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Enhancing Growth and Intestinal Health in Triploid Rainbow Trout Fed a Low-Fish-Meal Diet through Supplementation with *Clostridium butyricum*

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Abstract: This study evaluates the effects of dietary *Clostridium butyricum* on growth performance and intestinal health in triploid rainbow trout (*Oncorhynchus mykiss*). Administered in a 12-week trial, five isonitrogenous and isolipidic feeds contained different *C. butyricum* levels [G1 (0), G2 (1.6×10^6 CFU/g), G3 (1.2×10^7 CFU/g), G4 (1.1×10^8 CFU/g), and G5 (1.3×10^9 CFU/g)]. Significant enhancements in growth performance, including improved feed conversion ratios and specific growth rates in the G4 group, were indicative of enhanced nutrient utilization, corroborated by optimal digestive enzyme activity levels. Antioxidant capabilities were also enhanced in the G4 group, indicated by increased serum superoxide dismutase and catalase activities, along with a significant decrease in malondialdehyde levels. Gut microbiota analysis indicated a probiotic concentration-dependent modulation of microbial communities, with a marked enrichment in beneficial bacterial phyla like Firmicutes in the G4 group. This microbial shift correlated with significant downregulations in immune-related gene expressions, including interleukins (*IL-1β*, *IL-8*), *NF-κB*, *MyD88*, and *TNF-α*, highlighting an activated immune response. Correspondingly, serum lysozyme and immunoglobulin M contents were significantly elevated in the G4 group. Challenge tests with *Aeromonas salmonicida* exhibited the higher disease resistance of fish fed the G4 diet. In conclusion, the study shows the potential of dietary *C. butyricum*, especially at 1.1×10^8 CFU/g, in enhancing the growth, health, and disease resistance of triploid rainbow trout through modulating gut microflora and stimulating immune responses.

Keywords: triploid rainbow trout; *C. butyricum*; low-fish-meal diet; growth performance; intestinal health

Key Contribution: Dietary supplementation with *C. butyricum* at 1.1×10^8 CFU/g significantly enhances growth performance; stimulates immune responses, and improves intestinal health in triploid rainbow trout, offering a promising strategy for aquaculture nutrition optimization.

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Citation: Wang, C.; Li, F.; Wang, D.; Lu, S.; Han, S.; Gu, W.; Jiang, H.; Li, Z.; Liu, H. Enhancing Growth and Intestinal Health in Triploid Rainbow Trout Fed a Low-Fish-Meal Diet through Supplementation with *Clostridium butyricum*. *Fishes* **2024**, *9*, 178. https://doi.org/10.3390/fishes9050178

Academic Editor: Francisco J. Moyano

Received: 23 April 2024
Revised: 7 May 2024
Accepted: 11 May 2024
Published: 14 May 2024

1. Introduction

As global demand for aquacultured fish increases, the need for sustainable feed alternatives becomes critical [1,2]. With the continuous advancement of aquaculture technologies and economic benefits, the demand for fish meal, a high-quality protein source, has increasingly surged. Consequently, the search for suitable alternatives to fish meal in aquafeeds has become a topic of significant interest within the industry [3,4]. Soybean

meal, as a plant protein source, offers advantages such as relatively high protein content, widespread availability, and lower cost, positioning it as an excellent alternative to fish meal [5]. However, the presence of antinutritional factors in soybean meal, including protease inhibitors, antivitamins, and allergens, can impair aquatic animals' ability to absorb nutrients from feed, subsequently diminishing growth performance [6]. Research has shown that supplementing feed additives in diets where plant protein sources like soybean meal replace fish meal can effectively mitigate the adverse effects associated with plant protein substitution [7]. This approach enhances the utilization ratio of plant proteins in feeds, therefore contributing to more sustainable and cost-effective aquaculture practices.

Probiotics, as effective feed additives, have demonstrated advantages in promoting growth, enhancing feed utilization rates, and bolstering the immune system in fish. Despite these benefits, the literature reveals certain gaps, particularly in the specific applications of different probiotic strains and their optimal concentrations for different fish species. Many studies have generalized the effects of probiotics without delving into strain-specific responses or the ideal dosages required to elicit optimal health benefits in specific fish species [8]. Moreover, the mechanisms underlying the probiotic action, especially in relation to immune modulation and gut microbiota balance, are often not detailed, leaving a gap in the comprehensive understanding necessary for tailored probiotic applications in aquaculture.

Clostridium butyricum, capable of anaerobic growth and spore production, exhibits resilience to heat, acid, alkali, and high temperatures, categorizing it among the spore-forming probiotics [9]. *C. butyricum* has been identified as a superior probiotic in aquaculture due to its robust spore-forming ability that ensures survival under harsh conditions and its proven efficacy in enhancing growth, immune responses, and gut health, surpassing other commonly used probiotics [10,11]. Supplementing with appropriate amounts of *C. butyricum* has been shown to effectively maintain gut health in fish. On a cellular level, *C. butyricum* not only sustains cell viability but also increases mucin secretion; regarding the intestinal mucosa, it promotes repair and reduces permeability, aiding in the restoration of physiological functions [7]. This enhancement of mucosal barrier function by *C. butyricum* is crucial as it directly influences the composition and function of the gut microflora, establishing a more stable and beneficial microbial community that enhances overall gut health. The gut microflora's dysbiosis can lead to various diseases, while *C. butyricum* fosters the proliferation of beneficial bacteria such as bifidobacteria, lactobacilli, and pseudobacilli and inhibits the growth of harmful bacteria like *Staphylococcus*, *Candida*, *Klebsiella*, *Campylobacter*, *Pseudomonas*, *Escherichia coli*, *Shigella*, *Salmonella typhi*, and putrefactive bacteria, therefore reducing the production of harmful substances within the organism [12]. Studies in hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) [11] carp (*Cyprinus carpio*) [13] have revealed the significant role of *C. butyricum* in affecting aquatic animals' growth performance, physiological and biochemical indicators, modulation of gut microflora, and enhancement of immunity. Current research on *C. butyricum* primarily focuses on fish meal-based feeds, with limited studies in plant protein substitutes. The potential of *C. butyricum* and its metabolites to repair intestinal damage caused by plant protein substitutes and to improve growth performance, antioxidative capacity, and immunity under such conditions is less reported.

Oncorhynchus mykiss, classified under the order Salmoniformes, family Salmonidae, and genus *Oncorhynchus*, is a prominent species among cold-water fishes cultivated in China. Notably, triploid rainbow trout obtains consumer preference for its rapid growth, efficient feed conversion, high muscle yield, lack of intramuscular bones, and palatable flesh compared to its diploid [14]. Despite the rapid advancement in aquaculture, the industry confronts challenges such as the limited availability of high-quality protein sources for feed, environmental contamination affecting water quality, and the widespread occurrence of diseases. By optimizing feed formulations and supplementing effective additives, it is feasible to sustain optimal nutrition and growth in rainbow trout, even with reduced fish meal content. Previous nutritional assessments in our laboratory have shown that

triploid rainbow trout can maintain normal growth and development with a fish meal inclusion level of 15% [15,16]. However, research into the effects of *C. butyricum* supplementation in low-fish-meal diets, particularly its impact on the immune response, intestinal inflammation-related gene expression, and microflora in triploid rainbow trout, remains scarce. Therefore, this study investigates the effects of different levels of *C. butyricum* in low-fish-meal diets on the growth, digestive physiology, immune functions, and gut microflora of triploid rainbow trout. These findings aim to provide a basis for the dietary formulation of trout feeds.

2. Materials and Methods

2.1. Fish Management

The triploid rainbow trout (initial body weight: 259.73 ± 0.80 g) were sourced from the Benxi Aigemolin Rainbow Trout Farming Company in Liaoning Province (Benxi, China). At the start of the trial, trout were acclimatized to the experimental conditions for 15 days. Subsequently, the fish were randomly allocated into 15 tanks, each with a capacity of 500 L and 20 fish per tank. Water quality parameters were monitored once a day. During the 12-week trial, the tanks were maintained under a relatively constant dissolved oxygen level (>6.0 mg/L), temperature (18.0–20.0 °C), pH (6.5–6.8), ammoniacal nitrogen level (<0.02 mg/L), and photoperiod (12 h light: 12h dark). The fish were fed four times daily (6:00, 9:00, 14:00, 16:00) until apparent satiety.

2.2. Diets

Five isonitrogenous and isolipidic diets supplemented with different levels of *C. butyricum* [G1 (0), G2 (1×10^6 CFU/g), G3 (1×10^7 CFU/g), G4 (1×10^8 CFU/g), and G5 (1×10^9 CFU/g)] were formulated. The *C. butyricum*, provided by Hangzhou Huajia Biotechnology Co., Ltd. (Hangzhou, China), had a bacterial concentration of 1×10^9 CFU/g. This specific strain is referred to as *C. butyricum* CICC 24854. For feed preparation, the probiotic powder was first homogenized with a small quantity of the base feed to ensure even distribution. This premix was then thoroughly blended with the remaining feed components using a paddle mixer to maintain uniformity. The formulated diets utilized fish meal and soybean meal as the primary protein sources, supplemented with compound amino acids and brewer's yeast. Fish oil and soybean oil were utilized as lipid sources, while wheat middings served as the carbohydrate sources (Table 1). The diets were passed through a 250-μm sieve. The dry, finely ground feed components were completely blended with lipids and then homogenized using a blender (GYJ-250B; Dashiqiao Bao's Feed Machinery Factory; Yingkou, China). The dough was pelleted into 3.0-mm (diameter) granular feed using a lab pelletizer (SLP-45; Fishery Mechanical Facility Research Institute, Shanghai, China). The pellets were then air-dried to a moisture content of approximately 10% by weight, collected in vacuum-packed bags, and stored at −20 °C until use.

