Hindawi
Aquaculture Nutrition
Volume 2022, Article ID 6965174, 11 pages
https://doi.org/10.1155/2022/6965174

Research Article

Dietary *Clostridium butyricum* Improves Growth Performance and Resistance to Ammonia Stress in Yellow Catfish (*Pelteobagrus fulvidraco*)

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Received 28 March 2022; Revised 6 May 2022; Accepted 25 May 2022; Published 11 June 2022

Academic Editor: Erchao Li

The effects of dietary *Clostridium butyricum* (CB) on growth performance, intestinal morphology, tight junction proteins, and immune-related gene mRNA levels in *Pelteobagrus fulvidraco* were investigated. The fish were fed with diets containing 0 (control, CB0), 4.8×10^6 (CB1), 4.5×10^7 (CB2), 5.1×10^8 (CB3), and 3.6×10^9 (CB4) CFU/kg *Clostridium butyricum* for 56 days followed by a 72 h ammonia challenge. The results showed that significantly higher final weight, specific growth rate, body length, and intestinal weight were observed in fish fed with CB diets ($P < 0.05$). The fish fed with CB1, CB2, and CB3 diets had significantly higher intestinal length, propionic acid concentration, and alkaline phosphatase activity and significantly lower feed conversion ratio than those in CB0 ($P < 0.05$). Significantly higher concentrations of butyric acid and valeric acid and significantly lower malondialdehyde content were observed in CB4 than in CB0 ($P < 0.05$). Intestosomatic index, villus length, villus width, intestinal protease, Na^+/K^+-ATPase, and creatine kinase activities were significantly increased in CB2 or CB3 than in CB0 ($P < 0.05$). Fish in CB2 or CB3 had significantly lower content of interleukin 1β and interleukin 6 and relative expression of *interleukin 1* (*Il-1*), *interleukin 8 (Il-8*), and *nuclear transcription factor-κB* (*Nf-κb*) compared to that in CB0 ($P < 0.05$). Dietary CB significantly decreased the relative expression of myosin light chain kinase (*Mlck*) ($P < 0.05$). Significantly higher relative expressions of *claudin-1*, *zonula occludens protein-1*, and *occludin* were observed in CB2, CB3, and CB4 compared to CB0 ($P < 0.05$). Fish in CB0 had higher CMR than that in CB2, CB3, and CB4 under ammonia nitrogen stress for 48 and 72 h ($P < 0.05$). Dietary *Clostridium butyricum* improved growth performance and resistance to ammonia stress in yellow catfish by increasing intestinal short-chain fatty acid (SCFA) productions, upregulating genes encoding tight junction proteins, downregulating transcription of proinflammatory factors *Il-1* and *Il-8*, and inhibiting the Mlck/Nf-κb signaling pathway.

1. Introduction

The intestine is the first line of defense against the invasion of pathogens and harmful substances from the external environment [1]. Being continuously exposed to foreign substances including microbes, pathogens, and other toxic substances from food, the intestine is sensitive to environmental stress [2]. Therefore, intestinal development, immune response, and antioxidant status may be important in maintaining fish intestine health, thus benefiting the growth performance of fish.

Intestinal development is closely related to digestion and absorption of nutrients and structural integrity of intestine [2, 3]. Digestive enzymes (e.g., trypsin, chymotrypsin, lipase,

and amylase) and brush-border membrane enzymes (e.g., alkaline phosphatase, Na^+/K^+-ATPase, and creatine kinase) play vital roles in the food utilization [4, 5]. Intestinal physical integrity including villus height, villus width, and muscular thickness is critically important for efficient functioning and absorption capacity of the digestive system [6]. The tight junction (TJ) proteins are composed of transmembrane TJ proteins (occludin and members of the claudin superfamily) and cytosolic TJ proteins (e.g., zonula occludens-1 (Zo-1)), which could regulate the intercellular structural integrity in fish gut [7, 8].

Intestinal immune system can protect fish against potentially dangerous antigens, microorganisms, and poisonous elements [9]. Proinflammatory cytokines like interleukin 1 (IL-1), interleukin 8 (IL-8), and tumor necrosis factor-α (TNF-α) were involved in the regulation of immune function [10, 11]. The nuclear transcription factor-κB (NF-κB) signaling pathway plays a critical role in inflammation, which was involved in the regulation of inflammatory and proinflammatory cytokines [12]. Furthermore, the fish intestine is highly susceptible to oxidative damage caused by excess reactive oxygen species, and thus, antioxidant defense usually should be increased to prevent oxidative damage in fish [13, 14].

Dietary supplementation with probiotics is considered as an important way to enhance the intestinal health in fish [15]. *Clostridium butyricum* (CB) is a gram-positive obligate anaerobic bacillus, which exhibits positive effects on inhibition of inflammatory response and reparation of intestinal epithelium and reduction of pathogenic bacteria [16, 17]. In recent years, CB has been carried out in human, terrestrial, and some aquatic species to increase growth performance, promote nutrient utilization efficiency, maintain intestinal morphology, and improve disease resistance [18–20]. Feed supplemented with CB with a range of 10^7–10^{11} CFU/kg could improve growth performance, immune response, and intestine health of aquatic species, including large yellow croaker *Larimichthys crocea* [20], tilapia *Oreochromis niloticus* [21], tilapia *Oreochromis niloticus* × *O. aureus* [22], silver pomfret *Pampus argenteus* [23], giant freshwater prawn (*Macrobrachium rosenbergii*) [24], and shrimp *Litopenaeus vannamei* [25]. However, the effects of dietary CB on growth, intestinal health, immune response, and resistance to environmental stress in yellow catfish are still uncertain. Yellow catfish (*Pelteobagrus fulvidraco*) is an economic important omnivorous freshwater fish species in China. It is sensitive to environmental stressors such as ammonia [26], nitrite [27], hypoxia [28], and high temperature [29]. Therefore, the aim of this study was to investigate the effects of CB on growth performance, intestinal morphology, mRNA levels of tight junction proteins and immune-related genes, and resistance to ammonia stress in juvenile yellow catfish (*Pelteobagrus fulvidraco*).

