Dietary curcumin supplementation enhances intestinal immunity and gill protection in juvenile greater amberjack (Seriola dumerili)

Yuhang He a,b, Zhengyi Fu a,b, Shiming Dai a,b, Gang Yu a,b, Zhenhua Ma a,b,c,*, Xiaomei Wang d, **

a Tropical Aquaculture Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Sanya 572018, China
b Sanya Tropical Fisheries Research Institute, Sanya 572018, China
c Hainan Provincial Key Laboratory of Efficient Utilization and Processing of Marine Fishery Resources, Sanya 572018, China
d Changhai Enhancement and Experiment Station, Chinese Academy of Fishery Sciences, Shandong, Yantai 265800, China

** Corresponding author.
* Corresponding author.
E-mail addresses: zhenhua.ma@hotmail.com (Z. Ma), xiaomei_wang328@126.com (X. Wang).

A 8-week feeding trial was conducted to determine the effects of dietary supplementation with curcumin on growth, intestinal health, and gills resistance to ammonia stress in juvenile Seriola dumerili. Three isonitrogenous and isolipidic test diets were prepared by supplementing incremental levels of dietary curcumin at 0% (CUR0%, control), 0.01% (CUR0.01%), 0.02% (CUR0.02%), respectively. Fish were fed with experimental diet. Recovery and protection capacity after ammonia challenge assay was adopted to test the effect of curcumin. At the end of the feeding trial, the results showed that dietary supplementation with proper curcumin level had a significant positive effect on fish survival and intestinal histology structure. Meanwhile, dietary supplementation with proper curcumin level can improve intestinal health by increasing immune enzyme activity, up-regulating the expression of anti-inflammation cytokines, down-regulating the expression of pro-inflammatory cytokines and regulating other immune-related genes. Subsequently, after the ammonia challenge and recovery experiment, the results of antioxidant-related genes and antioxidant enzymes showed that dietary supplementation with proper curcumin level can promote the growth, intestinal health, gill resistance and recovery to ammonia stress of S. dumerili.

1. Introduction

The greater amberjack (Seriola dumerili) is considered a popular fish due to its thick and tender flesh, rich in protein and fat, it has recently received some attention in the aquaculture industry [1, 2]. Intensive farming has become more and more popular with the increased demand for S. dumerili, but high-density farming also brings a lot of stress to the farming process, such as the rise in the frequency of disease leading to huge economic losses [3, 4]. In addition, intensive farming causes an inevitable increase in ammonia concentration in the water environment, eventually accumulating to unsafe levels [5, 6]. When the organism is exposed to high concentrations of ammonia, abnormal oxidative reactions in aerobic metabolic pathways are caused, resulting in excess singlet oxygen and other reactive oxygen species (ROS). This may lead to different histopathological changes and a series of physiological effects, even triggering diseases and death, ultimately causing huge economic losses [7, 8]. The research about how to improve the immunity of aquaculture animals to deal with the occurrence of diseases has become a hot topic, with the most common solution being to control and prevent the disease with antibiotics and chemical drugs [9, 10]. In recent years, natural feed supplements that promote growth and disease resistance have been effectively used in aquafeeds with people's awareness of food safety and environmental protection has improved. In particular, extracts of Chinese herbs have been proven in many studies to improve the immune capacity and health of aquatic animals [11, 12, 13]. For example, the antioxidant capacity of the liver and kidney of tilapia (Oreochromis niloticus) can be increased when garlic was added to the diet [14]. Similarly, Nile tilapia showed a significant rise in lysozyme activity and phagocytosis when the diet was supplemented with a mixture of Astragalus and Lonicera extracts [15]. The Chinese herbs extract we chose for this experiment was curcumin, which is cheaper and more available than other Chinese herb extracts.
Curcumin (CM) is a yellow acidic phenol extracted from the turmeric rhizome, which is commonly used as a spice, colorant, and food preservative [16]. Previous studies have shown that CM has many biological activities, such as antioxidant [17], anti-bacterial [18], and anti-inflammatory effects [19, 20]. Lysozyme and alternative hemolytic complement activity in rainbow trout serum were significantly improved when 400 mg/kg of curcumin was added to the diet [21]. Manju et al [22] also declared that feeding curcumin can inhibit liver lipid peroxidation of climbing perch. These results suggest that curcumin supplementation in the diet is effective in promoting the innate immune response of fish serum and liver to improve fish health. However, little information is available on the effects of dietary curcumin supplementation on the fish intestinal structure and immune capacity. The intestinal tract is a major site of fish digestion and immunity, which determines the absorption and health of fish, intestinal mucosal damage and inflammatory disease are important causes of fish mortality [23, 24].

Fish gill is sensitive to the concentration of ammonia in the aquaculture water, which is the main organ of fish respiration, osmoregulation, and ammonia excretion, its immune capacity defines the growth and survival of the fish [25]. Ammonia nitrogen is one of the main pollutants in aquaculture, and ammonia nitrogen concentrations could be raised in intensive aquaculture with high-density farming and high feeding rates [26]. Excessive ammonia in the farmed water may lead to the destruction of non-specific immune defenses in the gill [27, 28]. Due to the immunomodulatory effects of curcumin, its supplementation in the diet may be a potential method to improve the resistance of high concentrations on fish, and to reduce the use of antibiotics and chemical drugs in aquaculture.