2.3. Sample Collection

The fish were starved for 24 h before sampling. Anesthesia was administered using 40 mg/L of MS-222. After that, samples from the blood, whole body, liver, and midsection of the intestine were collected. From each tank, four fish were chosen to analyze body composition and examine blood chemistry and liver antioxidant capacity. Three fish were selected from each tank for mid-intestine (the middle section of the intestine after the pyloric caecum) gene expression and digestive enzyme analysis. Blood was collected from each fish's tail vein and then left to stand for 4 h at 4 °C. Then, the blood was centrifuged in a pre-cooled centrifuge for 15 min at 5000× *g*. The supernatant was transferred to a centrifuge tube and stored at −80 °C. Excess lipids and inclusions were removed from the mid-intestines of fish and placed in cryovials. The tubes were then immediately submerged in liquid nitrogen for RNA extraction and digestive enzyme analysis. Additionally, whole bodies from each treatment group were labeled and stored at −80 °C for the determination of body composition. Another two intestinal samples were

collected under sterile conditions from two randomly selected fish per tank. The exterior of each fish was sanitized with 75% alcohol before the intestines were extracted using sterile scissors. Contents from the foregut to the hindgut were transferred into a sterile 2 mL centrifuge tube (RNase-Free, Axygen, Union City, CA, USA) using sterile tweezers and immediately frozen in liquid nitrogen for gut microflora high-throughput sequencing analysis.

Table 1. Composition and nutrient levels of the basal diet (air-dried basis, %).

Items	Contents
Wheat middings	19.60
Soybean meal	21.60
Compound amino acid	17.02
Fish meal	15.00
Beer yeast	6.00
Soybean oil	7.78
Fish oil	7.80
Premix	4.00
$Ca(H_2PO_4)_2$	1.00
Calcium propionate	0.20
Total	100.00
Nutritional level	
Gross energy (MJ/kg)	18.68
Moisture	9.24
Crude protein	40.16
Crude lipid	17.02
Crude fiber	2.65
Ash	9.25

Note: The concentrations of *C. butyricum* in the formulated diets were assessed using quantitative PCR methods to confirm that the concentrations of 1.6×10^6, 1.2×10^7, 1.1×10^8, and 1.3×10^9 CFU g^{-1} of feed for Groups G2, G3, G4, and G5, respectively. Premix 4.0% as described by Wang et al. (2022) [15]. Nutrient levels were measured values.

2.4. Nutrient Content

The diets and whole fish were analyzed following the AOAC protocols (2016) [17]. The moisture content of the samples was analyzed using the drying method. The samples were put in an oven at 105 ± 0.5 °C for 3 h until they reached a constant weight. The protein content of the whole fish was determined using automatic rapid nitrogen-fixing (rapid N exceed, Elementar, Langenselbold, Germany). The ash content was determined using the cauterization method. First, 2.0 ± 0.5 g of the sample was weighed precisely and placed in the crucible, which was then heated in the muffle furnace. The temperature was set to 350 °C for half an hour, followed by an adjustment to 600 °C for 2 h. The crude lipid content was determined using the Soxhlet extraction method with a Soxhlet extraction system (Extraction system-811, BUCHI, Flawil, Switzerland) and petroleum ether as the extraction solvent. The extraction process lasted for a duration of 10 h to ensure complete extraction of the lipid content from the sample. The determination of crude fiber was conducted using the acid-alkali digestion method, which involves sequential digestion of the sample with sulfuric acid and sodium hydroxide, followed by combustion of the insoluble residue to quantify fiber content using a fiber analyzer (TH-SF22, Tianhong Co., Weifang, China). The gross energy content was determined through the utilization of a bomb calorimeter (LRY-600A, Chuangxin Ltd., Shijiazhuang, China).

2.5. Digestive Enzyme

Liver and intestinal samples were thawed at 4 °C. Add pre-cooled physiological saline solution with a concentration of 0.86% to the thawed sample at a ratio of 1:9 between the sample and physiological saline solution. The samples were homogenized by employing a high-speed dispersive tissue homogenizer (FJ-200, Shanghai Instrument Co., Shanghai, China). Following this, the samples were placed into a low-temperature cen-

trifuge (H2050R, Shanghai Instrument Co., China) and centrifuged at a speed of 6000× *g* for 10 min to segregate the supernatant. Digestive enzyme analyses followed a protocol from the manufacturer, utilizing commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The lipase-specific substrate was decomposed, and lipase activity was determined by the rate of formation of red methyl halide. The product is measured at 580 nm (A054-2-1). α-Amylase (C016-1-1) hydrolyses starch to produce monosaccharides, which bind to unhydrolyzed starch by the addition of iodine solution to form a blue complex, the absorbance of which is measured at 660 nm. The intestinal protease activity was determined using the Folinphenol method. Casein (substrate) is broken down by protease to generate amino acids containing phenolic groups, which react with the Folinphenol reagent to produce a blue color. The absorbance of this color was measured at 680 nm to calculate protease activity.

2.6. Biochemical Analysis

Analyses of antioxidant and immune indicators were performed for superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT), lysozyme (LZM), and immunoglobulin (IgM) following the manufacturer's protocol, utilizing commercial kits from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) (Table 2).

Table 2. The chemical analysis kits were used in the experiment.

Items	Product Number	Method
SOD	A001-3	Based on the scavenging effect of SOD on superoxide radicals, one may gauge the activity of SOD indirectly through the measurement of the absorbance of the purple product (at 450 nm).
MDA	A003-1	MDA present in degradation products of lipid peroxides can react with thiobarbituric acid (TBA), and the amount of MDA can be determined by measuring the absorbance of the resulting red product (532 nm).
CAT	A007-2-1	Catalase activity is assessed by its capacity to decompose hydrogen peroxide (H_2O_2), reflected in a decrease in absorbance at 240 nm. This is executed by blending a specified volume of reagent one with a ten-fold volume of reagent two's working solution, aiming for an absorbance between 0.5 and 0.55. The assay is initiated by mixing 0.02 mL of the sample with 3 mL of this substrate solution, warmed to 25 °C, and measuring the absorbance immediately and after one minute. The activity is quantified by the change in absorbance, indicating H_2O_2 decomposition.
LZM	A050-1-1	At a specific concentration of opaque bacterial solution, the lysozyme hydrolyses the peptidoglycan on the bacterial cell wall, resulting in bacterial cleavage and reduced concentration. This leads to an increase in transmittance, which is measured at 530 nm to determine the lysozyme content.
IgM	H109-1-1	The IgM level can be determined by measuring the turbidity produced when IgM in the sample forms an immune complex with anti-IgM antibodies in the reagents. After incubation with the reagents at 37 °C, the absorbance is measured at 340 nm, and the concentration is calculated from a nonlinear calibration curve.

Note: SOD, superoxide dismutase; MDA, malondialdehyde; CAT, catalase; LZM, lysozyme; IgM, immunoglobulin M.

Biochemical parameters, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides (TG), total protein (TP), glucose (GLU), and albumin (ALB), were quantitatively analyzed using an MNCHIP Celercare V5 automatic biochemical analyzer (MNCHIP, Tianjin, China). Serum samples, collected by centrifuging blood at 4000× *g* for 15 min, were processed according to the manufacturer's guidelines to ensure accurate assessments of metabolic function indicators.

2.7. Real-Time Polymerase Chain Reaction (PCR) Analysis

The intestines were collected, an appropriate amount of liquid nitrogen was added, and ground in a cooled mortar until they became a fine powder. Total RNA was isolated from the powdered samples utilizing the TRIzol reagent method (Ambion, San Diego, CA, USA). To remove DNA, the sample was treated with Rnase-free DNase I (Thermo Scientific, Waltham, MA, USA). The mass of RNA was measured by agarose gel electrophoresis, for which a 1% agarose gel was prepared and run at 110 V for 30 min. The concentration and quality of the extracted RNA required for subsequent experiments were determined using an ultra-micro UV-visible spectrophotometer (Thermo Scientific NanoDrop 2000) featuring an A260 nm/A280 nm ratio ranging from 1.8 to 2.0. The conversion of RNA to cDNA was conducted with the PrimeScript^{TM} RT Reagent Kit (TaKaRa, Dalian, China) according to the kit instructions. The cDNA concentration was determined using an ultra-micro spectrophotometer, and samples with completed reverse transcription were diluted to 50 ng/μL for dispensing and storage.

Use the NCBI website to design primers, ensuring avoidance of repeated bases and adherence to a melting curve Tm value of 55–80 °C. It is typically recommended that primer length falls between 18 and 27 bp but should not exceed 38 bp, with a primer GC content of 30–80%. The length of the PCR amplification product should fall within the range of 80–250 bp, with a primer annealing temperature of around 60 °C (Table 3).

Table 3. Primers used for determining gene expression of rainbow trout.

