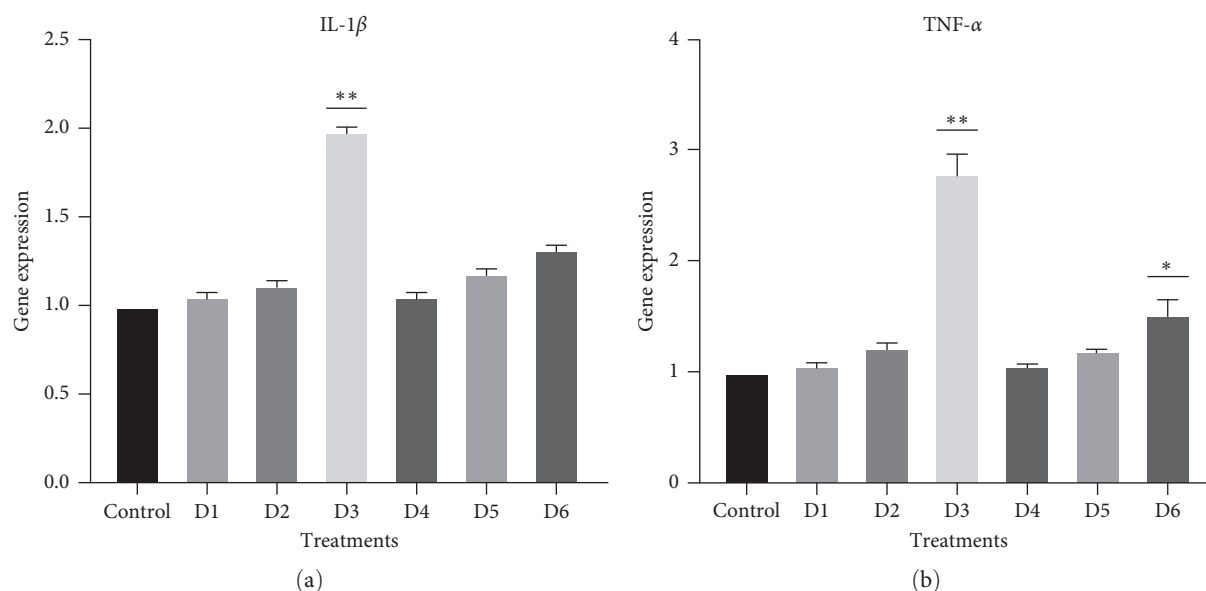


FIGURE 2: Relative mRNA expression of IL-1 β statistically significant ($p < 0.05$) in liver (a) and head kidney (b) of *Labeo rohita* across different experimental diets. IL-1 β , interleukin-1 β ; TNF- α , tumor necrosis factor α . In statistical graphs, asterisks denote significance levels: more stars indicate greater statistical significance. The results indicate that D3 showed the best outcomes, demonstrating statistical significance.

graphical representation of RBC and WBC count can be seen in Figure 1.

3.4. Gene Expression. The expression levels of immune-related genes, including IL-1 β and TNF- α , were significantly increased in the liver and head kidney tissues of fish administered *B. licheniformis* and *C. verum* in comparison to the control group (Figures 2 and 3). In the liver, using the control fish as a baseline, the D3 group exhibited an increase in IL-1 β by a factor of 3.2 and TNF- α by a factor of 2.5 ($p < 0.01$). In the fish D6 group, kidney tissues exhibited a 2.8-fold increase in IL-1 β expression levels and a 2.3-fold increase in TNF- α expression levels ($p < 0.01$).

3.5. Pathogenic Challenge and Survival Rate. Survival rates across all treatment groups following exposure to *A. hydrophila* exceeded those of the control groups (Figure 4). The D3 group exhibited a notable cytokine level, achieving a survival rate of $85.60\% \pm 1.20\%$, followed by the D6 group at $78.40\% \pm 1.50\%$. The control group recorded the lowest survival rate at $56.50\% \pm 2.00\%$.