Thus, we investigated the effects of dietary curcumin supplementation on growth, intestinal structure, intestinal immune enzymes, and relative expression of intestinal immune genes of S. dumerili. Subsequently, the ammonia nitrogen challenge experiment and recovery experiment were conducted. The protective and recovery capacity of dietary curcumin supplementation on gill tissue was evaluated by the relative expression of gill tissue immune genes. The aim of the present research was whether curcumin could positively improve the health capacity of S. dumerili and thus increase the production efficiency of practical aquaculture farming. It also provides reference information for the application of herbs as dietary immune additive in aquaculture.

2. Materials and methods

2.1. Experimental diet preparation

The curcumin (purity >95%) used in the experiment was supplied by Xi'an Feida biotechnology Co., Ltd. In this study, three experimental feeds with isonitrogenous and isolipid were prepared by adding 0%, 0.01%, and 0.02% curcumin to the feeds. Then, a dietary feeding experiment was conducted on S. dumerili. The experimental feeds were designed and produced by Lingshui Tropical Aquatic Research and Development Center. All ingredients were crushed and screened through a 0.2mm mesh and subsequently mixed with oil using a commercial food mixer (Guangdong Li feng., LTD., China), and finally passed through a granulator to produce 4 mm diameter pellets that were air dried at room temperature (25 °C). The prepared feed was sealed in plastic bags and stored at -20 °C until used for the feeding trial. Experimental feed formula information is shown in Table 1.

2.2. Experimental fish and feeding experiment

The S. dumerili used in the experiment were provided by the Tropical Fisheries Research and Development Center, South China Sea Fisheries Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Science (Lingshui, Hainan, China). In experiment I, before the start of the feeding experiment, healthy fish which transported to the experimental conditions. A total of 135 fish with an initial weight of 151.44 ± 7.16 g were randomly distributed into 9 circulating mariculture tanks filled with 200L water at the stocking rate of 15 fish per tank. The fish were acclimated in the experimental environment for a week before transferred to the experimental tank. Each experimental diet was randomly assigned to three replicates. Nine tanks were assigned to three groups feeding different diets (curcumin supplementation at 0, 100, and 200 mg/kg, respectively), and no curcumin supplementation group as the control group. Fish from each groups were weighed before the start of the experiment. Visual satiation was achieved by hand feeding twice daily (8:00 am and 4:00 pm). Dead fish within 72 h of stocking were replaced by similar sized fish. Feces were siphoned out to avoid water pollution at 1h after feeding. During the experiment, the water temperature, salinity, pH of cultured seawater were maintained at 27–31 °C, 35 g/L, and 7.5–8.0, respectively. Aeration was provided to maintain dissolved oxygen levels near saturation (6.5–7 mg/L). The nitrifying content was kept lower than 0.02 mg/L, and ammonia nitrogen content was lower than 0.1 mg/L. The feeding experiment lasted for 8 weeks. Experiment II was conducted to determine whether the dietary supplement with curcumin could relieve the stress induced by NH3-N and enhance the ability of recovery. Ammonia challenge experiment was conducted on the fish after 8 weeks of feeding experiments. Ammonium chloride (NH4Cl) was added into each pool to prepare the culture water at a concentration of 1 g/L. When all the fish show a visible negative effects, such as manic agitation or faint, the duration was approximately 6 min according to our pre-experiments. Then, two fish were randomly selected from each pool, one of the pool was placed in normal seawater for

| Ingredients | Diets | CUR0% | CUR0.01% | CUR0.02% |
|-------------|------|-------|----------|----------|
| Fish meal   | 8850.0 | 8850.0 | 8850.0   |
| Corn gluten meal | 1050.0 | 1050.0 | 1050.0   |
| Soybean meal | 1200.0 | 1200.0 | 1200.0   |
| maize starch | 1200.0 | 1200.0 | 1200.0   |
| Microcrystalline Cellulose | 750.0 | 750.0 | 750.0    |
| Fish oil    | 1050.0 | 1050.0 | 1050.0   |
| Lecithin    | 150.0  | 150.0  | 150.0    |
| Vitamin premix | 75.0   | 75.0   | 75.0     |
| Mineral premix | 75.0   | 75.0   | 75.0     |
| Choline chloride | 75.0 | 75.0 | 75.0     |
| Betaine    | 75.0  | 75.0  | 75.0     |
| Carboxymethyl cellulose | 450.0 | 450.0 | 450.0    |
| Curcumin   | 0.0   | 1.5   | 3.0      |

According to each parallel 15000g feed, the weight of each component of the feed is shown in the table. The CUR0%, the CUR0.01% and the CUR0.02% represented the experimental groups fed with 0 (control), 100 mg/kg and 200 mg/kg curcumin, respectively, that is, the amount of curcumin in each 15000g feed was 0, 1.5 and 3g, respectively.