2. Material and Methods

2.1. Experimental Diets. The CB with a count of 6×10^{12} colony-forming units (CFU)/g was obtained from Organic-Biotech Biotech Co., Ltd., China. Juvenile yellow catfish were fed with five isonitrogenous (42%) and isolipidic (9%) diets, which were supplemented with CB at 0 (CB0), 2 (CB1), 20 (CB2), 200 (CB3), and 2000 (CB4) mg/kg of diet, respectively. Five different concentrations of CB (0, 1.2×10^7, 1.2×10^8, 1.2×10^9, and 1.2×10^{10} CFU/kg) were selected based on results obtained in previous studies [20–23]. The final CB concentrations in the five diets were 0, 4.8×10^6, 4.5×10^7, 5.1×10^8, and 3.6×10^9 CFU/kg, which were determined by the plate count method [30]. CB was mixed with water sources and then mixed with the other feedstuffs. All diets were prepared and pelleted into 1.5 mm diameter by twin screw extruder (SLX-80, South China University of Technology, China). After drying at the temperature of 55°C for 6 h, diets were stored at −20°C until being used. The ingredients and proximate composition of experimental diets are presented in Table 1.

2.2. Fish Feeding and Management. The fish were purchased from Guangzhou, China, and were acclimated with the control diet for 2 weeks in an indoor recirculating aquaculture system. Six hundred fish (5.55 ± 0.01 g) were stocked into fifteen cylindrical fiberglass tanks (water volume 300 L) at 40 fish per tank to conduct the experiment. Five experimental diets were randomly allocated to triplicate groups of fish. During the 56-day feeding trial, the water temperature ranged from 27 to 32°C, pH 7.5-7.9, and dissolved oxygen > 6.0 mg/L. Fish were reared under 12 h light : 12 h dark dial cycle photoperiod. All the fish were manually fed to satiation with the experimental diets two times daily (8:30 and 18:30).

2.3. Sample Collection. At the termination of the feeding trial, fish in each tank were individually weighed and counted after 24 h fasting period. The fish were anesthetized in tricaine methanesulfonate before sampling. Six fish from each tank were dissected, and the body weight, body length, intestinal weight, and intestinal length were used to determine intestosomatic index (ISI) and intestine length index (ILI). The intestine of another six fish was quickly removed and frozen in liquid nitrogen and then stored at −70°C until analysis. The proximal intestines of another three fish from each tank were collected to measure intestinal morphology according to Cheng et al. [31].

2.4. Challenge Test. Thirty fish from each dietary group (10 fish per tank) were transferred to 100 L water with NH_4Cl for challenge test. The fish were challenged with 2.65 mg/L unionized ammonia for 72 h [26]. Mortality was recorded every 24 h during the stress test, and dead fish were removed.

The cumulative mortality rate (CMR) was calculated as follows:

$$\mathrm{CMR}\,(\%) = 100 \times \frac{\text{number of death fish after challenge test}}{\text{number of fish before challenge test}}. \quad (1)$$

2.5. Intestinal Morphology. Intestinal samples were fixed in the 4% buffered formalin solution for 24 h and then transferred to 70% ethanol. Subsequently, the fixed specimens were processed following the conventional histological

Table 1: The ingredients and proximate composition of experimental diets (g/kg dry matter).

Diets	Dietary CB concentrations (mg/kg)				
	CB0	CB1	CB2	CB3	CB4
Ingredients					
Peru fish meal	250.0	250.0	250.0	250.0	250.0
Soybean meal	300.0	300.0	300.0	300.0	300.0
Rapeseed meal	90.0	90.0	90.0	90.0	90.0
Corn gluten meal	60.0	60.0	60.0	60.0	60.0
Wheat flour	223.0	223.0	223.0	223.0	223.0
Fish oil	25.0	25.0	25.0	25.0	25.0
Soybean oil	25.0	25.0	25.0	25.0	25.0
Vitamin premix*	1.0	1.0	1.0	1.0	1.0
Mineral premix†	5.0	5.0	5.0	5.0	5.0
$Ca(H_2PO4)_2$	15.0	15.0	15.0	15.0	15.0
Vitamin C ester	1.0	1.0	1.0	1.0	1.0
Choline chloride	3.0	3.0	3.0	3.0	3.0
Microcrystalline cellulose	2.0	1.75	1.5	1.0	0
CB	0.0	0.25	0.5	1.0	2.0
Proximate nutrition composition					
Crude protein	426	423	420	425	422
Crude lipid	90.1	87.7	90.1	85.6	84.5
Ash	82.5	81.9	81.9	83.0	84.2
Moisture	77.9	86.9	85.7	80.7	81.8

*One kilogram of vitamin premix contained the following: VA 3,200,000 IU, VB_1 4 g, VB_2 8 g, VB_6 4.8 g, VB_{12} 0.016 g, VD 1,600,000 IU, VE 16 g, VK 4 g, nicotinic acid 28 g, calcium pantothenate 16 g, folic acid 1.28 g, inositol 40 g, biotin 0.064 g. Moisture ≤ 10%. †One kilogram of mineral premix contained the following: $MgSO_4 \cdot H_2O$ 12 g, $Ca(IO_3)_2$ 9 g, KCl 36 g, Met-Cu 1.5 g, $ZnSO_4 \cdot H_2O$ 10 g, $FeSO_4 \cdot H_2O$ 1 g, Met-Co 0.25 g, $NaSeO_3$ 0.003 6 g. Moisture ≤ 10%.

methods. The tissues were sliced, embedded in paraffin, and stained with hematoxylin and eosin. The tissue sections were examined using light microscopy (Eclipse E100, Tokyo, Japan) with Image-Pro Plus 6 software (Media Cybernetics, Maryland, USA). Villus length, villus width, and muscular thickness were measured.

2.6. Intestine Biochemical Analysis. Whole intestine samples were homogenized and centrifuged (6000g, 20 min, 4°C), and the supernatant was collected for analysis. Commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used to determine intestinal enzyme activities of protease, lipase, amylase, alkaline phosphatase (ALP) and Na^+/K^+ ATPase, superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity (TAOC), and concentrations of malondialdehyde (MDA), following the corresponding manufacturer's instructions. Concentration of intestinal complement 3 (C3), tumor necrosis factor (TNF-α), interleukin 6 (IL-6), and interleukin 1β (IL-1β) was determined by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, Minnesota, USA). Intestinal short-chain fatty acid (SCFA) concentration was measured by gas chromatography, according the method of Weitkunat et al. [32].