Genes	Forward Primer Sequences (5′–3′)	Reverse Primer Sequences (5′–3′)	Accession Number	Amplification Efficiency
β-Actin	GGACTTTGAGCAGGAGATGG	ATGATGGAGTTGTAGGTGGTCT	CP137043.2	95.72%
IL-1β	ACATTGCCAACCTCATCATC	GTTCTTCCACAGCACTCTCC	AJ278242.2	97.89%
IL-2	GAAACCCAATTCCCAGACTC	TCCGTTGTGCTGTTCTCCT	NM_001164065.2	96.43%
IL-8	GAATGTCAGCCAGCCTTGTC	TCCAGACAAATCTCCTGACCG	XM_021625342.2	98.56%
IL-10	CGACTTTAAATCTCCCATCGAC	GCATTGGACGATCTCTTTCTTC	LR584431.1	98.01%
MyD88	CCCGAGAAACACTGTGGCA	TCTTCCGTGTTGGGTCCTG	HG325726.1	95.95%
TNF-α	GGTGATGGTGTCGAGGAGGAA	TGGAAAGACACCTGGCTGTA	XM_059555332.1	97.26%
NF-κB	CAGGACCGCAACATACTGGA	GCTGCTTCCTCTGTTGTTCCA	XM_036989126.1	96.87%
Occludin	CAGCCCAGTTCCTCCAGTAG	GCTCATCCAGCTCTCTGTCC	NM_001190446.1	98.68%
ZO-1	AAGGAAGGTCTGGAGGAAGG	CAGCTTGCCGTTGTAGAGG	XM_052462150.1	95.34%

RT-PCR was performed using the TaKaRa TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) Kit. The expression of *β-actin* was used as housekeeping genes. Real-time PCR assays were performed on an ABI 7500 Real-Time instrument (Applied Biosystems, Foster City, CA, USA). Three replicates of each amplification reaction were used for comparison (Table 4). Relative gene expression level was determined by the $2^{-\Delta\Delta CT}$ method [18].

Table 4. Real-time PCR amplification procedure.

Items	PCR Reaction Solution Preparation			PCR Amplification Procedure	
	Reagent	**Consumption**	**Concentration**	**Procedure**	**Instrument**
RT-PCR	TB Green Premix Ex Taq II (Tli RNaseH Plus)	10 μL	2×	Step 1: Reps: 1 95 °C 30 s Step 2: Reps: 40 95 °C 5 s 60 °C 34 s	7500 Real-TimePCR System; Applied Biosystems, Waltham, MA, USA
	ROX Reference Dye II	0.4 μL	50×		
	PCR Forward Primer	0.8 μL	10 μM		
	PCR Reverse Primer	0.8 μL	10 μM		
	cDNA	2 μL	50 ng/μL		
	DEPC H_2O	6 μL			

2.8. Intestinal Microflora Analysis

The assessment of gut microflora composition utilized the approach reported by Poolsawat et al. (2020) [11]. These samples were analyzed using 16S rRNA gene sequencing. Total DNA was extracted from 0.2 g of intestinal feces using a DNA Extraction Kit (Beijing Tiangen Biochemical Technology Co. Ltd., Beijing, China), following the manufacturer's protocol for total DNA extraction from intestinal microorganisms. DNA isolation from the extracted samples utilized the specific primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′), as described by Sun et al. (2020) [19]. PCR amplification was performed under optimized conditions: an initial denaturation at 95 °C for 5 min, followed by 27 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 45 s, with a final extension at 72 °C for 10 min. The PCR amplicons were purified using a 0.7% agarose gel, quantified with a Qubit dsDNA Broad Range Assay Kit (Life Technologies, Carlsbad, CA, USA), and pooled at an equal concentration of 20 ng/μL. Sequencing was conducted on an Illumina MiSeq platform (300 bp paired-end reads) at Shanghai Meiji Biopharmaceutical Technology Co. Ltd., Shanghai, China. The raw sequencing data were deposited in the NCBI Sequence Read Archive (SRA) database under the accession number PRJNA1093281. An Operational Taxonomic Unit (OTU) was defined based on sequence samples demonstrating more than 97% similarity within their 16S rRNA gene sequences, analyzed using the QIIME platform managed by Shanghai Meiji Biopharmaceutical Technology Co. Ltd. (Shanghai, China).

2.9. Challenge with Aeromonas salmonicida

For the challenge experiment with *A. salmonicida*, the strain used was *A. salmonicida* subsp. *salmonicida* ATCC 33658, originally isolated from a diseased salmonid. Ten fish from each tank were selected after 12 weeks of cultivation. The *A. salmonicida* culture was grown in TSB medium at 28 °C for 24 h. The optical density (OD) of the culture was measured at 600 nm using a UV-vis spectrophotometer to calculate the concentration of the bacterial suspension. Following gradient dilution, a preliminary experiment was conducted to infect triploid rainbow trout and determine the 72-h median lethal dose (LD_{50}) concentration of the bacterial strain. For the challenge, the bacterial culture was freshly agitated, and its concentration was adjusted to the LD_{50} value for infection. At the end of the cultivation period, each fish was intraperitoneally injected with 0.1 mL of *A. salmonicida* at an LD_{50} concentration of 6.8×10^8 CFU/mL. Subsequently, mortality rates were closely monitored for 72 h.

2.10. Calculations and Statistical Analysis

The assessment of growth performance in the study was carried out using the following parameters:

Survival rate (SR, %) = (total no. fish harvested/total no. fish cultured) × 100%;

Weight gain rate (WGR, %) = $((W_1 - W_2)/W_2) \times 100\%$;

Specific growth rate (SGR, %/d) = $(\ln(W_1/W_2)/D) \times 100\%$;

Feed conversion ratio (FCR) = W_3/total biomass gain (g);

Hepatosomatic index (HSI, %) = $(W_4/W_1) \times 100\%$;

Viscerasomatic index (VSI, %) = $(W_5/W_1) \times 100\%$;

Where W_1, W_2, and D mean the wet fish weight (g), initial fish weight (g), and feeding period (d), respectively. W3, W4, and W5 mean the dry feed intake (g), liver weight (g), and viscera weight (g), respectively.

Data were statistically analyzed using SPSS 22.0 (Chicago, IL, USA), and Levene's test for equality of variances was applied to assess homogeneity of variance. The data were analyzed with one-way ANOVA, and mean differences were analyzed through Tukey's multiple-range test. Images were drawn using GraphPad Prism 9.0 (Graph Pad Software, San Diego, CA, USA). Data are expressed as means ± S.E., and differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Growth Performance

The growth performance of triploid rainbow trout fed diets with different *C. butyricum* levels (Table 5). SR ranged from 89.67% to 94.33%. There were significant effects of dietary treatments on FBW, WGR, FCR, SGR, and HSI ($p < 0.05$). Notably, G4 demonstrated the highest growth performance, achieving the most efficient FCR of 1.08 ± 0.02 and the highest SGR of 1.61 ± 0.03. Furthermore, a significant difference was observed in HSI, with G2 exhibiting a larger liver size relative to body weight than the other groups ($p < 0.05$).

Table 5. The growth performance of triploid rainbow trout fed diets containing different *C. butyricum* levels.

Items	Groups				
	G1	G2	G3	G4	G5
IBW/g	260.33 ± 1.53	258.33 ± 1.53	260.00 ± 0.00	260.00 ± 1.00	260.00 ± 0.00
FBW/g	935.23 ± 19.34 ^{a}	918.23 ± 21.48 ^{a}	911.13 ± 50.50 ^{a}	1002.82 ± 12.46 ^{b}	896.35 ± 44.32 ^{a}
WGR/%	259.23 ± 7.46 ^{a}	255.46 ± 1.52 ^{a}	250.54 ± 20.15 ^{a}	286.35 ± 5.13 ^{b}	245.89 ± 17.15 ^{a}
FCR	1.19 ± 0.04 ^{ab}	1.21 ± 0.05 ^{b}	1.24 ± 0.10 ^{b}	1.08 ± 0.02 ^{a}	1.27 ± 0.09 ^{b}
SGR/%·d^{-1}	1.52 ± 0.03 ^{a}	1.51 ± 0.05 ^{a}	1.49 ± 0.10 ^{a}	1.61 ± 0.03 ^{b}	1.47 ± 0.09 ^{a}
VSI/%	13.97 ± 1.11	16.77 ± 2.02	16.01 ± 3.76	14.35 ± 1.01	15.75 ± 1.07
HSI/%	1.36 ± 0.28 ^{a}	1.97 ± 0.43 ^{b}	1.62 ± 0.21 ^{ab}	1.29 ± 0.34 ^{a}	1.34 ± 0.19 ^{a}
SR/%	93.00 ± 0.00	94.33 ± 2.31	89.67 ± 5.77	94.33 ± 2.31	94.33 ± 2.31

Note: Each value represents mean ± S.E. ($n = 3$). Values in the same row with different superscript letters are significantly different ($p < 0.05$). The details are as presented in the table below. Initial mean body weight (IBW, g); Final mean body weight (FBW, g); Survival rate (SR, %); Weight gain rate (WGR, %); Specific growth rate (SGR, %·d^{-1}); Feed conversion ratio (FCR); Viscerasomatic index (VSI, %); Hepatosomatic index (HSI, %).

3.2. Body Composition

Dietary *C. butyricum* affected the whole-body composition of triploid rainbow trout, significantly altering moisture, ash, crude lipid, and crude protein content ($p < 0.05$) (Table 6). Notably, G5, which had the highest crude protein content at 17.32 ± 1.52%, was significantly different from G1, which contained the lowest protein content at 16.32 ± 2.28 ($p < 0.05$). There were no significant differences in moisture, ash, and crude lipid among the groups ($p > 0.05$).

Table 6. Body composition of triploid rainbow trout fed diets containing different *C. butyricum* levels (%).