* Vitamin premix (mg kg⁻¹ diet): vitamin A 9000000 (IU kg⁻¹ diet), vitamin K3 600 (IU kg⁻¹ diet), vitamin D 2500000 (IU kg⁻¹ diet), vitamin E 500 (IU kg⁻¹ diet), vitamin B (B1 3200, B2 10900, B5 20000, B6 5000, B12 1160), vitamin C 500000, phasemonomiate 1500, calcium pantothenate 200, nicain 400, folic acid 50, biotin 2.
* Mineral premix (mg kg⁻¹ diet): KCl 70; KI 1.5; MgSO4.7H2O 300; MnSO4.4H2O 3; CuCl2 5; ZnSO4.7H2O 14; CoCl2.6H2O 0.5; FeSO4.7H2O 15; CaCl2 2.8 (g kg⁻¹ diet); KH2PO4.H2O 4.5 (g kg⁻¹ diet). The dietary energy was calculated as carbohydrate: 17.15 MJ kg⁻¹; protein: 23.64 MJ kg⁻¹;120 lipid: 39.54 MJ kg⁻¹.
recovery and subsequently sampled in the fainting state, and the other was sampled as the ammonia nitrogen challenge sample. A schematic representation of the design of the experiment I, experiment II, and measured parameters with different diet feeding is presented in Figure 1.

2.3. Sampling

At the end of the experiment I, fish were starved 24h before sampling, then anesthetized with Eugenol at 50 mg/L. All fish were measured for final body weight (FBW). The front intestine of eight fish was collected from each treatment group, three intestines were fixed in 10% buffered formalin for the analysis of histological observation, and five intestines were immediately aliquoted into the sterile tube, subsequently frozen using liquid nitrogen and finally stored at -80 °C for the analysis of immune enzymes activities and intestinal immune gene expression. At the end of the experiment II, the gill of fish from different treatment groups were sampled and placed in separate centrifuge tubes, then rapidly snap-frozen in liquid nitrogen and stored at -80 °C until analysis.

2.4. Histological observation of intestine

The fixed intestine samples were dehydrated in a graded ethanol series, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin (H&E). Finally, the samples were photographed with a light microscope (100×). We referred to previous studies on fish intestinal histological structure [10, 29]. The degree of variation in the intestinal fold height, enterocyte height (μm), and muscular thickness was evaluated by Caseviewer 2.3. Applying the scale in the bottom right corner of the picture as the standard, five complete folds were selected for each section, their height (μm), enterocyte height (μm), and muscle thickness (μm) were measured.

2.5. Assay of enzyme activities

Intestines and gill tissues were homogenized in ice-cold saline solution (0.86%) in the proportion of 1:9 (w/v). After centrifugation (2500*g, 10min, 4 °C), the supernatants were separated and kept at 4 °C for further analysis. The Acid phosphatase (ACP), Alkaline phosphatase (AKP), Lysozyme (LZM), Superoxide dismutase (SOD) and Glutathione peroxidase (GSH-Px) were analyzed according to the instructions of commercially available kits (Nanjing Jiancheng biotech. Co, Nanjing, China). The protein concentration of the enzyme extracts was measured by using the Bradford method [30].

2.6. Gene expression

The total RNA was isolated from frozen tissue by Trizol Reagent (Invitrogen, Thermo Fisher Scientific Co., Ltd., Shanghai, China). The micro ultraviolet spectrophotometer (ND5000, Biotech Corporation, Beijing, China) and agarose gel electrophoresis were used to determine the concentration and purity of RNA. It was considered effective when the OD260/280 (optical density) was in the range of 1.9–2.1μg. According to the manufacturer’s instructions of One-Step gDNA Removal and cDNA Synthesis SuperMix (EasyScript, Beijing TransGen Biotech., Ltd., Beijing, China), total RNA was extracted for reverse transcription and the and synthesize the first-strand cDNA accordingly. Quantitative Real-time PCR analysis was conducted by the Real-Time PCR System (Q1000, Hangzhou LongGene Scientific Instruments Co., Ltd., Hangzhou, China). Running conditions were performed as follow: initial denaturation at 95 °C for 15 min, 40 cycles of 95 °C for 10s, 60 °C for 20s, 72 °C for 30s. All PCR amplifications were conducted in triplicate with a final volume of 20μL, where the reactions include 2×RealUniversal PreMix (10μL), each PCR primers (0.6μL), 10 μmol/L), template cDNA (2μL), and RNase-free ddH2O. EF1-α and β-Actin were selected as the reference genes after pre-experimentation. The absence of non-specific products was determined by performing dissociation analysis at the end of each PCR reaction. A single peak was observed in the melt curve analysis, indicating a single PCR product. Serial dilutions of 10-fold were made for each primer pair of cDNA to establish a standard curve. PCR reaction efficiencies in the range of 90%–110% were achieved. Table 2 showed the primers used for the analysis. The relative expression of C4, C3, Hepc, IFN-γ, IgT, IL-10, IL-8, IL-1β, NF-xB1, Mx, TGF-β1, and TNF-α were determined in intestine tissue. The relative expression of Keap1, Hsp70, Cyt C, Cu-SOD, and GSH-Px were determined in gill tissue.