2.7. RNA Extraction and Real-Time Quantitative PCR Analysis. Total RNA was extracted from the whole intestine of yellow catfish using Trizol Reagent (Takara Biotech, Dalian, China), following the protocol of the manufacturer, and electrophoresed on a 1.2% denaturing agarose gel to test the integrity. The RNA reverse was then transcribed to cDNA using PrimeScript RT reagent kit (Takara, Japan). The qPCR assay was carried out using an ABI Viia7 real-time PCR machine (Applied Biosystems, USA). The amplification was carried out in a 10 μL reaction volume containing 5 μL SYBR Green Master Mix, 2 μL of each respective primer (2 μM), 2 μL cDNA product, and 1 μL RNA-free water. The specific primers and housekeeping gene (β-actin) were designed using primer 5.0 (PREMIER Biosoft International, Palo Alto, CA, USA) based on the sequences obtained from the published sequences of yellow catfish (Table 2). β-Actin was used as a nonregulated reference gene to normalize target gene transcript levels in yellow catfish studies [26, 33]; furthermore, β-actin gene expression of the intestine was also stable and was not significantly affected by dietary CB in our present research. All reactions were performed in duplicate, and each assay was repeated three times. The gene expression levels were analyzed using the $2^{-\Delta\Delta CT}$ method [34].

2.8. Statistical Analysis. Statistical analyses were performed with the software SPSS 20.0 (Chicago, USA). One-way analysis of variance (ANOVA) and a multiple range test (Tukey's HSD test) were used to determine significant variation ($P < 0.05$). Data were expressed as means ± standard error (S.E.).

3. Results

3.1. Growth Performance, Feed Efficiency, and Intestinal Growth. The final weight, SGR, SR, FCR, body length, intestinal length, intestinal weight, ILI, and ISI are showed in Table 3. Final weight, SGR, body length, and intestinal weight in CB1, CB2, CB3, and CB4 were significantly higher than those in CB0 ($P < 0.05$). Significantly higher intestinal length and lower FCR were found in CB1, CB2, and CB3 compared to CB0 ($P < 0.05$). ISI in CB2 and CB3 groups showed significant increase compared with that in CB0 level ($P < 0.05$). SR was 100% in all groups, and there were no significant differences among groups ($P > 0.05$). ILI did not show significant changes among the groups ($P > 0.05$).

3.2. Intestinal SCFA Contents and Enzyme Activities. The effects of dietary CB on intestinal SCFA contents are presented in Table 4. Acetic acid, propionic acid, butyric acid, and valeric acid contents in CB groups were higher than those in CB0. Acetic acid content ($P < 0.05$) was significantly elevated in the CB1 group, but not the CB2, CB3, and CB4 treatments ($P > 0.05$). Propionic acid content was significantly ($P < 0.05$) increased in all groups except the CB4 treatment, and the highest level was observed in the CB2 group. Butyric acid and valeric acid contents in the CB4 group showed significantly increase compared with CB0 level ($P < 0.05$).

Table 2: Primers used in real-time PCR.

Target	Forward primer (5′-3′)	Reverse primer (5′-3′)	Size (bp)
Claudin-1	ACGCTAACAACGGCTCAGA	CCTTACATTCAGACACCACCTT	171
Zo-1	CGGAGCCACCCAAACAGTAT	TTAGAGTCTCCGCCTCTGCT	129
Occludin	CGAGCGAGAGACTACGACAC	TCCAGGAATTGTGGGCTTCC	239
MLCK	GCCACGAAGACCAAAAGCTC	AACCAAGGGCCAGAGACTTG	118
IL-1	AAACCAGCATCTCCAGTGTC	GAGCAAAGGCTGTTCCGTAT	168
IL-8	CACTCACCAAGCCAGCAATG	AGACAACCCAAGACTTCACC	228
TNF-α	ATAACCCACGCCTATGACTG	GGCTATGACTCGCAACACTT	207
NF-κB	AGCCGTCTTGGCTAACAGTC	AGAGGCGTAGAGCCGTCATA	198
β-Actin	TCCTGACCGAGAGAGGCTAC	TCCAGAGAGGAGGAAGAGGC	136

Zo-1: zonula occludens protein-1; MLCK: myosin light chain kinase; IL-1: interleukin 1; IL-8: interleukin 8; TNF-α: tumor necrosis factor-α; NF-κB: nuclear transcription factor-κB.

Table 3: Growth performance, feed efficiency, and intestinal growth of juvenile yellow catfish fed diets containing different levels of CB for 56 days (means ± S.E.M., $n = 3$).

Items	Dietary CB concentrations (mg/kg)				
	CB0	CB1	CB2	CB3	CB4
Initial weight (g)	5.55 ± 0.01	5.55 ± 0.00	5.50 ± 0.06	5.55 ± 0.00	5.56 ± 0.00
Final weight (g)	19.79 ± 0.67^{a}	27.71 ± 1.23^{b}	26.09 ± 1.67^{b}	28.71 ± 1.39^{b}	24.74 ± 1.43^{b}
SGR	2.25 ± 0.06^{a}	2.83 ± 0.09^{bc}	2.70 ± 0.12^{bc}	2.90 ± 0.08^{c}	2.62 ± 0.10^{b}
SR	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
FCR	1.41 ± 0.1^{b}	1.11 ± 0.08^{a}	0.98 ± 0.02^{a}	1.04 ± 0.05^{a}	1.14 ± 0.15^{ab}
Final body length (cm)	10.88 ± 0.13^{a}	12.23 ± 0.19^{b}	11.78 ± 0.24^{b}	12.30 ± 0.19^{b}	11.93 ± 0.25^{b}
Intestinal length (cm)	4.61 ± 0.28^{a}	5.71 ± 0.35^{b}	5.68 ± 0.34^{b}	5.74 ± 0.22^{b}	5.38 ± 0.22^{ab}
Intestinal weight (g)	0.19 ± 0.02^{a}	0.27 ± 0.02^{b}	0.31 ± 0.02^{bc}	0.35 ± 0.02^{c}	0.28 ± 0.03^{b}
ILI	0.44 ± 0.03	0.46 ± 0.02	0.48 ± 0.02	0.47 ± 0.02	0.45 ± 0.02
ISI (%)	0.95 ± 0.08^{a}	0.97 ± 0.06^{a}	1.21 ± 0.05^{b}	1.23 ± 0.06^{b}	1.11 ± 0.05^{ab}

Different superscript letters within a row indicate significance ($P < 0.05$). Specific growth rate (SGR, %/day) = 100 × [Ln final weight (g) − Ln initial weight (g)]/number of days; survival rate (SR, %) = 100 × (finial number of fish)/(initial number of fish); intestine length index (ILI) = intestinal length (cm)/total body length (cm); intestosomatic index (ISI, %) = 100 × intestinal weight (g)/body weight (g).