Groups	Moisture	Ash	Crude Lipid	Crude Protein
G1	73.13 ± 1.04	2.49 ± 0.13	8.58 ± 0.56	16.32 ± 2.28 ^{a}
G2	73.47 ± 1.43	2.36 ± 0.16	9.21 ± 1.64	16.94 ± 1.21 ^{ab}
G3	72.82 ± 1.52	2.43 ± 0.10	9.75 ± 1.60	17.05 ± 0.52 ^{ab}
G4	72.81 ± 1.26	2.45 ± 0.10	9.16 ± 0.30	16.93 ± 1.09 ^{ab}
G5	73.77 ± 0.83	2.37 ± 0.07	8.40 ± 0.12	17.32 ± 1.52 ^{b}

Note: Value represents mean ± S.E. ($n = 3$). Values in the same row with different superscript letters are significantly different ($p < 0.05$).

3.3. Digestive Enzyme

There were significant effects of dietary *C. butyricum* on lipase, protease, and amylase activities among the groups ($p < 0.05$) (Table 7). Lipase activity showed a rise, ranging from G1 at 42.79 ± 8.96 U/mgprot to peak in G4 at 126.55 ± 27.90 U/mgprot. Similarly, protease activity showed significant variations, with G1 showing minimal activity at 940.23 ± 151.04 U/mgprot, and G4 showing maximal at 1310.29 ± 233.30 U/mgprot. Conversely, amylase activity exhibited a decreasing trend, starting at 2.51 ± 0.05 U/mg protein in both G1 and G3 and reaching its lowest at 1.59 ± 0.03 U/mg protein in G5.

Table 7. Effects of dietary *C. butyricum* supplementation on digestive enzyme activity of triploid rainbow trout.

Groups	Lipase (U/mgprot)	Protease (U/mgprot)	Amylase (U/mgprot)
G1	42.79 ± 8.96 ^{a}	940.23 ± 151.04 ^{a}	2.51 ± 0.05 ^{b}
G2	83.55 ± 10.22 ^{b}	1015.49 ± 147.98 ^{ab}	2.22 ± 0.04 ^{b}
G3	79.12 ± 9.29 ^{b}	1224.72 ± 272.36 ^{bc}	2.51 ± 0.04 ^{b}
G4	126.55 ± 27.90 ^{c}	1310.29 ± 233.30 ^{c}	1.73 ± 0.04 ^{a}
G5	117.20 ± 30.58 ^{c}	945.00 ± 85.02 ^{a}	1.59 ± 0.03 ^{a}

Note: Value represents mean ± S.E. (n = 3). Values in the same row with different superscript letters are significantly different ($p < 0.05$).

3.4. Antioxidant Capacity and Immune Response

There were significant improvements in liver antioxidative capacity among the groups (Table 8) ($p < 0.05$). SOD activity increased significantly, peaking in G4 and G5.

Table 8. The antioxidant capacity of triploid rainbow trout fed diets containing different *C. butyricum* levels.

Groups	SOD (U/mL)	MDA (nmoL/mL)	CAT (U/mL)
Serum			
G1	16.40 ± 2.23 ^{a}	21.67 ± 2.40 ^{c}	13.96 ± 1.24 ^{a}
G2	21.46 ± 2.71 ^{a}	17.52 ± 0.79 ^{b}	17.63 ± 1.71 ^{ab}
G3	30.37 ± 6.09 ^{b}	15.15 ± 1.17 ^{b}	20.74 ± 3.83 ^{b}
G4	41.48 ± 10.75 ^{c}	10.11 ± 1.14 ^{a}	24.46 ± 1.81 ^{c}
G5	20.72 ± 3.97 ^{a}	8.48 ± 0.97 ^{a}	15.81 ± 2.93 ^{ab}
Liver			
G1	226.26 ± 6.46 ^{a}	11.36 ± 0.22 ^{c}	12.17 ± 0.46 ^{a}
G2	229.80 ± 7.90 ^{a}	10.77 ± 0.89 ^{c}	12.31 ± 0.40 ^{a}
G3	329.85 ± 11.49 ^{b}	8.45 ± 0.34 ^{b}	16.90 ± 0.99 ^{b}
G4	356.41 ± 8.31 ^{c}	7.44 ± 0.54 ^{a}	18.53 ± 1.79 ^{c}
G5	355.39 ± 3.29 ^{c}	7.02 ± 0.66 ^{a}	18.45 ± 0.78 ^{c}

Note: Value represents mean ± S.E. (n = 3). Values in the same row with different superscript letters are significantly different ($p < 0.05$). SOD, superoxide dismutase; MDA, malondialdehyde; CAT, catalase.

There were significant differences in serum antioxidative and oxidative stress markers among the groups ($p < 0.05$). SOD activity exhibited an increase from G1 at 16.40 ± 2.23 U/mL to the highest in G4 at 41.48 ± 10.75 U/mL. MDA levels showed a decrease from G1 to the lowest in G5 at 8.48 ± 0.97 nmoL/mL. Additionally, CAT activity peaked in G4 at 24.46 ± 1.81 U/mL.

Moreover, different levels of *C. butyricum* in the diet significantly impacted the activities of LZM and IgM in serum ($p < 0.05$), with peak activities observed in G4 (Figure 1).

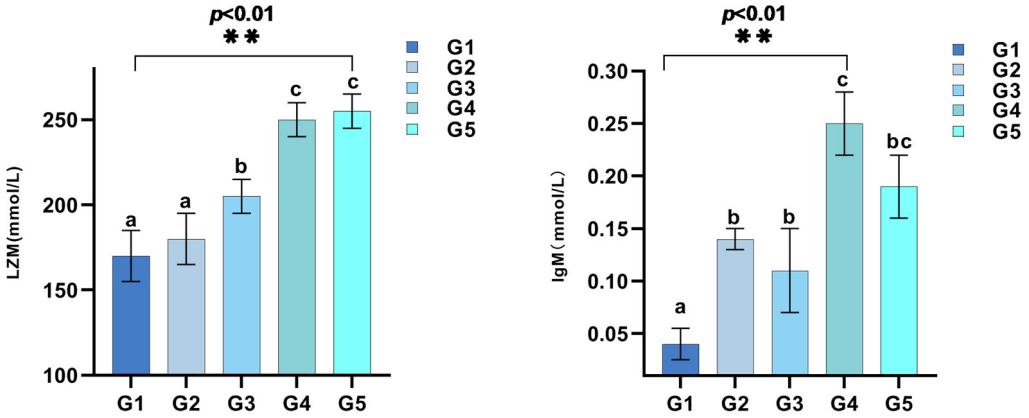

Figure 1. Lysozyme (LZM) and immunoglobulin M (IgM) responses in triploid rainbow trout by dietary *C. butyricum* supplementation (n = 3). Values in the columns with different superscript letters are significantly different ($p < 0.05$). ** indicates $p < 0.01$, representing statistically significant differences.

3.5. Serum Metabolic Markers

There were significant differences in serum AST levels among the groups ($p < 0.05$) (Table 9), with G5 showing the lowest AST at 360.65 ± 70.15 U/L and G2 showing the highest at 400.18 ± 80.36 U/L. Despite the differences in GLU, TP, ALB, ALT, and TG, these variations did not exhibit significant changes among the groups ($p > 0.05$).

Table 9. The serum indices of triploid rainbow trout fed diets containing different *C. butyricum* levels.

Groups	GLU (mmol/L)	TP (g/L)	ALB (g/L)	ALT (U/mL)	AST (U/mL)	TG (mmol/L)
G1	5.65 ± 0.95	27.55 ± 6.54	11.20 ± 1.58	17.25 ± 3.34	360.65 ± 70.15 ^{a}	4.95 ± 2.61
G2	5.40 ± 0.67	30.80 ± 4.17	11.70 ± 1.52	18.15 ± 4.52	400.18 ± 80.36 ^{b}	5.35 ± 1.46
G3	5.57 ± 0.78	30.35 ± 3.14	11.75 ± 1.63	18.85 ± 3.22	395.82 ± 15.92 ^{b}	5.28 ± 2.44
G4	5.70 ± 0.75	29.15 ± 3.18	11.52 ± 1.37	18.60 ± 1.85	385.89 ± 83.98 ^{ab}	5.15 ± 2.05
G5	5.82 ± 1.22	29.35 ± 8.21	11.80 ± 2.55	18.30 ± 2.38	397.25 ± 47.92 ^{b}	4.82 ± 2.20

Note: Value represents mean ± S.E. (n = 3). Values in the same row with different superscript letters are significantly different ($p < 0.05$). GLU, glucose; TP, total protein; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TG, triglycerides.

3.6. Relative Gene Expression in the Intestine

As shown in Figures 2 and 3, supplementation with different levels of *C. butyricum* significantly affected the expression of intestinal immune-related genes in triploid rainbow trout, such as interleukins (*IL-1β*, *IL-8*, *IL-10*), *NF-κB*, and *TNF-α* ($p < 0.05$). Notably, *IL-1β* expression reached its peak in G3, with no significant differences between other groups and the control group ($p > 0.05$). The expression levels of *IL-8*, *NF-κB*, and *MyD88* initially increased and then decreased as *C. butyricum* levels increased, with the peak expressions observed in G3. In contrast, *IL-10* expression progressively rose with increasing *C. butyricum* level, significantly higher than in the control group ($p < 0.05$). *TNF-α* expression, however, exhibited a declining trend. Meanwhile, *IL-2* expression remained consistent ($p > 0.05$), although a gradual decrease was observed.

Intestinal *occludin* expression levels revealed no significant disparities between groups G1 through G4 and a minor enhancement in G5 (Figure 4). The subsequent graph demonstrates an increased trend in *ZO-1* expression from G1 to G4 ($p < 0.05$).