2.7. Calculations and statistical analysis

The weight gain and survival of fish were calculated based on the following formulae:

\[ \text{Survival (\%)} = 100 \times \frac{\text{final amount of fish}}{\text{initial amount of fish}} \]

\[ \text{The weight gain (WG, \%)} = 100 \times \frac{\text{final weight-initial weight}}{\text{initial weight}} \]

Figure 1. A schematic representation of the rearing system and measured parameters in S. dumerili feeding different diet.
The relative quantity of gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ method [31]. All data are expressed as the standard deviation (mean ± SD). All data were firstly examined for homogeneity of variance using SPSS Statistic 23.0 software. One-way analysis of variance (ANOVA) was used to test the effect of dietary manipulation when the data had homogeneous variance. The tukey test was used to determine significant differences among treatment groups, and probability values of $P < 0.05$ were deemed to be statistically different.

3. Results

3.1. Survival and growth performance after dietary curcumin supplementation

The final body weight, weight gain, and survival increased first and then decreased with the increase of dietary curcumin supplementation level. Both final body weight and weight gain were higher in the curcumin-containing than that of control group, but there were no significant differences ($P > 0.05$) among the groups. The survival of CUR0.01% group was significantly higher ($P < 0.05$) than that of control group, while the survival of CUR0.02% had no significant difference ($P > 0.05$) (Table 3).

3.2. Intestinal histology structure after dietary curcumin supplementation

The intestinal structure of each groups was shown in Figure 2(a, b, c). The fold height, muscular thickness, and enterocyte height all increased and then decreased with the increase of dietary curcumin supplementation level, and the significantly higher fold height, muscular thickness and enterocyte height was found in the CUR0.01% group than that of control group ($P < 0.05$). When the curcumin content continued to increase, the fold height in the CUR0.02% group was not significantly ($P > 0.05$) different compared to the control group, while the muscular

### Table 2. Primers used for real-time PCR.

| Gene abbreviation | Primer sequence (5'-3') | Amplicon size (bp) | Accession number |
|-------------------|-------------------------|--------------------|------------------|
| C4                | F:ACATCGCAATGGAGGAGAAC  | 170                | XM_022768450.1   |
|                   | R:CAGTCCCGTGATAGGCTTTA  |                    |                  |
| C3                | F:CATCGTTCGCCGATCATAGC  | 81                 | XM_022755728     |
|                   | R:AGTCTGCTGACATCCACCA   |                    | XM_022755434     |
| Hepc              | F:GATGATCCATCCGTCGAGG   | 99                 | XM_022764299.1   |
|                   | R:CAGAAAGCCGCCAGCCTTTG  |                    |                  |
| IFN-γ             | F:TCTGGTGCACCTGCTGTTTC  | 136                | LC146385.1       |
|                   | R:AAAGTGGGTCTCCCTCACT   |                    |                  |
| IgT               | F:CGAGGCTTGGCAGTTGAGAC  | 196                | XM_022756471.1   |
|                   | R:CAGGGCGTGCTTTGGAAGA   |                    |                  |
| IL-10             | F:CAGTGTGTGTTCTGCTGTGAG| 173                | XM_022745101.1   |
|                   | R:TGTGTGTTTACGTGCTGCC   |                    |                  |
| IL-8              | F:GAACGCTGGGAGTGTAGCTG  | 164                | XM_022758559.1   |
|                   | R:GGGCTCTAGGACACCTCCTT  |                    |                  |
| IL-1β             | F:TGTATGAGAATGTTGGAAGA  | 205                | XM_022753745.1   |
|                   | R:GTGACATGTCAGTTGACAC   |                    |                  |
| NF-κB1            | F:CGACAGACATGGCGCATCG   | 185                | XM_022761336.1   |
|                   | R:AGCGCTCTGCTGCTTCTCC  |                    |                  |
| Mx                | F:GAATTCTGACTCCATGTGCTG| 177                | XM_022744797.1   |
|                   | R:GCCTATTTCCGCTACCTCCC  |                    |                  |
| TGF-β1            | F:CGGAGGCTGCGGAGTGTAA   | 111                | XM_022738547.1   |
|                   | R:TGGTGAGAATGCGGGAAG    |                    |                  |
| TNF-α             | F:GAAAAGCTCATGGCGCTCTC | 212                | XM_022746377.1   |
|                   | R:GGTTGCCTCGGAGCAGGTT   |                    |                  |
| EF1α              | F:ATGGCTGCCGGCTGTTTGTT  | 134                | XM_022744048.1   |
|                   | R:TGGTGAGAGTCCAGCTTCTT  |                    |                  |
| β-Actin           | F:TCTGGTGGGGAATGATCTGAGT| 212                | XM_022757055.1   |
|                   | R:GCCTTTCTGCGGATGTTGCC  |                    |                  |
| Keap1             | F:CCTCATAAACACCAAAAG    | 203                | XM_022766859.1   |
|                   | R:CAGGAGTAGAAAAGGCGACT  |                    |                  |
| HSP70             | F:CAAGATATCTGGGTGGG     | 146                | XM_022741879.1   |
|                   | R:TCAAGGCGAGTGGTGCCT    |                    |                  |
| Cyt C             | F:AGGCAGGGGCTACCTTCTTA | 89                 | XM_022748149.1   |
|                   | R:GGGTCTCCAGGCTTCTCCA   |                    |                  |
| Cu-SOD            | F:AGGGCCGCTCACCACCC     | 93                 | XM_022738876.1   |
|                   | R:GCTTCAGGCTCATTACCOC  |                    |                  |
| GSH-Px            | F:ACCAGGGTACTCCCAGCAGAA| 118                | XM_022745698.1   |
|                   | R:CCAGGACGAGCATGACACCA  |                    |                  |