4. Discussion

This research aimed to assess the impact of incorporating *B. licheniformis* and *C. verum* in the diet on the growth performance, immune modulation, gene expression, and

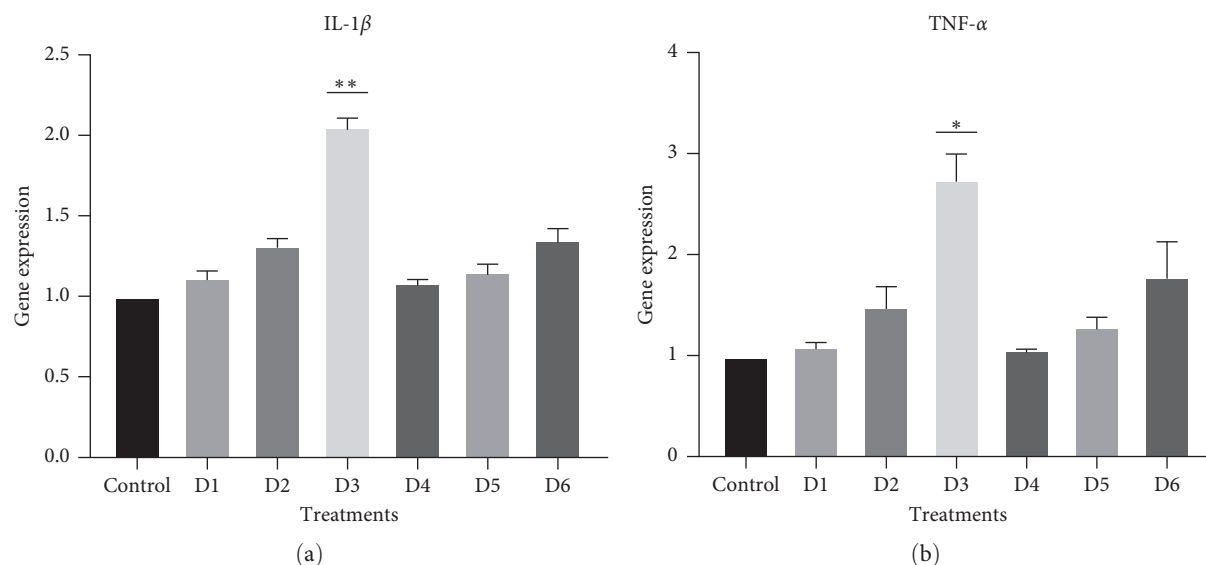


FIGURE 3: Relative mRNA expression of TNF- α statistically significant ($p < 0.05$) in the liver (a) and head kidney (b) of *Labeo rohita* across different experimental diets. IL-1 β , interleukin-1 β ; TNF- α , tumor necrosis factor α . Statistically significant results were observed for D3 which showed the most notable improvements. * indicates a lower level of statistical significance, suggesting minimal difference. ** denote moderate statistical significance, indicating notable differences.

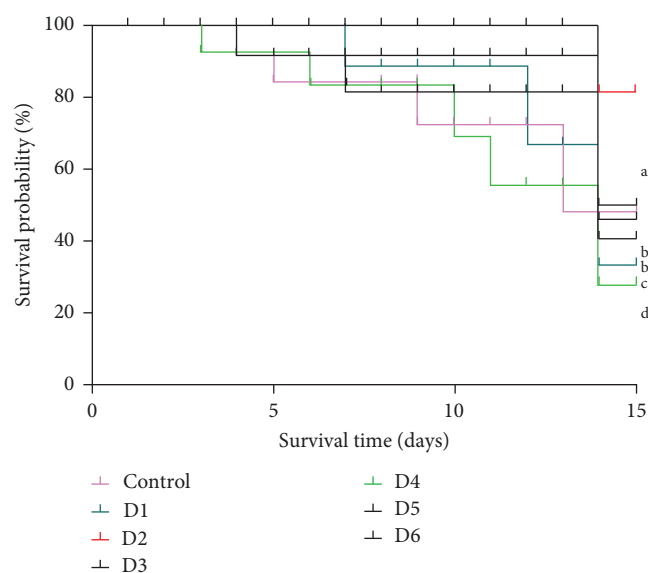


FIGURE 4: Kaplan-Meier % survival probability curve of *Labeo rohita* following *Aeromonas hydrophila* challenge for 12 weeks. The statistical significance of the results is indicated by the letters, with a being the most significant, followed by b, c, and d. More statistical significance means less mortality.

disease resistance of *L. rohita* fingerlings. The results indicated that *B. licheniformis* and *C. verum* improved growth, immunity, and disease resistance with optimal outcomes observed when high doses of both additives were administered concurrently. The results align with recent studies advocating for probiotic and phytobiotic supplementation in aquaculture.