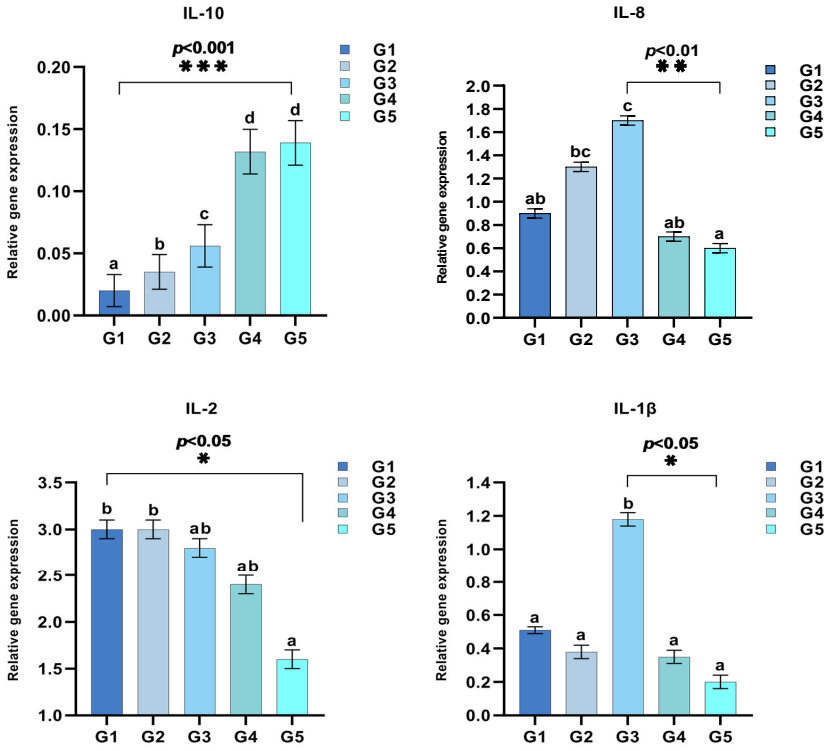

Figure 2. Modulation of interleukin profiles in triploid rainbow trout by dietary *C. butyricum* supplementation (n = 3). Values in the columns with different superscript letters are significantly different ($p < 0.05$). * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$, representing statistically significant differences.

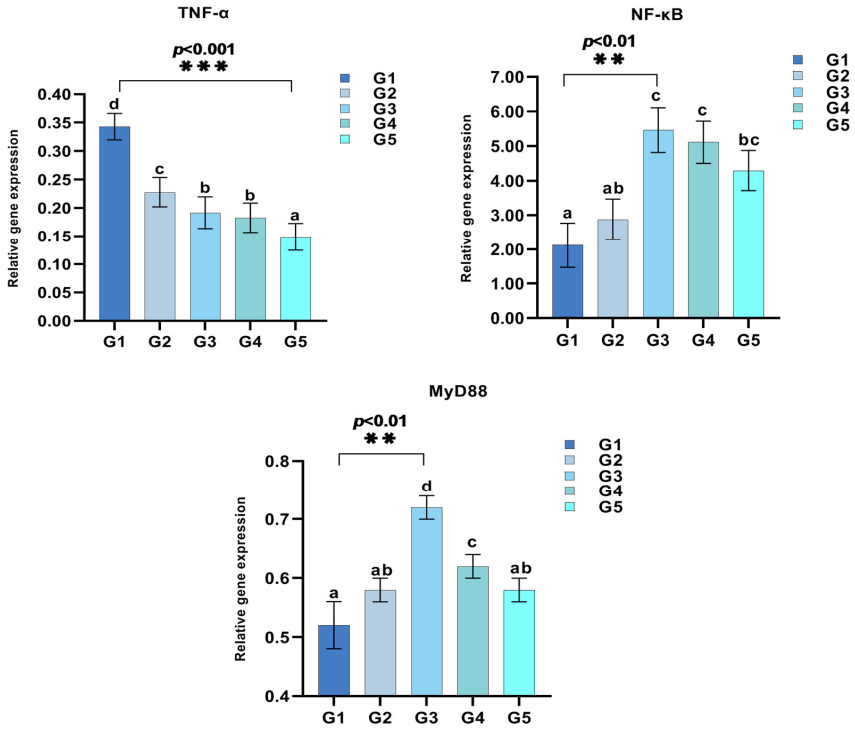

Figure 3. Impact of *C. butyricum* supplementation on key inflammatory gene expression in triploid rainbow trout (n = 3). Values in the columns with different superscript letters are significantly different ($p < 0.05$). ** indicates $p < 0.01$, and *** indicates $p < 0.001$, representing statistically significant differences.

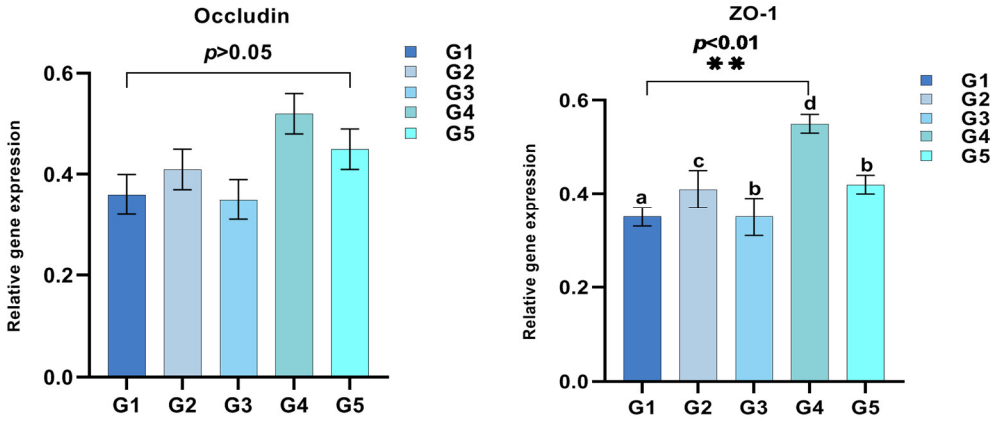

Figure 4. Effects of different concentrations of *C. butyricum* on triploid rainbow trout intestinal *occludin* and *ZO-1* expression (n = 3). ** indicates $p < 0.01$, representing statistically significant differences.

3.7. Intestinal Microflora

The addition of different levels of *C. butyricum* significantly impacted the gut microflora of triploid rainbow trout among the groups, as depicted in a Venn diagram (Figure 5). A core microbiome of 121 operational taxonomic units (OTUs) common to all groups suggests a stable microbial community across different supplementation levels. Despite different *C. butyricum* levels, there was no significant impact on the Chao1, ACE, Shannon, and Simpson diversity indices (Table 10).

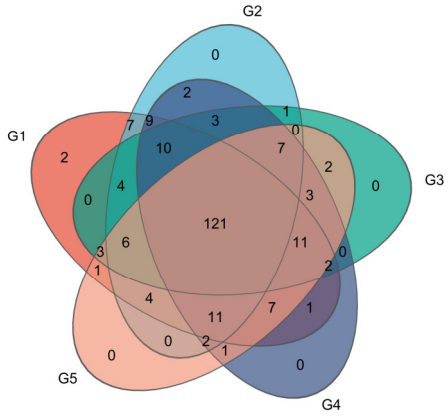

Figure 5. Venn diagram of shared and unique operational taxonomic units (OTUs) among triploid rainbow trout gut microflora subjected to different levels of *C. butyricum* supplementation (n = 3).

Table 10. The intestinal microflora diversity index of triploid rainbow trout fed diets containing different *C. butyricum* levels.

Groups	Chao1	ACE	Shannon	Simpson
G1	118.79 ± 19.04	120.20 ± 20.17	1.34 ± 0.24	0.49 ± 0.23
G2	125.56 ± 17.67	122.18 ± 15.16	1.19 ± 0.13	0.57 ± 0.06
G3	115.14 ± 22.18	128.15 ± 10.50	1.28 ± 0.39	0.58 ± 0.20
G4	121.91 ± 20.94	122.23 ± 19.10	1.14 ± 0.24	0.60 ± 0.12
G5	131.00 ± 34.66	123.41 ± 33.74	1.26 ± 0.30	0.58 ± 0.09

Note: Value represents mean ± S.E. (n = 3).

Barplot analyses showed the predominance of Proteobacteria and Firmicutes among all groups, with fluctuations in Firmicutes abundance (Figure 6). The presence of Actinobacteriota, Cyanobacteria, and other minor phyla contributed to microbial diversity. At the genus level, it illustrates a diverse bacterial community with genera like *Burkholderia*–

Caballeronia–Paraburkholderia, *Pseudomonas*, and *Clostridium* present in different proportions across groups G1 to G5.