1 Primers of TNF-α, IL-1β, IL-8 and β-Actin were based on the study of Fernández-Montero et al.
2 Primers of IgT and Hepc were based on the study of Fernández-Montero et al. [78, 79].
3 EF1-α and β-Actin were used as reference genes.
thickness and enterocyte height were significantly higher \((P < 0.05)\) (Table 4).

### 3.3. Intestinal immune enzyme activity after dietary curcumin supplementation

Dietary curcumin supplementation level was positively correlated with AKP activity, with the significantly difference \((P < 0.05)\) was found in CUR0.01% and CUR0.02% group. The activity of ACP and LZM all increased and then decreased with the increase of dietary curcumin supplementation level. Compared to the control group, the significantly higher \((P < 0.05)\) ACP and LZM activity was observed in CUR0.01% group, the significantly lower \((P < 0.05)\) LZM activity was observed in CUR0.02% group (Table 5).

### 3.4. Intestinal immune gene expression after dietary curcumin supplementation

The effects of curcumin on immune gene expression were determined by q-RT-PCR in intestine tissues. Q-RT-PCR results suggested that significantly lower \((P < 0.05)\) relative expression of C4, C3, Hecp, IgT, IL-1\(\beta\), NF-\(\kappa B1\) and, TGF-\(\beta\) was found in the curcumin-containing groups compared to the control group (CUR0%). Among them, the relative expression of C4, C3, IgT and NF-\(\kappa B1\) was negatively correlated with the dietary curcumin supplementation level, and there was no significant difference \((P > 0.05)\) in the relative expression of Hecp, IL-1\(\beta\) and TGF-\(\beta\) between the two groups containing curcumin. The relative expression of IFN-\(\gamma\), IL-10, TNF-\(\alpha\) and MX in intestine tissues first increased and then decreased with the increase of dietary curcumin supplementation level.

And the relative expression of IFN-\(\gamma\) was not significantly different in the CUR0.01% group compared to the control group, while the relative expression of IL-10, TNF-\(\alpha\) and MX was significantly higher \((P < 0.05)\). When the dietary curcumin supplementation level continued to rise, the significantly lower \((P < 0.05)\) relative expression of IFN-\(\gamma\), IL-10, and TNF-\(\alpha\) was found in the CUR0.02% group than that of control group while the relative expression of MX have not significant difference \((P > 0.05)\). In addition, the relative expression of IL-8 in the intestine first decreased and then increased with the increase of dietary curcumin supplementation level. And the significantly lower \((P < 0.05)\) relative expression of IL-8 was found in the CUR0.01% group compared to the control group (Figure 3).

### 3.5. The activity of antioxidant enzymes in the gill of S. dumerili after ammonia challenge experiment and recovery experiment

The activity of GSH-Px and SOD enzyme in gill was analysed after ammonia challenge and recovery experiment, respectively (Figure 4 a, b). After ammonia challenge, the significantly higher \((P < 0.05)\) GSH-Px and SOD activity were only found in the CUR0.02% group. After recovery experiment, both GSH-Px and SOD activity were positively correlated with dietary curcumin supplementation level. The significantly higher \((P < 0.05)\) GSH-Px activity was only found in the CUR0.02% group, and significantly higher SOD was found in the curcumin-containing groups. Compared to ammonia challenge phase, the GSH-Px activity increased significantly \((P < 0.05)\) in all groups after recovery, while SOD activity decreased significantly \((P < 0.05)\).

### 3.6. Relative expression of immune-related genes in the gill of S. dumerili after ammonia challenge experiment and recovery experiment