Table 4: Intestinal SCFA concentration (μg/100 g) of juvenile yellow catfish fed diets containing different levels of CB for 56 days (means ± S.E.M., $n = 3$).

Items	Dietary CB concentrations (mg/kg)				
	CB0	CB1	CB2	CB3	CB4
Acetic acid	305.80 ± 38.06^{a}	544.37 ± 62.35^{b}	445.11 ± 46.68^{ab}	425.94 ± 37.76^{ab}	389.15 ± 72.52^{ab}
Propionic acid	11.95 ± 0.43^{a}	17.61 ± 1.96^{b}	21.70 ± 1.31^{b}	20.16 ± 1.88^{b}	12.07 ± 0.86^{a}
Butyric acid	5.85 ± 0.96^{a}	7.85 ± 1.80^{ab}	7.29 ± 0.53^{ab}	7.60 ± 1.42^{ab}	15.97 ± 5.78^{b}
Valeric acid	2.84 ± 0.71^{a}	4.06 ± 0.55^{ab}	3.06 ± 0.30^{ab}	3.01 ± 0.56^{ab}	4.76 ± 0.53^{b}

Different superscript letters within a row indicate significance ($P < 0.05$).

The effects of dietary CB on intestinal activities of protease, lipase, amylase, ALP, CK, and Na^+/K^+-ATPase are presented in Table 5. Intestinal protease and ALP activities increased with increasing CB levels up to CB3 ($P < 0.05$). The activities of protease and ALP in CB2 and CB3 were significantly higher than those in CB0 ($P < 0.05$). Na^+/K^+-ATPase activity was the lowest for fish in CB0 and the highest for fish in CB3 ($P < 0.05$). CK activity was the lowest for fish in CB0 and the highest for fish in CB2 ($P < 0.05$). However, the other intestinal enzyme activities were not influenced by dietary CB levels ($P > 0.05$).

3.3. Intestinal Morphology. The proximal intestinal morphology is presented as Figure 1. Table 6 shows the effects of dietary CB on villus length, villus width, and muscular thickness of the intestine. There was a significant increase of villus length in CB2 and CB3 compared with CB0, CB1, and CB4 ($P < 0.05$). Significantly higher value of villus width

Table 5: Intestinal enzyme activities (U/mgprot) of juvenile yellow catfish fed diets containing different levels of CB for 56 days (means ± S.E.M., $n = 3$).

Items	Dietary CB concentrations (mg/kg)				
	CB0	CB1	CB2	CB3	CB4
Trypsin	10.92 ± 0.85^{a}	13.36 ± 2.02^{ab}	15.38 ± 2.66^{b}	15.47 ± 1.47^{b}	13.04 ± 2.46^{ab}
Lipase	20.35 ± 0.89	27.08 ± 2.35	23.95 ± 3.62	27.06 ± 6.49	26.09 ± 2.94
Amylase	1.18 ± 0.06	1.31 ± 0.03	1.35 ± 0.16	1.26 ± 0.02	1.27 ± 0.11
ALP	498.54 ± 22.45^{a}	592.93 ± 26.96^{b}	623.78 ± 26.47^{b}	627.96 ± 15.67^{b}	508.04 ± 25.48^{a}
CK	0.83 ± 0.16^{a}	1.33 ± 0.14^{ab}	1.54 ± 0.37^{b}	1.19 ± 0.17^{ab}	0.85 ± 0.09^{a}
Na^{+}/K^{+}-ATPase	2.86 ± 0.92^{a}	4.09 ± 0.18^{ab}	3.86 ± 0.26^{ab}	4.84 ± 1.36^{b}	3.49 ± 0.79^{ab}

Different superscript letters within a row indicate significance ($P < 0.05$). Abbreviation: ALP: alkaline phosphatase; CK: creatine kinase.

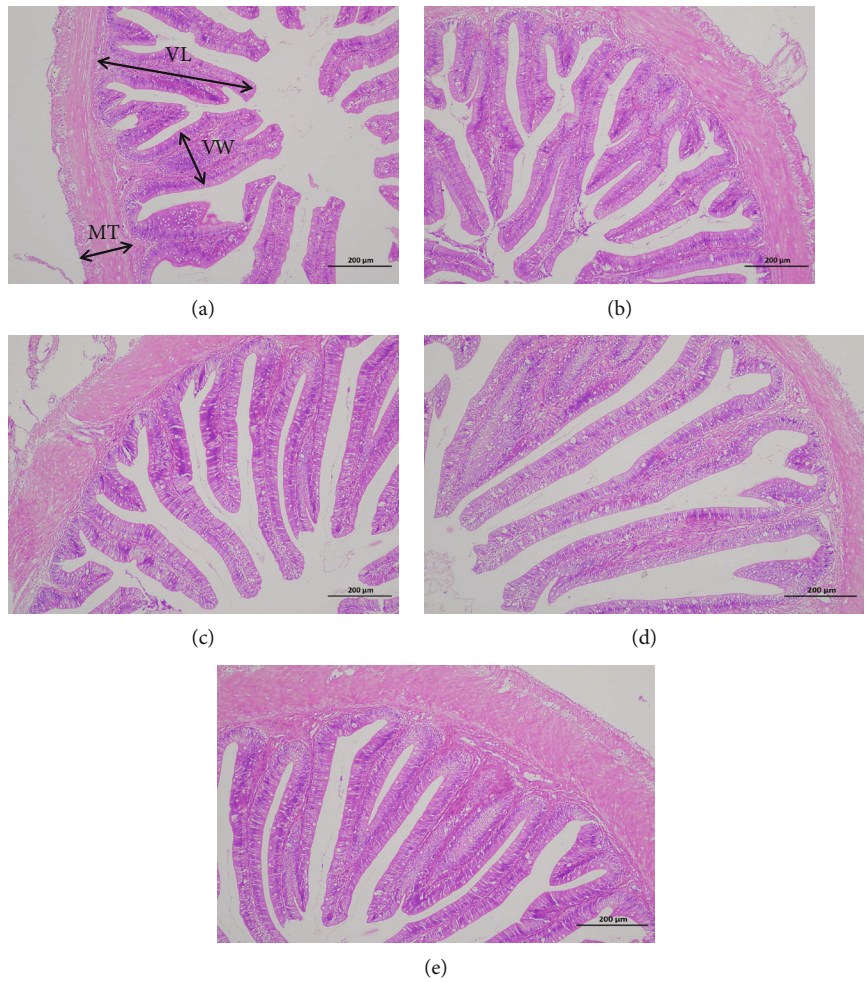

Figure 1: Proximal intestinal morphology of juvenile yellow catfish fed diets containing different levels of CB for 56 days: (a) CB0; (b) CB1; (c) CB2; (d) CB3; (e) CB4. VL: villus length; VW: villus width; MT: muscular thickness (HE staining; original magnification ×100).