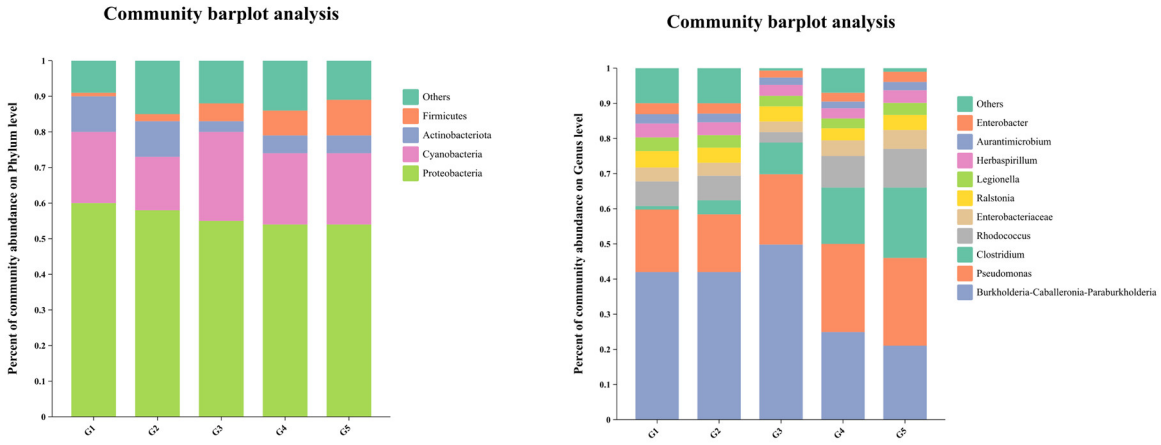

Figure 6. Comparative analysis of microbial community functions on phylum and genus level in triploid rainbow trout: the role of *C. butyricum* in low-fish meal diets (n = 3).

Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) showed distinct microbial profiles among the groups, with G1 showing a unique composition (Figure 7). This separation shows the potential of *C. butyricum* supplementation to modulate gut microflora at the genus level, with G4 exhibiting the greatest variance post-supplementation. Principal Coordinates Analysis (PCoA) and Non-metric Multidimensional Scaling (NMDS) revealed that *C. butyricum* supplementation subtly alters gut microflora without causing significant overall structural changes (PCoA: PC1, 21.09%, PC2, 11.23%, R = 0.0290, p = 0.327; NMDS stress: 0.188) (Figure 8). G1 presented a unique microbial profile, while G2, G3, and G4 showed overlapping clusters, and G5 showed greater variability.

The COG functional classification revealed that the supplementation levels might influence the gut microbiome's functional potential, with categories related to carbohydrate transport and metabolism, translation, ribosomal structure and biogenesis, and amino acid transport and metabolism being significantly affected (Figure 9). This suggests that *C. butyricum* not only alters microbial composition but also affects the functional capabilities of the trout's gut microflora.

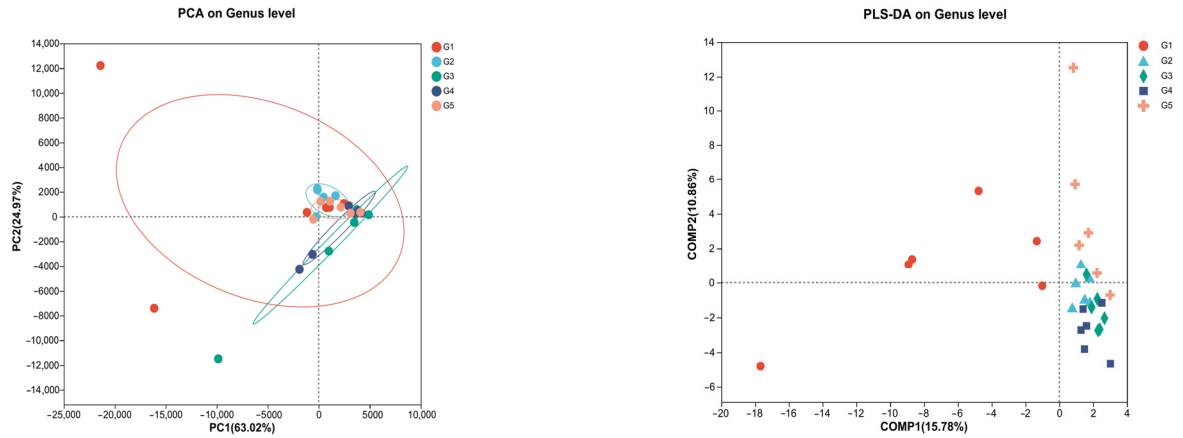

Figure 7. Principal component analysis (PCA) and Partial least squares discriminant analysis (PLS-DA) on genus level of triploid rainbow trout gut microflora response to *C. butyricum* supplementation (n = 3).

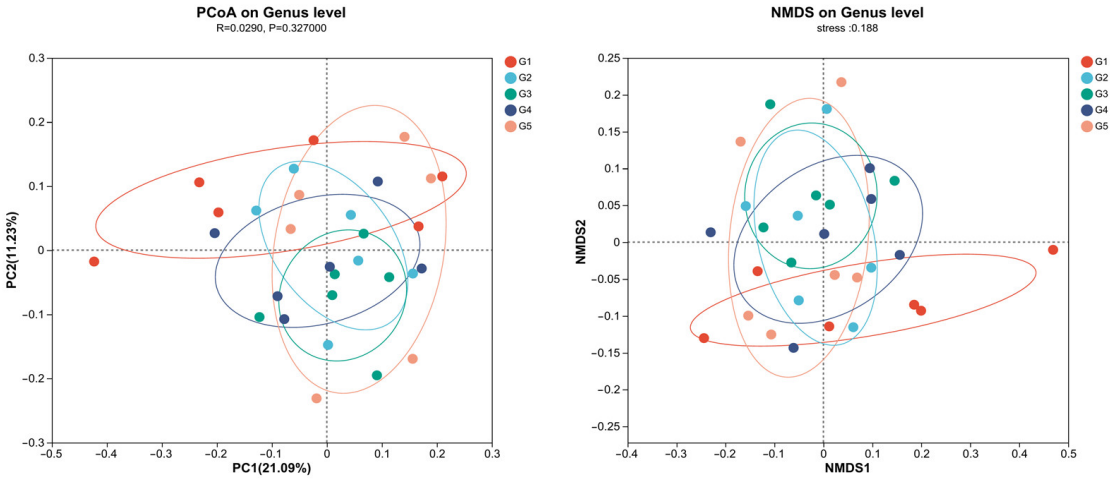

Figure 8. Principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS) analysis: evaluating the *C. butyricum* impact on triploid rainbow trout microflora composition (n = 3).

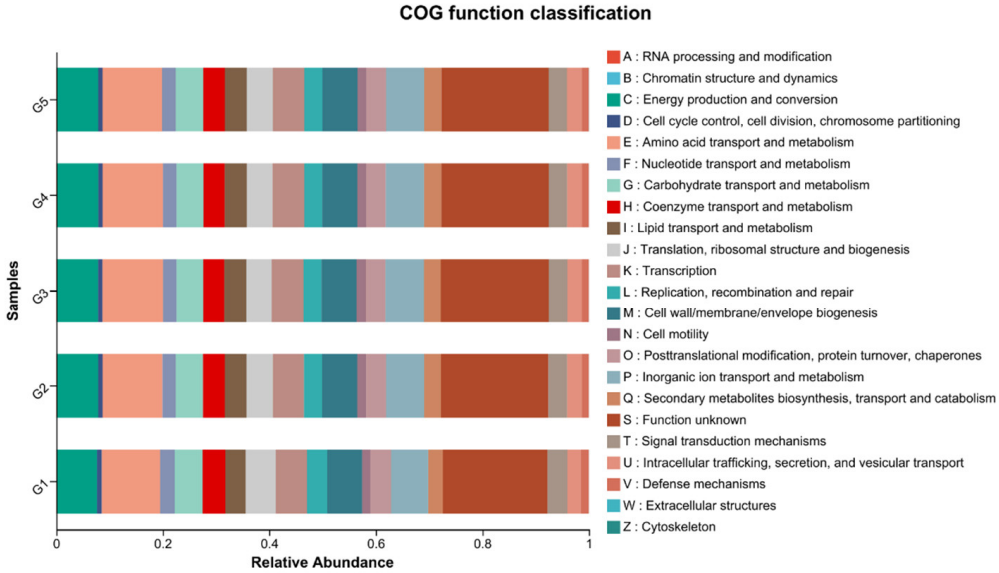

Figure 9. Cluster of orthologous groups (COG) functional classification in triploid rainbow trout microflora influenced by *C. butyricum* supplementation (n = 3).

3.8. Challenge with A. salmonicida

The graph presents the SR of triploid rainbow trout over 72 h after being exposed to *A. salmonicida*, following 12 weeks of cultivation with different levels of *C. butyricum* supplementation (Figure 10). Initially, all groups exhibited an SR of 100%. However, with time, G3 and G1 showed earlier reductions in survival, with G3 experiencing a more apparent decline. By the conclusion of the 72-hour observation period, G4 recorded the highest SR (66.67%). Similarly, G5 and G3 showed higher SR, with G5 achieving 63.33% and G3 achieving 60.00%. G1 and G2 exhibited SR of 50% and 53.33%, respectively.