The fact that growth parameters were much better in the D3 group (109 CFU/g *B. licheniformis*) and the D6 group (75 mg/kg

C. verum) supports the idea that probiotics and phytobiotics can help animals absorb and use nutrients better in their food. Comparable improvements in FCR and SGR have been documented in many aquaculture studies involving the use of *Bacillus* species. As evidenced in this study, *Oreochromis mossambicus* showed a greater growth performance when fed with higher concentrations of probiotic *B. licheniformis* in their diet, which agrees with the results of this current study [22]. This rise in growth may be due to the improvement of gut health, adjustment of the digestive system, and the rise of beneficial microbes in the GI tract attained through the use of probiotics [23]. Ramesh et al. [24] demonstrated the immunostimulatory effect of *B. licheniformis* in *L. rohita*, which aligns with our observation of enhanced growth and immunological responses under diets supplemented with *B. licheniformis*. This correlation emphasizes the potential of *B. licheniformis* as a beneficial probiotic in aquaculture.

As for *C. verum*, studies have demonstrated that it contains powerful antioxidants and has anti-inflammatory effects that help reduce oxidative stress in fish that are kept in intensive aquaculture systems. Oxidative stress has long been recognized as an inhibitory factor in the growth of fish, especially under conditions of high-density rearing where metabolic activities are high, causing high levels of ROS within the fish [12]. It can be noted that the markedly improved growth characteristics recorded in this study among the experimental D6 group may be related to the property *C. verum* possesses lowering the oxidative stress levels and thereby, enhancing the overall metabolic performance of the animals, similar to the findings of Zeeshan et al. [25] who observed the same results in broiler chickens. Gupta et al. [26] highlighted the significant role of probiotics and herbal supplements in immunonutrition, focusing on their ability to counteract the negative effects of chemotherapeutics in fish farming.

The upregulation of immune genes such as IL-1 β and TNF- α and their expression in the liver and kidney tissues of the fish on *B. licheniformis* and *C. verum* shows increased immune modulation. Both IL-1 β and TNF- α are pro-inflammatory cytokines involved in the first line of an immune response, enabling an organism to adapt and fight pathogens [8]. This increase in the concentration of immune genes, especially in the D3 group, strengthens the notion that *B. licheniformis* like probiotics augment immune capacity through cytokine production [27]. Additionally, dietary supplementation with *B. licheniformis* was found to enhance immune response in sea cucumbers through upregulation of immune-related genes as we also showed for *L. rohita* [28].

Cinnamomum verum has been shown to have some immune modulation effects in the said group D6. This may be due to its bioactive components, for instance, cinnamaldehyde, which has been demonstrated to be an effective antibacterial and anti-inflammatory agent. These compounds also help in regulating the immune response through inhibition of inflammation and boosting cellular immune effector cells like macrophages and lymphocytes [11]. The levels of serum IgM and complement C3, which were found to increase in the serums of the study participants, are additional evidence of *C. verum*'s capacity to enhance humoral immunity, which agrees with the findings of Sukul et al. [5], who reported enhancement of immune function of *L. rohita* when fed diets rich in phytobiotics. Nazeemashahul et al. [29] discussed the broader applications of herbal-based products in aquaculture, emphasizing their role in growth promotion and immune stimulation. Although specific quantitative data were not provided, their review supports the general trend observed in our study, where herbal supplementation (specifically *C. verum*) significantly enhanced growth and immune responses.

It was found that when *B. licheniformis* is assisted by *C. verum*, as gained in groups D3 and D6, there are better parameters for growth, immune response, and disease prevention as compared with control and other experimental groups. This means that probably two additives influence each other positively when included. *Bacillus licheniformis* supplements are effective in modulating gut symbiotic bacteria by eliminating pathogens, while phytobiotics such as *C. verum* possess additional properties that foster gut health by being antioxidative and antimicrobial [30]. This combination improves the homeostasis of the gut environment, thereby, enhancing prey capture and immune response.