Table 6: Intestinal morphology of juvenile yellow catfish fed diets containing different levels of CB for 56 days (means ± S.E.M., $n = 3$).

Item	Dietary CB concentrations (mg/kg)				
	CB0	CB1	CB2	CB3	CB4
Villus length	632.32 ± 114.01^{a}	727.41 ± 46.29^{a}	1186.05 ± 239.85^{b}	1110.97 ± 192.16^{b}	767.09 ± 123.43^{a}
Villus width	105.24 ± 3.05^{a}	106.95 ± 20.16^{ab}	132.49 ± 13.2^{ab}	138.48 ± 15.54^{c}	111.57 ± 9.73^{bc}
Muscular thickness	122.72 ± 24.41	147.02 ± 6.85	142.01 ± 13.16	154.68 ± 61.75	172.92 ± 29.92

Different superscript letters within a row indicate significance ($P < 0.05$).

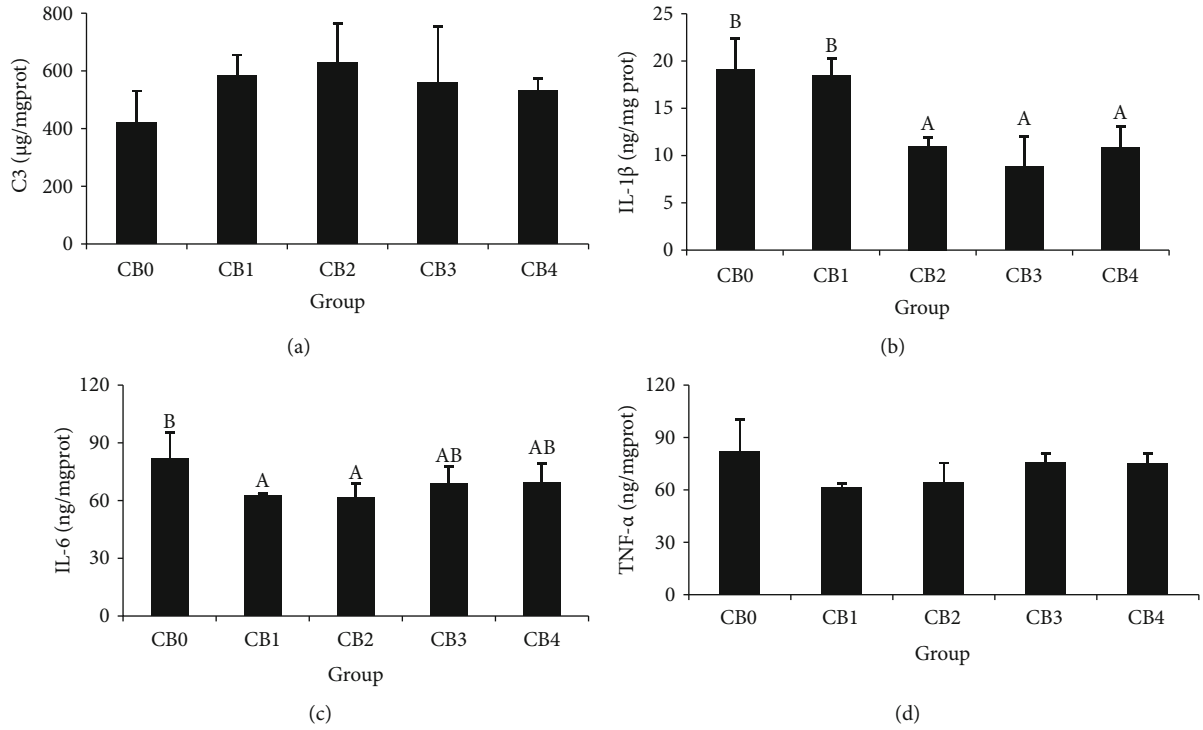

Figure 2: Effects of dietary CB administration on (a) C3, (b) IL-1β, (c) IL-6, and (d) TNF-α levels in the intestine of yellow catfish. [a,b,c]Bars with different superscripts represent significant difference ($P < 0.05$). Data presented are means ± S.E.M. of 3 replicates. Abbreviation: C3: complement 3; TNF-α: tumor necrosis factor; IL-1β: interleukin 1β; IL-6: interleukin 6.