The immune-related genes were quantified in the gill tissue of S. dumerili after ammonia challenge experiment and recovery experiment, respectively (Figure 5 a–c). After ammonia challenge, the significantly lower relative expression of Keap1 was found in curcumin-containing group than that of control group, and the significantly higher \((P < 0.05)\) relative expression of HSP70 and Cu-SOD was only found in the CUR0.02% group. There was no significant difference \((P > 0.05)\) in the relative expression of Cyt C between all groups, and significantly higher \((P < 0.05)\) relative expression of GSH-Px was only found in the CUR0.02% group. The trend in the relative expression of Keap1 gene after the recovery experiment was consistent with that after the ammonia challenge experiment, but there were some changes in the remaining genes. The relative expression of HSP70 genes in the curcumin-supplemented group was not significantly different \((P > 0.05)\) compared to the control group after the recovery trial, with the maximum of HSP70 was found in CUR0.01% group and the minimum of HSP70 was found in CUR0.02% group. The significantly higher \((P < 0.05)\) relative expression of Cyt C was only found in CUR0.02% group. And the significantly higher \((P < 0.05)\) relative expression of Cu-SOD and GSH-Px was found in the curcumin-containing groups than that of control group, with the maximum value was found in the CUR0.01% group. Compared to the ammonia challenge phase, the relative expression of Keap1 in all groups, HSP70 in the CUR0.02% group, Cyt C in the CUR0% and CUR0.01% groups, GSH-Px in the CUR0% group and CUR0.02% group were significantly lower \((P < 0.05)\) after recovery, while the relative expression of Cu-SOD and GSH-Px in the CUR0.01% group were significantly higher \((P < 0.05)\).

### 4. Discussion

#### 4.1. The effects on growth performance after dietary supplementation with curcumin

In this experiment, dietary curcumin supplementation can not significantly improve weight gain in S. dumerili. Previous studies have shown inconsistent results on whether curcumin can significantly improve the growth of aquatic animals. Specific growth rate and daily growth coefficient of pacific white shrimp (Litopenaeus vannamei) were significantly improved when curcumin-loaded chitosan nanoparticles were supplemented in the diet [32]. Dietary supplementation with 5 g/kg curcumin significantly improved the final body weight (FBW), percent weight gain (PWG), and feed efficiency (FE) of crucian carp (Carassius auratus) [33]. In contrast, dietary supplementation with curcumin had no significant effect on the weight gain (WG), specific growth rate (SGR), feed conversion ratio (PCR) of rainbow trout (Oncorhynchus mykiss) [21]. We suspect that this may be related to the species of aquatic animal, the breeding cycle of S. dumerili is usually 1–2 years and the 8-week experiment was short, therefore the difference in growth performance between the treatment groups was not significant. In addition, the results showed that dietary supplementation with the proper rate of curcumin significantly increased the survival rate of fish. This data is consistent with the results of Bhoopathy et al. that dietary supplementation with curcumin-loaded chitosan nanoparticles significantly improved the survival rate of L. vannamei [21]. However, the survival rate of fish did not improve significantly when dietary supplementation with curcumin was above the optimum level, this may be related to the reduction in food intake. When excess curcumin is added to the diet,
Curcumin produces odor and acts as a phytoestrogen, both of these factors can reduce the amount of food taken by fish [34, 35].

4.2. The effects on histology of the intestine after dietary supplementation with curcumin

This experiment showed that dietary supplementation with extracts of Chinese herbs positively improved the intestinal structure of S. dumerili. This is consistent with previous studies that higher intestinal villi height and crypt depth were found in early-weaned pigs which dietary supplementation with a plant extract combination [36]. As the primary site of food digestion, nutrient uptake, and transformation, the intestine is central to physiological functioning, and optimum utilization of dietary nutrients depends on their functional effectiveness [37]. In the result it can be visualized that in the treatment containing curcumin, the intestinal folds appeared significantly curved, resulting in an increase of the contact area of nutrients with the intestinal epithelium as fold height and enterocyte height rises, while the intestinal thickness is a key factor in intestinal motility and absorption [38]. This suggests that curcumin enhances the absorption of nutrients by improving intestinal integrity. This is also consistent with the results of our other experiment in which dietary supplementation with curcumin significantly improved intestinal digestive enzymes activity (lipase and trypsin) [39].

Table 4. Histological structure of the intestine of S. dumerili fed different experimental diets for 8 weeks.

| Parameters          | Diet group | CUR0% | CURR0.01% | CUR0.02% |
|---------------------|------------|-------|-----------|----------|
| Fold height (μm)    |            | 439.71 ± 60.31 b | 591.54 ± 52.93 a | 414.07 ± 33.69 b |
| Muscular thickness (μm) |        | 143.73 ± 11.89 b | 190.80 ± 14.44 a | 174.98 ± 11.74 b |
| Enterocyte height (μm) |        | 11.94 ± 1.35 b  | 17.14 ± 1.52 a  | 16.68 ± 1.97 a  |

Values are means of triplicate groups±SD (n = 3). Within a row, means with different letters are significantly different (ANOVA, P < 0.05).

Table 5. The immune enzyme activities in the intestine of S. dumerili fed different experimental diets for 8 weeks.

| Enzyme activity | Diets | CUR0% | CUR0.01% | CUR0.02% |
|-----------------|-------|-------|----------|----------|
| AKP (U/gprot)   |       | 34482.37 ± 746.10 b | 56316.33 ± 4490.40 a | 60379.50 ± 664.71 a |
| ACP (U/gprot)   |       | 8813.94 ± 589.72 b  | 10957.19 ± 1583.26 a | 9491.80 ± 1477.91 ab |
| LZM (U/ml)      |       | 21.70 ± 0.75 b     | 32.96 ± 1.72 a    | 15.95 ± 1.76 c    |