This combination enhances gut homeostasis, thereby, improving prey capture and immune response. The elevated survival rates of fish in D3 (85.6%) and D6 (78.4%) following the *A. hydrophila* challenge demonstrate the benefits of incorporating probiotics and phytobiotics for enhancing disease resistance in fish. The modulation of the immune system and the inhibition of pathogenic bacteria by *B. licheniformis* have been documented in earlier studies, indicating that these activities are not novel. Zhang et al. [27] demonstrated that the incorporation of *B. licheniformis* in broilers mitigated the effects of necrotic enteritis by decreasing inflammation and improving gut barrier function, which may have contributed to the high survivorship observed in this study. Probiotics

function by synthesizing digestive enzymes and promoting beneficial intestinal microorganisms that aid in the digestion of complex nutrients [23]. According to Yu et al. [8], inclusion of *B. licheniformis* improved digestive enzyme activity in weaning piglets. Khan et al. [10] reported that the herb *C. verum* enhances digestion by stimulating the production of bile and digestive enzymes. Prior research has utilized cinnamon oil to combat fish infections attributed to the bacterium *Streptococcus iniae* in Tilapia [31] and *A. hydrophila* infections in Nile Tilapia [32]. Both studies demonstrated a significant inhibitory effect on the pathogen, indicating its potential as an alternative to antibiotics in aquaculture. Our study similarly found that *B. licheniformis* and cinnamon from *C. verum* enhanced survival of *L. rohita* against *A. hydrophila*. This study demonstrates that the inclusion of *B. licheniformis* and *C. verum* in the diet of *L. rohita* fingerlings enhances growth performance, modulates the immune system, and increases disease resistance. The optimal combinations of the two additives yielded the most favorable results, indicating their potential as effective antibiotic alternatives in aquaculture. This paper discusses the potential of probiotic-phytobiotic combinations to enhance fish health and productivity. Improvement of these additives is necessary to enhance their benefits and evaluate their safety for fish, as well as the sustainability of fish farming practices.

5. Conclusion

The evaluated combinations of *C. verum* and *B. licheniformis* may serve as a sustainable and cost-effective alternative to antibiotics. The study demonstrated that the incorporation of 10⁹ CFU/g of *B. licheniformis* and 75 mg/kg of *C. verum* into the diet of *L. rohita* fingerlings significantly improves growth, feed efficiency, and immune response. Significant elevation of immune function genes, such as TNF- α and IL-1 β , was observed in both liver and head kidney tissues. *Bacillus licheniformis* enhances fish health and performance, and our findings elucidate its molecular mechanism. *Bacillus licheniformis* presents a viable option for aquaculture antibiotics, demonstrating enhanced fish survival rates against *A. hydrophila*, as well as improvements in fish health, productivity, and disease resistance.

Data Availability Statement

The data are available on request.

Ethics Statement

Ethical approval for this study was obtained from the Animal Ethics Committee, with approval reference number GCU/2022 dated 13 Sept 2022. All experimental procedures were carried out following the guidelines of the committee to ensure the welfare of the fish during the study. All handling was performed under anesthesia, and all exertions were made to reduce animal stress.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Iqra Majeed: Conceived and planned the project, carried out experimental and laboratory work, and wrote the manuscript. Atif Yaqub: Supervised the project and approved the final version to be published. Muhammad Ayub: Cosupervised the project and contributed to resources and methodology.