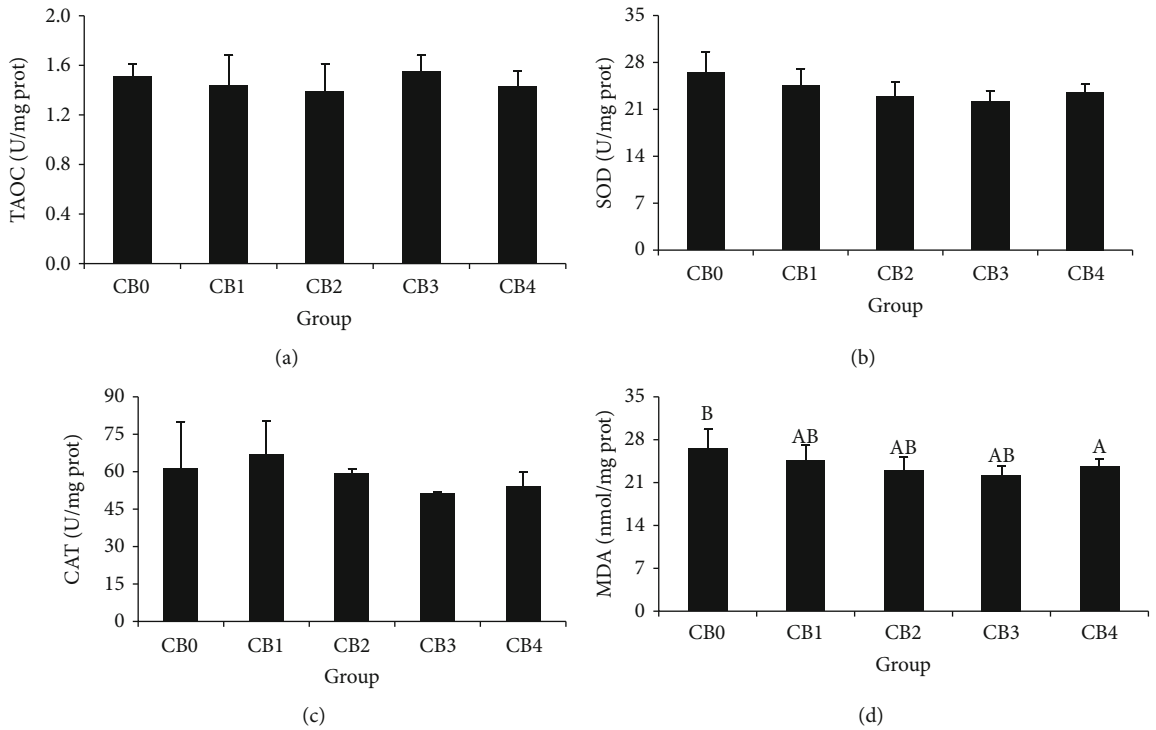

Figure 3: Effects of dietary CB administration on (a) TAOC, (b) SOD, (c) CAT, and (d) MDA levels in the intestine of yellow catfish. [a,b,c]Bars with different superscripts represent significant difference ($P < 0.05$). Data presented are means ± S.E.M. of 3 replicates. Abbreviation: TAOC: total antioxidant capacity; SOD: superoxide dismutase; CAT: catalase; MDA: malondialdehyde.

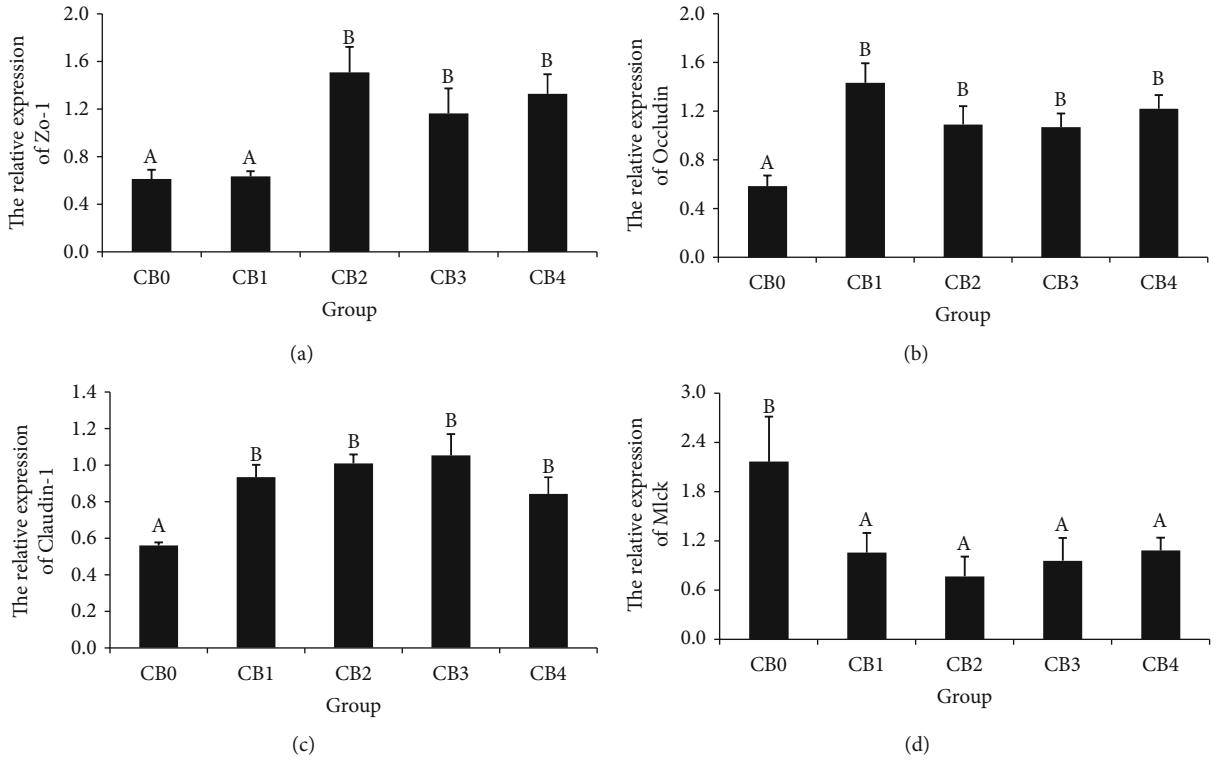

Figure 4: Effects of dietary CB administration on (a) *Zo-1*, (b) *occludin*, (c) *claudin-1*, and (d) *Mlck* mRNA levels in the intestine of yellow catfish. [a,b,c]Bars with different superscripts represent significant difference ($P < 0.05$). Data presented are means ± S.E.M. of 3 replicates. Abbreviation: *Zo-1*: zonula occludens protein-1; *Mlck*: myosin light chain kinase.