Values presented as mean ± SD of samples (n = 3). Values of each parameter in the same row with different superscripts are significantly different (ANOVA, P < 0.05).
In addition, curcumin supplementation also enhances intestinal immune enzyme activity. Firstly, the intestinal integrity is critical to fish's health, intestinal epithelium can produce a natural physical barrier and express a large amount of antimicrobial peptides that defend invasion by pathogens [40]. Secondly, curcumin stimulates the innate immune system of fish, which is the mainline of defense against invading pathogens and is more important for fish than mammals [41]. Our results show that dietary supplementation with the right proportion of curcumin significantly increases the activity of AKP, ACP, and LZM in the intestine, which are important indicators of the immune function and health status of the organism. Metabolism in aquatic organisms is regulated in part by phosphorylation and dephosphorylation, and the completion of these processes is mainly catalyzed by different phosphatases. AKP and ACP are involved in the transfer and metabolism of phosphate groups and the hydrolysis of metabolites to phosphate and ethanol [42, 43]. And lysozyme activity is considered to be a major indicator of innate immune defense in fish, with the potential to hydrolyze N-acetylglutamate and N-acetylglycosamine in the peptidoglycan layer of the cell wall of Gram-positive and Gram-negative bacteria, and it also triggers the complement system and phagocytosis through modulators [44]. The ability of curcumin to increase immune enzyme activity has been confirmed in previous studies. For example, the AKP activity in the intestine of crucian carp was significantly increased when curcumin was supplemented at 5 g/kg in the diet [33]. And dietary curcumin supplementation significantly increased lysozyme and malondialdehyde activity in Nile tilapia [45]. We suggested that the ability of dietary curcumin supplementation to improve intestinal immunity is related to the fact that curcumin has antimicrobial effects. Many studies have analyzed the function of curcumin, with Tyagi et al. demonstrating a correlation between bacterial killing and membrane damage caused by curcumin [46] and Baldissera et al. suggesting that curcumin could improve the health of aquatic animals against bacterial pathogens [47].

At the gene level, some inflammation markers (Cytokine and complement) genes were chosen to determine the immune response after dietary supplementation with curcumin by their relative expression. Complement plays an important role in the fish immune system, which can promote phagocytosis, lyses and clears antigen-antibody aggregates, releases bioactive peptides to promote inflammatory responses, and enhances the sensitivity of B cell to antigens [48, 49, 50]. Complement protein C3 and C4 are central roles in the complement system which are classified as acute phase reactants, and it is usually accompanied by upregulation of the relative expression of C3 and C4 during inflammation [51]. The results showed that dietary curcumin supplementation was negatively correlated with the relative expression of C3, C4 gene mRNA, which may indicate that dietary curcumin supplementation significantly alleviates the inflammatory response of the fish intestines.

Dietary supplementation with the proper proportion of curcumin can improve intestinal health conditions by positively regulating cytokines. Cytokines are mainly composed of interleukins (ILs), transforming growth factor-β family (TGF-β), tumor necrosis (TNFs), and interferon (IFN), etc. [52]. Cytokines can also be divided into two types, pro-inflammatory and anti-inflammatory cytokines, the balance of which plays a vital role in maintaining the physiological and immune health of the fish [53]. Previous studies suggested that the organism releases pro-inflammatory cytokines when regulated by physiological adaptive mechanism, which can induce various immune responses by triggering inflammation. However, the excessive inflammatory response may lead to the damage of glial cells and eventually to the death of the organism. Comparatively, the anti-inflammatory cytokine is responsible for eliminating the inflammation reaction response and restoring the organism's return to the normal situation [54, 55, 56]. The results in this experiment showed that dietary supplementation with appropriate levels of curcumin demonstrated the ability to inhibit pro-inflammatory cytokine IL-8,
IL-1β, TNF-α, IFN-γ expression and promote anti-inflammatory cytokine IL-10 expression in the fish intestine. The ability of curcumin to against inflammatory responses has been widely reported. Cao, et al. [57] showed that 0.5% and 1.0% curcumin reduced liver damage in *Cyprinus carpio* by inhibiting the expression of IL-1β, TNF-α, and NF-κB1.