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References

- [1] M. G. Rasul and B. C. Majumdar, "Abuse of Antibiotics in Aquaculture and Its Effects on Human, Aquatic Animal and Environment," *The Saudi Journal of Life Sciences* 2, no. 3 (2017): 81–88.
- [2] C. Rodgers and M. Furones, "Antimicrobial Agents in Aquaculture: Practice, Needs and Issues," in *Options Méditerranéennes, Series A, Mediterranean Seminars*, 86, (CIHEAM, 2009): 41–59.
- [3] M. Kamran, H. Razzaq, M. Noorullah, M. Ahmad, and A. Zuberi, "Comparative Analysis of Genetic Diversity, Growth Performance, Disease Resistance and Expression of Growth and Immune Related Genes Among Five Different Stocks of *Labeo rohita* (Hamilton, 1822)," *Aquaculture* 567 (2023): 739277.
- [4] K. Amoah, X. H. Dong, B. P. Tan, et al., "Effects of Three Probiotic Strains (*Bacillus coagulans*, *B. licheniformis* and *Paenibacillus polymyxa*) on Growth, Immune Response, Gut Morphology and Microbiota, and Resistance Against *Vibrio harveyi* of Northern Whittings, *Sillago sihama* Forsskål, 1775," *Animal Feed Science and Technology* 277 (2021): 114958.
- [5] T. Sukul, Z. A. Kari, G. Téllez-Isaías, and K. Ghosh, "Autochthonous *Bacilli* and *Fructooligosaccharide* as Functional Feed Additives Improve Growth, Feed Utilisation, Haemato-Immunological Parameters and Disease Resistance in Rohu (*Labeo rohita*)," *Animals* 13, no. 16 (2023): 2631.
- [6] S. I. Paul and M. M. Rahman, "Draft Genome Sequence of, *Bacillus subtilis*, YBS29, a Potential Fish Probiotic that Prevents Motile *Aeromonas* Septicemia in *Labeo rohita*," *Microbiology Resource Announcements* 11, no. 10 (2022).
- [7] Y. Xu, Y. Yu, Y. Shen, et al., "Effects of *Bacillus subtilis* and *Bacillus licheniformis* on Growth Performance, Immunity, Short Chain Fatty Acid Production, Antioxidant Capacity, and Cecal Microflora in Broilers," *Poultry Science* 100, no. 9 (2021): 101358.
- [8] X. Yu, Z. Dai, G. Cao, et al., "Protective Effects of *Bacillus licheniformis* on Growth Performance, Gut Barrier Functions, Immunity, and Serum Metabolome in Lipopolysaccharide-Challenged Weaned Piglets," *Frontiers in Immunology* 14 (2023): 1140564.
- [9] N. P. Pezsa, D. Kovács, B. Rácz, and O. Farkas, "Effects of *Bacillus licheniformis* and *Bacillus subtilis* on Gut Barrier Function, Proinflammatory Response, ROS Production, and Pathogen Inhibition Properties in IPEC-J2—*Escherichia coli*/*Salmonella Typhimurium* Co-Culture," *Microorganisms* 10, no. 5 (2022): 936.
- [10] M. I. R. Khan, D. Kamilya, T. G. Choudhury, and G. Rathore, "Dietary Administration of a Host-Gut Derived Probiotic *Bacillus amyloliquefaciens* Modulates Immune-Biochemical Response, Immune-Related Gene Expression, and Resistance of *Labeo rohita* to *Aeromonas hydrophila* Infection," *Aquaculture* 546 (2022): 737390.
- [11] K. Ghosh, A. Mukherjee, D. Dutta, et al., "Endosymbiotic Pathogen-Inhibitory Gut Bacteria in Three Indian Major Carps Under Polyculture System: A Step Toward Making a Probiotics Consortium," *Aquaculture and Fisheries* 6, no. 2 (2021): 192–204.
- [12] D. García-Medel, C. Angulo, R. Escamilla-Montes, J. A. Fierro-Coronado, G. Diarte-Plata, and C. Gámez-Jiménez, *Bacillus licheniformis*, BCR 4-3 Increases Immune Response and Survival of *Litopenaeus vannamei* Challenged With *Vibrio parahaemolyticus* (Aquaculture International, 2020).
- [13] H. Ramirez-Olea, B. Reyes-Ballesteros, and R. A. Chávez-Santoscoy, "Potential Application of the Probiotic *Bacillus licheniformis* as an Adjuvant in the Treatment of Diseases in Humans and Animals: A Systematic Review," *Frontiers in Microbiology* 13 (2022): 993451.
- [14] A. Yaqub, M. N. Awan, M. Kamran, and I. Majeed, "Evaluation of Potential Applications of Dietary Probiotic (*Bacillus licheniformis*) on Growth, Digestive Enzyme Activity, Hematological, Biochemical, and Immune Response of *Tilapia* (*Oreochromis mossambicus*)," *Turkish Journal of Fisheries and Aquatic Sciences* 22, no. 5 (2021).
- [15] S. S. Habib, S. Naz, A. I. Batool, et al., "Effect of Different Anaesthetics on Hematology and Blood Biochemistry of *Labeo rohita*," *Aquaculture Studies* 24, no. 1 (2023): AQUAST1195.
- [16] B. J. Barbara, B. Imelda, and L. A. Michael, *Dacie and Lewis Practical Haematology* (Elsevier, 12th edition, 2017).
- [17] A. G. Gornall, C. J. Bardawill, and M. M. David, "Determination of Serum Proteins by Means of the Biuret Reaction," *Journal of Biological Chemistry* 177 (1994): 751–766.
- [18] A. Ullah, A. Zuberi, M. Ahmad, et al., "Dietary Administration of the Commercially Available Probiotics Enhanced the Survival, Growth, and Innate Immune Responses in Mori (*Cirrhinus mrigala*) in a Natural Earthen Polyculture System," *Fish & Shellfish Immunology* 72 (2018): 266–272.
- [19] D. P. Anderson and A. K. Siwicki, *Basic Hematology and Serology for Fish Health Programs* (Fish Health Section, Asian Fisheries Society, Manila, Philippines, 1995): 185–202.
- [20] S. F. Gonzalez, K. Buchmann, and M. E. Nielsen, "Real-Time Gene Expression Analysis in Carp (*Cyprinus carpio* L.) Skin: Inflammatory Responses Caused by the Ectoparasite *Ichthyophthirius multifiliis*," *Fish & Shellfish Immunology* 22, no. 6 (2007): 641–650.
- [21] R. Harikrishnan, C. Balasundaram, and M.-S. Heo, "Impact of Plant Products on Innate and Adaptive Immune System of Cultured Finfish and Shellfish," *Aquaculture* 317, no. 1–4 (2011): 1–15.
- [22] M. Abinaya, S. Shanthi, J. Palmy, K. Al-Ghanim, M. Govindarajan, and B. Vaseeharan, "Exopolysaccharides-Mediated ZnO Nanoparticles for the Treatment of Aquatic Diseases in Freshwater Fish *Oreochromis mossambicus*," *Toxics* 11, no. 4 (2023): 313.
- [23] L. Li, X. Lv, X. Han, et al., "The Effects of *Bacillus licheniformis* on Growth Performance and Microbiota Diversity of Pekin Ducks," *Frontiers in Veterinary Science* 9 (2022): 832141.
- [24] D. Ramesh, A. Vinothkanna, A. K. Rai, and V. S. Vignesh, "Isolation of Potential Probiotic *Bacillus* spp. and Assessment of