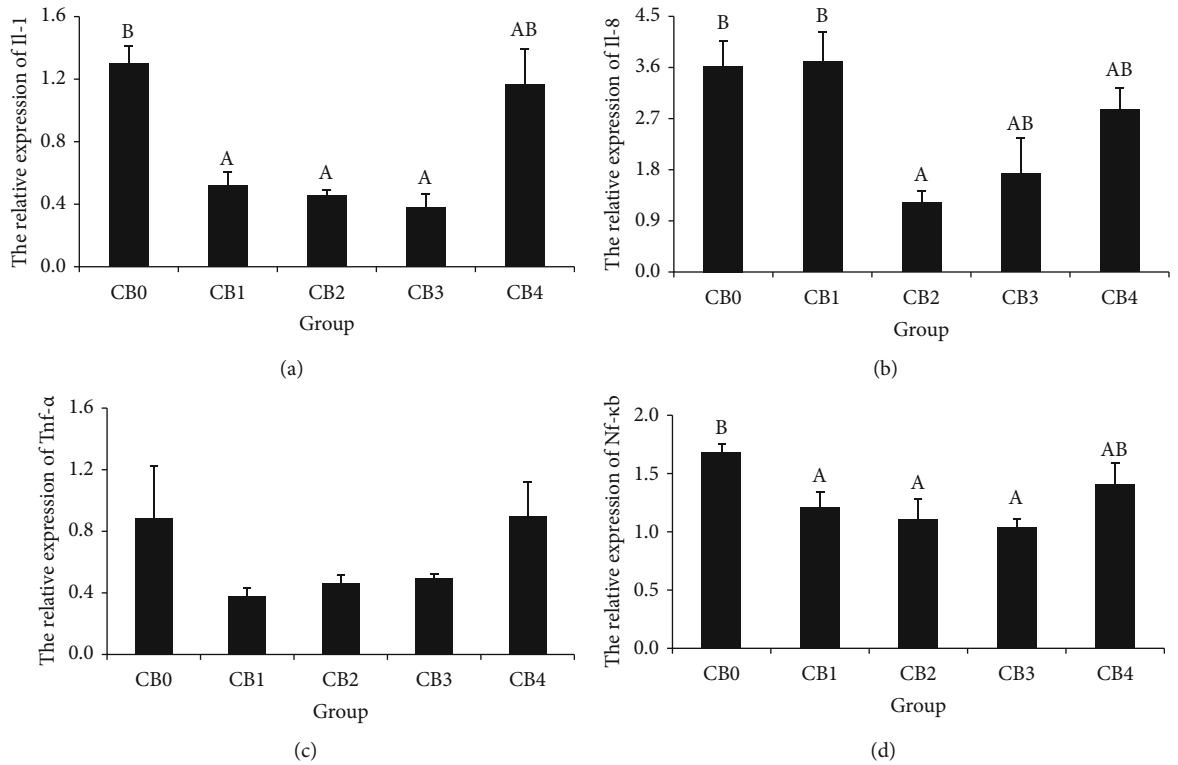

Figure 5: Effects of dietary CB administration on (a) *Il-1*, (b) *Il-8*, (c) *Tnf-α*, and (d) *Nf-κb* mRNA levels in the intestine of yellow catfish. [a,b,c]Bars with different superscripts represent significant difference ($P < 0.05$). Data presented are means ± S.E.M. of 3 replicates. Abbreviation: *Il-1*: interleukin 1; *Il-8*: interleukin 8; *Tnf-α*: tumor necrosis factor-α; *Nf-κb*: nuclear transcription factor-κb.

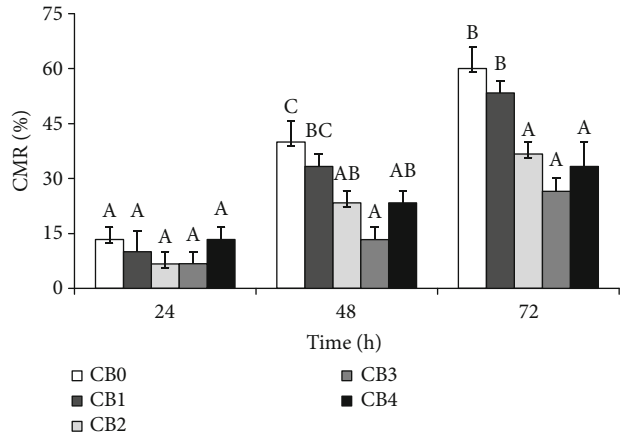

FIGURE 6: Effects of dietary CB administration on CMR of yellow catfish under ammonia stress at 24, 48, and 72 h. [a,b,c]Bars with different superscripts represent significant difference ($P < 0.05$). Data presented are means ± S.E.M. of 3 replicates. Abbreviation: CMR: cumulative mortality rate.

was observed in CB3 and CB4 than in CB0 ($P < 0.05$). Muscular thickness was significantly higher in CB3 than in other groups ($P < 0.05$).

3.4. Immune Parameters. The levels of C3, IL-1β, IL-6, and TNF-α of juvenile yellow catfish are presented in Figure 2. Significantly lower level of IL-1β was observed in CB2, CB3, and CB4 compared to CB0 and CB1 ($P < 0.05$). Intestinal IL-6 content in CB1 and CB2 was significantly lower than that in CB0 ($P < 0.05$). The contents of C3 and TNF-α were similar among all experimental groups ($P > 0.05$).

3.5. Antioxidative Parameters. The effects of dietary CB on TAOC, SOD, CAT, and MDA are presented in Figure 3. The highest MDA occurred in CB0 among all groups ($P < 0.05$). The values of SOD, CAT, and TAOC were not significantly affected by dietary CB levels ($P > 0.05$).

3.6. Tight Junction Proteins and Immune-Related Gene mRNA Levels in the Intestine

3.6.1. Tight Junction Proteins and MLCK mRNA Levels. The effects of dietary CB on tight junction proteins and *Mlck* mRNA levels are shown in Figure 4. Dietary CB levels significantly decreased the relative expression of MLCK ($P < 0.05$). Compared with those in CB0, the relative expressions of *claudin-1*, *Zo-1*, and *occludin* were significantly increased in CB2, CB3, and CB4 ($P < 0.05$).

3.6.2. Expression Levels of Immune-Related Genes. The effects of dietary CB on expression levels of immune-related genes are presented in Figure 5. Fish in CB1, CB2, and CB3 had lower relative expressions of *Il-1* and *Nf-κb* than that fed CB0 ($P < 0.05$). Dietary CB significantly decreased the relative expression of *Il-8* in CB2 compared with CB0 and CB1 ($P < 0.05$). Among the experimental groups, the mRNA expression of *Tnf-α* showed no significant differences ($P > 0.05$).

3.7. The Resistance to Ammonia-Nitrogen Stress. CMR under ammonia exposure for 24, 48, and 72 h are presented in Figure 6. The fish fed with CB diets had significantly lower mortality after exposure to ammonia nitrogen. Fish in CB0 had higher CMR than that in CB2, CB3, and CB4 under stress for 48 and 72 h ($P < 0.05$). The lowest CMR was observed in CB3 under stress for 48 and 72 h ($P < 0.05$). No significant differences were found in CMR among the groups under stress for 24 h.