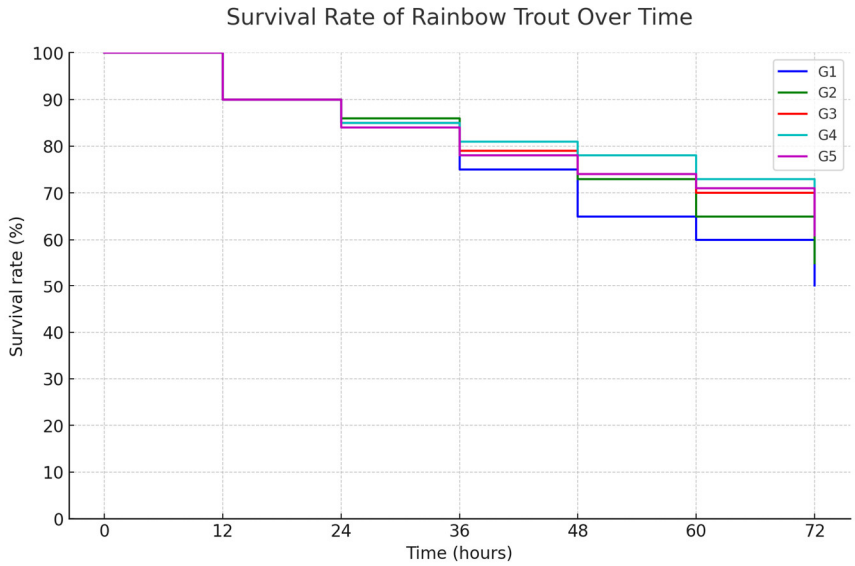

Figure 10. Survival rate of triploid rainbow trout over time post-*A. salmonicida* infection with different levels of *C. butyricum*.

4. Discussion

4.1. Growth Performance

C. butyricum, as a feed additive, has been shown to significantly enhance growth performance, feed utilization rates and intestinal health in a variety of fish species (Table 11), including croaker (*Miichthys miiuy*) [20], tilapia [11], large yellow croaker (*Larimichthys crocea*) [21], pompano (*Trachinotus ovatus*) [12], and carp [13]. Similarly, findings by Lan (2019) indicated that *C. butyricum*, could improve weight gain in European eel (*Anguilla anguilla*), with insignificant effects on HSI and VSI [22]. These align with our results, showing that as dietary levels of *C. butyricum* increased, so did the WGR and SGR in triploid rainbow trout, with the lowest FCR observed at a supplementation concentration of 1×10^8 CFU/g. The presence of antinutritional factors in high soybean meal proportions could reduce nutrient absorption and utilization in triploid rainbow trout. However, our study found that adding *C. butyricum* to low-fish meal diets can counteract the growth-inhibitory effects of soybean meal and enhance feed efficiency to some extent. These findings suggest that *C. butyricum* facilitates better nutrient utilization and feed efficiency, potentially through the production of short-chain fatty acids (SCFAs) like butyrate, which enhance digestive processes and reduce gut inflammation. The production of SCFAs in the intestine is primarily by dietary *C. butyricum* supplementation, butyrate, and acetate. Butyrate serves as a fundamental energy source for epithelial cell growth and can release H^+ into the neutral cytoplasm to reduce intracellular pH, potentially utilizing the remaining H^+ in metabolism to lower cell mortality rates [23]. This process aids in maintaining epithelial tissue integrity, facilitating nutrient absorption. Additionally, butyrate may enhance feed utilization by balancing the gut's microecological environment [24].

4.2. Body Composition

In our study, the whole-body composition of triploid rainbow trout showed no significant differences in moisture, crude lipid, and ash content, with an increase observed in crude protein levels. This indicates that *C. butyricum* supplementation is beneficial for enhancing protein deposition in triploid rainbow trout. Research by Huang (2017) found that juvenile tiger grouper (*Epinephelus fuscoguttatus* × *E. lanceolatus*) fed diets containing *C. butyricum* exhibited increased crude protein content without affecting ash [25]. Within the intestines, *C. butyricum* engages in anaerobic fermentation, producing specific metabolites such as butyrate, which is known to enhance intestinal health by strengthening the gut barrier and modulating immune responses. Additionally, the enzymes and amino acids

released by *C. butyricum* help in the efficient breakdown and assimilation of proteins, supporting muscle growth and protein deposition in fish. This direct interaction of butyrate and enzymatic activity not only corroborates the growth-promoting effects of *C. butyricum* but also shows its potential to improve overall fish health and feed efficiency.

Table 11. Comparative growth metrics of various fish species administered with *C. butyricum* as a feed additive.

Species	Dose (CFU/g)	SGR (%/day)	FCR	SR (%)
Croaker (*Miichthys miiuy*) [20]	Control: Basal diet, CB1: 10^3, CB2: 10^5, CB3: 10^7, CB4: 10^9	Control–CB4: 0.31 ± 0.01 ^{b}, 0.43 ± 0.03 ^{ab}, 0.45± 0.10 ^{ab}, 0.55 ± 0.17 ^{ab}, 0.58 ± 0.09 ^{a}	Control–CB4: 3.40 ± 0.25^{b}, 2.64 ± 0.11 ^{ab}, 2.93 ± 0.93 ^{ab}, 2.18 ± 0.68 ^{ab}, 1.89 ± 0.39 ^{a}	All: 100
Tilapia (*Oreochromis niloticus* × *O. aureus*) [11]	Control, C-1: 0.75 × 10^8, C-2: 1.5 × 10^8, C-3: 3 × 10^8, C-4: 6 × 10^8, C-5: 1.2 × 10^9	Control–C-5: 3.54 ± 0.02 ^{d}, 3.56 ± 0.02 ^{cd}, 3.65 ± 0.03 ^{ab}, 3.69 ± 0.05 ^{a}, 3.63 ± 0.03 ^{abc}, 3.59 ± 0.03 ^{bcd}	Control–C-5: 1.14 ± 0.02 ^{d}, 1.12 ± 0.01 ^{cd}, 1.06 ± 0.02 ^{a}, 1.03 ± 0.03 ^{a}, 1.07 ± 0.02 ^{abc}, 1.10 ± 0.02 ^{bcd}	All: 100
Large yellow croaker (*Larimichthys crocea*) [21]	Control: (0.00%), CB1: 5 × 10^6, CB2: 1 × 10^7, CB3: 2 × 10^7	Control–CB3: 9.95 ± 0.10 ^{b}, 10.92 ± 0.09 ^{a}, 10.60 ± 0.21, 10.60 ± 0.17 ^{ab}	-	All: Control: 15.26 ± 1.87; CB1: 23.20 ± 2.60; CB2: 20.35 ± 1.77; CB3: 19.83 ± 2.05
Pompano (*Trachinotus ovatus*) [12]	C1: 0, C2: 2.5 × 10^6, C3: 5 × 10^6, 1 × 10^7, C4: 2 × 10^7, C5: 4 × 10^7	C1–C5: 3.54 ± 0.02 ^{b}, 3.56 ± 0.04 ^{abc}, 3.57 ± 0.05 ^{abc}, 3.59 ± 0.02 ^{bc}, 3.62 ± 0.03 ^{c}, 3.53 ± 0.02 ^{ab}	C1–C5: 1.47 ± 0.01, 1.49 ± 0.04, 1.54 ± 0.02, 1.50 ± 0.03, 1.49 ± 0.06, 1.50 ± 0.04	C1–C4: 100, C5: 98.67 ± 2.31
Carp (*Cyprinus carpio*) [13]	C0: 0, C1: 3 × 10^7, C2: 3 × 10^8, C3: 3 × 10^9	C0–C3: 4.10 ± 0.08 ^{ab}, 4.23 ± 0.08 ^{a}, 4.17 ± 0.06 ^{ab}, 4.08 ± 0.07 ^{b}	C0–C3: 1.53 ± 0.04 ^{ab}, 1.47 ± 0.04 ^{b}, 1.51 ± 0.03 ^{ab}, 1.52 ± 0.04 ^{ab}	All: 100

Note: Value represents mean ± S.E. Values in the same row with different superscript letters are significantly different ($p < 0.05$).

4.3. Digestive Enzyme

In this study, as the level of *C. butyricum* increased, the activities of lipase and protease in the intestines of triploid rainbow trout significantly rose compared to the control group, reaching their peak at a concentration of 1.1 × 10^8 CFU/g, while the activity of α-amylase significantly decreased. Similar results were observed in studies on *Cherax quadricarinatus*, where the lipase and amylase activities in the *C. butyricum* treatment group significantly increased by 165.84% and 18.75%, respectively, compared to the control group [26]. Additionally, a feeding trial with juvenile hybrid grouper (*Epinephelus fuscoguttatus* × *E. lanceolatus*) also found that diets supplemented with *C. butyricum* not only enhanced gastric digestive enzyme activities but also increased intestinal trypsin, lipase, and amylase activities [27]. These findings suggest the need for detailed dose-response studies to identify the optimal concentrations of *C. butyricum* that maximize digestive efficiency without compromising the digestive processes. This could involve systematic variations of probiotic dosages in feeding trials to map out a full spectrum of enzymatic responses and establish a

threshold beyond which no further benefits are observed, or adverse effects may begin to appear.

4.4. Antioxidant Capacity

The butyrate produced by *C. butyricum* plays a crucial role in maintaining the antioxidant capacity of the colonic epithelium. Dietary *C. butyricum* and *Bacillus* coagulans in rainbow trout diets have been shown to effectively increase serum total antioxidant capacity T-AOC and SOD activity while reducing MDA content, whether used singly or in combination [28]. In this study, increasing levels of *C. butyricum* enhanced the antioxidant capacity in the serum and liver of triploid rainbow trout. Studies on tilapia also found that dietary *C. butyricum* increased CAT, SOD, and hemoglobin activities, enhancing resistance to *A. hydrophila* infection [29]. Hasan et al. (2018) found elevated SOD activity in flounder (*Paralichthys olivaceus*) after probiotic supplementation [30]. Likewise, probiotics have been shown to boost SOD and CAT activities in various species, such as Japanese eel (*Anguilla japonica*) [31] and grouper (*Epinephelus coioides*) [32]. Thus, *C. butyricum*, as a probiotic, can enhance the antioxidant capacity of aquatic animals, therefore improving their disease resistance. Excessive oxidative stress and inflammatory responses can lead to the oxidation of lipids and proteins, with oxidized low-density lipoprotein (LDL) being internalized by macrophages to form foam cells, stimulating the production of pathogenic factors [33]. The antioxidant effects of *C. butyricum* may relate to the butyrate and NADH produced, potentially clearing reactive oxygen species (ROS) through NADPH generation, thus reducing oxidative stress [34].