- Their Subcellular Components to Induce Immune Responses in *Labeo rohita* against *Aeromonas hydrophila*,” *Fish & Shellfish Immunology* 45, no. 2 (2015): 268–276.
- [25] M. Zeeshan, H. Zaneb, S. Masood, et al., “Morphological Modulation of Broiler Organs in Response to an Organic Acid–Phytogen Composite in Healthy Broilers,” *Agriculture* 12, no. 6 (2022): 791.
- [26] A. Gupta, S. K. Gupta, M. Priyam, et al., “Immunomodulation by Dietary Supplements: A Preventive Health Strategy for Sustainable Aquaculture of Tropical Freshwater Fish, *Labeo rohita* (Hamilton, 1822),” *Reviews in Aquaculture* 13, no. 4 (2021): 2364–2394.
- [27] Z. Zhang, Y. Fu, H. Zhao, and X. Zhang, “Social Enrichment Affects Fish Growth and Aggression Depending on Fish Species: Applications for Aquaculture,” *Frontiers in Marine Science* 9 (2022): 1011780.
- [28] L. Wang, C. Gao, B. Wang, C. Wang, G. Sagada, and Y. Yan, “Methionine in Fish Health and Nutrition: Potential Mechanisms, Affecting Factors, and Future Perspectives,” *Aquaculture* 568 (2023): 739310.
- [29] S. Nazeemashahul, F. J. Fawole, B. R. Am, et al., “Herbal Immunomodulators for Aquaculture,” in *Immunomodulators in Aquaculture and Fish Health*, (CRC Press, 2024): 119–135.
- [30] S. Stoyanova, G. Zhelyazkov, I. Sirakov, K. Velichkova, and Y. Staykov, “Influence of Dietary *Cinnamomum verum* Extract on the Growth and Economic Efficacy of Common Carp (*Cyprinus carpio* L.), Reared in a Recirculation System,” *Trakia Journal of Sciences* 16, no. 4 (2018): 307–312.
- [31] P. Rattanachaikunsopon and P. Phumkhachorn, “Potential of Cinnamon (*Cinnamomum verum*) Oil to Control *Streptococcus iniae* Infection in Tilapia (*Oreochromis niloticus*),” *Fisheries Science* 76, no. 2 (2010): 287–293.
- [32] M. H. Ahmad, A. M. El Mesallamy, F. Samir, and F. Zahran, “Effect of Cinnamon (*Cinnamomum zeylanicum*) on Growth Performance, Feed Utilization, Whole-Body Composition, and Resistance to *Aeromonas hydrophila* in Nile Tilapia,” *Journal of Applied Aquaculture* 23, no. 4 (2011): 289–298.