4. Discussion

CB supplementation increased final weight and SGR and reduced FCR indicating that CB could improve the growth performance of yellow catfish in this study. Consistent with our results, the beneficial influences of CB on growth have been observed in several fish species, including large yellow croaker [20], tilapia [22], and silver pomfret [23]. The improvement of weight gain and feed utilization might be associated with the increase in SCFA stimulated by CB in the intestine. In the present study, CB supplementation could increase the concentration of intestinal SCFAs, including acetic acid, propionic acid, butyric acid, and valeric acid. The increased intestinal SCFAs resulted in lower pH and higher activities of digestive enzymes [35]. In this study, the supplementation of CB in the diet enhanced intestinal trypsin and lipase activities. Similar results were reported for tilapia and shrimp in which activities of intestinal amylase, lipase, and trypsin were improved in fish fed with CB [21, 25]. Yin et al. [20] reported that dietary CB increased lipase activity of large yellow croaker (*Larimichthys crocea*) larvae. The brush-border membrane enzymes play important roles in the nutrient absorption process of the intestine [5]. In the present study, dietary CB significantly enhanced intestinal ALP, CK, and Na^+/K^+-ATPase activities of yellow catfish. The enhanced digestive and absorption abilities might lead to the increase of growth performance in yellow catfish.

The growth, development, and integrity of fish intestine are crucial for the maintenance of normal intestinal functions [36]. In this study, the intestine length, weight, and ISI showed a similar trend with the intestinal enzyme activities, suggesting that CB stimulated intestinal growth and development of yellow catfish. A previous study has revealed that the increase in small intestinal weight of suckling pigs was caused by a rapid increase in the gut thickness of all segments of small intestine in relation to an increase in absorptive area [37]. In the present study, CB supplementation could increase the sizes of villus length, villus width, and muscular layer thickness, which contribute to increase the surface area of intestine for nutrient absorption. Similar results were reported that the villus height, enterocyte height, and muscular thickness in large yellow croaker larvae significantly enhanced when dietary CB was added [20]. Intestinal tight junction protein (e.g., *occludin*, *claudin-1*, and *Zo-1*) participates in maintaining the stability and permeability of intestinal epithelial barrier [38]. Xiao et al. [39] showed that CB protected the intestinal barrier function by upregulating *Zo-1*, *claudin-1*, and *occludin* expression. In

this study, a significant increase in mRNA expression levels of *claudin-1*, *occludin*, and *Zo-1* was found in the intestine of yellow catfish fed with CB diets. These results revealed that CB could improve intestinal development of yellow catfish by maintaining intestinal integrity and promoting intestinal epithelial proliferation.

TJ structure could be regulated by proinflammatory cytokines such as interleukin-1 (IL-1), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α) [40, 41]. In the present study, CB supplementation significantly lowered the concentration of the proinflammatory cytokine (IL-1β, IL-6, and TNF-α) and the expression levels of the proinflammatory cytokines (*Il-1*, *Il-8*, and *Tnf-α*), which revealed that optimal dietary CB levels suppressed the intestinal inflammatory response. Zhao et al. [42] showed that dietary CB could downregulate the expression level of *Ifn-γ*, *Il-1β*, *Il-8*, and *Tnf-α* in intestinal tissues and intestinal epithelial cells of chickens with *Salmonella* infection. Yin et al. [20] showed that dietary CB could decrease the expression levels of *Ifn-γ*, *Il-1β*, *Il-6*, and *Il-8* to protect large yellow croaker from pathogens. TNF-α could induce TJ structure damage by regulating myosin light chain kinase (*Mlck*) expression via the Nf-κb signaling pathway [43]. The expression of *Mlck* is positively correlated with the expression of *Tnf-α* and inversely proportional to the expression of TJ proteins [44]. In this study, dietary CB decreased the expression levels of the *Mlck* and showed a negative correlation to the expression levels of *Zo-1*, *claudin-1*, and *occludin*, which is ascribed to inhibiting *Nf-κb* mRNA expression. The CB-induced downregulation of proinflammatory cytokines *Mlck* and *Nf-κb* may explain the upregulated mRNA expression of genes encoding TJ proteins.

Inflammatory response and oxidative stress are tightly associated processes that trigger fish cell damage [45]. Antioxidant enzymes and antioxidant substances play important roles in reducing oxidative stress [46]. Malondialdehyde (MDA) reflects lipid peroxidation degrees [47]. In the current study, CB supplementation could decrease intestinal MDA content, which was consistent with previous studies on *Oreochromis niloticus* [27], *Macrobrachium rosenbergii* [24], and *Litopenaeus vannamei* [25]. Depressed MDA content reflected the protection against free radical attack in yellow catfish. Intriguingly, no significant differences in antioxidant enzymes such as activities of SOD and CAT were observed among dietary treatments. He et al. [48] found CB could increase SOD, catalase, and glutathione peroxidase in hybrid grouper, but no significant differences were observed between the CB treatment group and the control diet group. Combining the results of our research, CB could effectively decrease inflammatory factor and MDA levels to protect yellow catfish from pathogens.

The present study showed that the cumulative mortality rate of fish fed the control diet and exposed to 100 mg/L total ammonia nitrogen for 72 h (60%) was significantly higher than those fed 4.5×10^7, 5.1×10^8, and 3.6×10^9 CFU/kg CB diets (26–36%). Ammonia exposure could impair the cell function via triggering oxidative stress and inducing the inflammatory response [49, 50]. The present study revealed that the dietary CB improved stress tolerance of yellow catfish against ammonia exposure by downregulating proinflammatory factors IL-1, IL-8, and TNF-α and inhibiting the MLCK/NF-κB signaling pathway. Similar to our study, dietary CB could induce the antioxidant and immune function to improve resistance against ammonia stress and *Vibrio parahaemolyticus* of *Litopenaeus vannamei* [25, 51]. In gibel carp, dietary CB could significantly improve the innate immune response to enhance the resistance against *Carassius auratus* herpesvirus [52].

5. Conclusions

Oral administration of CB at 4.5×10^7 (CB2), 5.1×10^8 (CB3), and 3.6×10^9 (CB4) CFU/kg in yellow catfish diets resulted in improved growth, promoted intestine development, and enhanced stress resistance against ammonia exposure. Dietary CB improved growth performance and stress resistance by increasing intestinal SCFA concentrations, upregulating of tight junction proteins, downregulating proinflammatory factors, and inhibiting the MLCK/NF-κB signaling pathway.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

Conflicts of Interest

The authors declare no competing conflicts of interest.

Authors' Contributions

Dongqiang Hou and Peijia Li contributed equally to this work.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (31402307) and the Science and Technology Planning Project of Guangdong Province (KTP20210322).

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