4.5. Serum Metabolites

Feeding aquatic animals with diets substituting fish meal with plant proteins introduces antinutritional factors like protease inhibitors, phytic acid, and gossypol from plant proteins into the animal's tissues via intestinal digestion and absorption, leading to stress responses that can alter serum biochemistry profiles. These changes include disruptions in protein digestion, mineral deficiencies, and increased oxidative stress markers, highlighting the critical need for optimized diet formulations to mitigate these adverse effects in aquaculture practices. Studies, such as those by Abdel-Tawwab et al. (2021), have shown no significant differences in AST and ALT activities when *Macrobrachium rosenbergii* was fed diets supplemented with *C. butyricum* [35]. In our study, an increase in the level of *C. butyricum* supplementation was associated with a gradual decrease in AST levels. Similarly, in carp [13], serum AST and ALT activity decreased, indicating an improvement in fish health with *C. butyricum*-enriched diets. The reduction in transaminase levels with higher *C. butyricum* supplementation suggests that adding *C. butyricum* to diets replacing fish meal with soybean meal can provide some protection against liver damage caused by plant proteins in triploid rainbow trout.

4.6. Immunity

C. butyricum, specifically, can regulate innate and adaptive immunity, enhancing the animal's defense against microbes and maintaining immunological homeostasis [36]. Immunomodulation and suppression of excessive immune responses and inflammation are considered mechanisms through which probiotics exert therapeutic effects [37]. Experiments by Xiao et al. (2019) have shown that *C. butyricum* can lower *IL-8* expression and increase *IL-10* expression in yellow catfish (*Pelteobagrus fulvidraco*) suffering from acute pancreatitis [38]. In our study, the addition of *C. butyricum* significantly influenced the expression of intestinal genes *IL-1β*, *IL-8*, *IL-10*, *MyD88*, *NF-κB*, and *TNF-α* in triploid rainbow trout. This suggests that *C. butyricum* not only activates essential immune pathways but also regulates inflammatory responses, which is crucial for maintaining immune homeostasis and improving disease resistance in triploid rainbow trout. The immune system is activated upon exposure to adverse factors, with various cytokines being moderately expressed to protect the organism; however, excessive immune responses can lead to inflammation. The

addition of *C. butyricum* in this study not only promoted the expression of pro-inflammatory cytokines but also anti-inflammatory cytokines, regulating pathways related to cytokine expression and modulating the immune response in triploid rainbow trout. Treatment with *C. butyricum* in mice with ulcerative colitis reduced the expression of cytokines such as *IL-1β*, *TNF-α*, and *IL-6* [39]. Feeding carp with lactobacillus strains increased the expression of pro-inflammatory (*TNF-α*, *IL-1β*, *IL-6*, *IL-12*) and anti-inflammatory (*IL-10*) cytokine genes [40], enhancing *IL-8* gene expression in grouper (*Epinephelus coioides*) intestines [41] and *TNF-α* expression in the small intestine of hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) [42]. This suggests that probiotics can induce a sensitizing effect in the host by increasing pro-inflammatory cytokines (like *IL-8*, *IL-6*, and *TNF-α*) and provide beneficial effects through the synthesis of anti-inflammatory cytokines (like *IL-10*).

After being fed probiotics, fish showed changes in the secretion of IgM and lysozyme (LZM). Gong et al. (2019) found that *Pediococcus pentosaceus*, a type of lactic acid bacteria, could increase the expression of IgM and complement protein C3 genes in the liver and spleen of grass carp (*Ctenopharyngodon idellus*) [43]. In this study, as the level of *C. butyricum* in the diet increased, serum LZM activity gradually rose, with G4 and G5 groups significantly higher than the control group; serum IgM content also gradually increased, peaking in the G4 group. This aligns with the findings of Song et al. (2006), who observed increased serum LZM activity and IgM content in *Lateolabrax japonicus* fed with *C. butyricum* [20]. This may be due to *C. butyricum* or its products activating the immune system, producing various immune factors, and stimulating LZM and IgM [44], therefore enhancing the immune function of triploid rainbow trout, protecting body tissues from damage, and maintaining health.

This observation implies enhanced pathogen resistance, likely due to the positive impact of *C. butyricum* on gut health and the subsequent strengthening of the immune response [32]. G5 also showed a higher SR, suggesting advantageous effects at this specific level of supplementation. These findings emphasize the critical role of optimizing *C. butyricum* supplementation levels to improve resistance against infections in triploid rainbow trout, proposing that an appropriate supplementation approach could serve as an effective strategy to improve the health and survivability of triploid rainbow trout raised on diets low in fish meal.

4.7. Intestinal Barrier

As the level of *C. butyricum* increases, there is an upregulation in the expression of the *occludin* and *ZO-1* genes. This protective role of *C. butyricum* in enhancing intestinal barrier function via the modulation of tight junction (TJ) proteins is proved by previous research. For example, Fu et al. (2023) have shown that butyrate, a short-chain fatty acid produced by *C. butyricum*, strengthens the intestinal barrier by elevating the expression of TJ proteins such as *occludin* and *ZO-1*, thus reducing inflammation and epithelial damage [45]. Similarly, Peng et al. (2009) found that butyrate improves intestinal barrier function through a mechanism that includes the upregulation of TJ proteins, highlighting the critical role of microbial metabolites in maintaining gut health [46]. Moreover, the activity of *C. butyricum* in boosting the expression of *occludin* and *ZO-1* suggests a viable mechanism for its positive effects in preventing and alleviating gastrointestinal disorders, such as inflammatory bowel disease (IBD), where compromised barrier function is a key issue. Bertiaux-Vandaële et al. (2011) noted that disturbances in TJ protein expression are linked to increased intestinal permeability, a characteristic feature of IBD pathology, highlighting the therapeutic value of adjusting TJ protein expression via probiotic supplementation [47].

4.8. Intestinal Microflora

The resilience of *C. butyricum* to gastric acids and temperature variations enables it to reach the intestines unaffected by stomach acids and various digestive enzymes. This characteristic aids in understanding its role in modulating gut microflora abundance [48]. The alpha diversity indices, including Chao1 and ACE, indicate microbial abundance, while

Shannon and Simpson indices represent community diversity, with higher Shannon values indicating greater diversity and higher Simpson values indicating lower diversity [49]. Our results suggest that Chao1 and ACE indices show no significant difference among the groups. Conversely, Shannon and Simpson indices suggest that high levels of *C. butyricum* may reduce microbial diversity. This could be due to *C. butyricum*'s inhibitory effect on harmful bacteria, reducing the diversity by lowering the survival chances of harmful microbes [50]. Additionally, the presence of categories related to inorganic ion transport and metabolism and coenzyme transport and metabolism further highlights the diverse metabolic capabilities of the trout microflora under different treatment conditions. Consistent with previous research, these findings support the concept that gut microflora plays a crucial role in influencing nutrient utilization and energy harvesting, significantly contributing to the improved growth performance and health status of fish under low-fish-meal dietary conditions [51].

5. Conclusions

This study demonstrates that supplementing a low fishmeal diet with *C. butyricum* (1.1×10^8 CFU/g) significantly increases growth and gut health in triploid rainbow trout. The probiotic *C. butyricum* boosts populations of beneficial bacteria such as Firmicutes, enhancing digestive enzyme activities and antioxidant defenses, resulting in improved nutrient utilization and reduced oxidative stress. Furthermore, this supplement strengthens immune defenses by regulating key immune genes and increasing serum immunoglobulin levels, therefore enhancing resistance to *A. salmonicida*. These benefits suggest that *C. butyricum* could be a valuable dietary supplement in aquaculture, promoting sustainable growth, improved gut health, and disease resistance. Integrating *C. butyricum* could also offer economic advantages by reducing the use of fishmeal, the most expensive component of fish feed, potentially leading to significant cost savings in commercial aquaculture. Future studies should explore the effects of this probiotic across different species and aquacultural environments, and an economic analysis might illuminate its financial viability.

Author Contributions: Conceptualization, C.W.; methodology, C.W.; resources, H.J. and S.H.; data curation, C.W.; writing—original draft preparation, F.L.; writing—review and editing, F.L., S.L. and C.W.; visualization, D.W., Z.L. and W.G.; supervision, C.W. and H.L.; project administration, C.W. and H.L.; funding acquisition, C.W. and H.L. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the National Key Research and Development Program of China (2023YFD2400400-5), the China Agriculture Research System of MOF and MARA (CARS-46), the Key Scientific Research Project of Heilongjiang Province (JD22A017), the Natural Science fund of Heilongjiang Province (LH2023C057), the Central Public-interest Scientific Institution Basal Research Fund, CAFS (2023TD60; 2023TD96; HSY202202M), and the National Postdoctoral Fund (2022MD713817).

Institutional Review Board Statement: The animal study was reviewed and approved by the Committee for the Welfare and Ethics of the Laboratory Animals of Heilongjiang River Fisheries Research Institute, CAFS (20200615). Written informed consent was obtained from the owners for the participation of their animals in this study.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article, and further inquiries can be directed to the corresponding authors.

Acknowledgments: The authors thank the participants who gave their time to the trial.

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

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