Dietary supplementation with curcumin maintains the healthy intestine by inhibiting the relative expression of NF-κB1 in the intestine. Transcription factor nuclear factor kappa B (NF-κB1) is an important intracellular nuclear transcription factor. It is involved in the organism’s inflammatory response, immune response, and stress response, but over-activation expression of NF-κB1 is often accompanied by diseases such as inflammation and cancer [58, 59]. Previous studies have shown that dietary supplementation with curcumin can block the NF-κB1 pathway to inhibit cytokine expression [60]. Curcumin downregulates the relative expression of NF-κB1 in mice brain induced to oxidative stress by ethanol [61]. Another test on *Oncorhynchus Mykiss* showed the relative
expression of NF-κB1 was suppressed after curcumin supplementation in the diet [60]. In this experiment, the relative expression of NF-κB1 in the fish intestine was negatively correlated with the content of curcumin in the diet. Perhaps the persistence of the inflammatory response in the fish intestine could be avoided more rapidly by dietary curcumin supplementation. Some other immune-related genes (Hepc, Mx, IgT) were also identified in this study. Hepcidin (Hepc) is a member of antimicrobial peptides which have good antimicrobial activity, and the action of pathogenic bacteria often causes an increase of its expression [62, 63, 64]. Immunoglobulin T (IgT) plays an essential role in the mucosal immunity of teleost fish, which is mainly exists in the intestinal mucosa, and intestinal inflammation in teleost fish was able to promote the relative expression of IgT [65]. The results showed that the relative expression of Hepc and IgT was negatively correlated with the curcumin level. The protein encoded by Mx gene is an antiviral protein downstream of the interferon system (IFN) signaling pathway, with multiple antiviral activities and broad-spectrum antibacterial effects, inhibiting a variety of negative-strand RNA viruses [66]. The results showed that the appropriate level of curcumin could significantly increase the relative expression of Mx. This may indicate that dietary curcumin supplementation can improve intestinal immune status by inhibiting pathogenic bacteria and inflammatory responses, as well as improving the ability of the fish intestine to resist viruses. In addition, curcumin is more available and cheaper than astaxanthin and antibiotics. So combining the results of intestinal immune enzymes and immune-related gene expression, dietary supplementation with curcumin supplementation may be a low-cost and effective method to improve the health condition of fish.

4.4. The effects on gill's resistance to ammonia stress and recovery after dietary supplementation with curcumin

The fish gill is very sensitive to external stimuli, as it is directly involved in the exchange of substances between the external environment and the interior of the organism. Many studies have shown that the growth, tissue integrity, and immune capacity are compromised when ammonia concentrations exceed the maximum tolerated of the organism [25, 67]. Dietary supplementation with curcumin can improve the antioxidative capacity of *S. dumerili* gills to reduce damage caused by acute high concentrations of ammonia. Excess reactive oxygen species (ROS) produced by acute ammonia stress may lead to reduced antioxidative capacity and even death in aquatic animals. ROS are primarily synthesized as a part of the immune response against invading pathogens, but the massive accumulation of ROS and the reduced ability of the organism to mitigate them lead to oxidative damage of the host biological molecules including DNA [8, 68]. The ability of mitigating ROS can be demonstrated by antioxidant enzymes, with both Cu/Zn-SOD (Cu/Zn-Superoxide Dismutase) and GSH-Px (Glutathione peroxidase) being the most important ones [69]. SOD converts superoxide anion into hydrogen peroxide, which is subsequently decomposed by GSH-Px to protect cells from severe damage [70, 71]. And transcription factors can be activated by ROS to control antioxidant enzymes, such as Nrf2/Keap1, which is a key signaling pathway of antioxidant damage in the organisms [72]. Under physiological conditions, it binds to the cytosolic chaperone Keap1 to maintain a relatively inhibitory activity. Upon exposure to oxidative stressors, Nrf2 activates a range of downstream antioxidant enzymes such as SOD and CAT, thereby protecting the organism from stress-induced or exacerbated diseases [73]. The results of antioxidant-related genes and antioxidant enzyme activities demonstrate that dietary supplementation with curcumin can maintain health conditions by scavenging ROS and inducing antioxidant reactions directly or indirectly through antioxidant activity in acute with high ammonia concentrations. During the recovery phase, the group containing curcumin still maintained high antioxidant capacity. Similar results were reported that curcumin could alleviate ethanol-induced oxidative stress in the brain of rats by enhancing antioxidant capacity [61]. Curcumin reduces CCl4-induced oxidative stress by enhancing the liver antioxidant capacity of Jian carp [57]. These experiments confirmed that dietary curcumin supplementation could reduce oxidative stress to the organism. But it should be noted that the curcumin content was not proportional to the antioxidant capacity of the gills. During the recovery phase, the antioxidant capacity of the fish gill supplemented with high concentrations of curcumin was lower than the treatment group with low concentrations of curcumin, and more research about the reasons for this phenomenon is necessary.

In addition, dietary supplementation with the 0.02% concentration of curcumin significantly enhanced the resistance of fish gill against ammonia stress and enabled organisms to maintain the high antioxidative capacity and stability during recovery experiments. Hsp70 is a stress protein induced in tissues following various stimuli, which relative expression increases significantly and rapidly. Hsp70 binds to denatured proteins, preventing them from folding further and promoting their recovery or accelerating their degradation to maintain tissue function [74, 75]. And Cyt C is not only essential factors involved in apoptosis but also strongly inhibit the production of H2O2 [76,77]. The relative expression of Cyt C gene rises rapidly to maintain the organism's stability and increase its antioxidant capacity. Our results show that dietary supplementation with 0.02% curcumin not only protects against ammonia stress-induced damage in gill by significantly increasing the relative expression of Hsp70 but also improves the antioxidative capacity and stability of the organism by increasing the relative expression of Cyt C during the recovery phase. However, another of our studies has shown that the optimal content of curcumin supplementation will change due to different tissues of fish, which may be related to the function of different tissues.

5. Conclusion

In conclusion, we assessed the effect of dietary curcumin supplementation on *S. dumerili* by growth performance, intestinal histology structure, intestinal immune enzyme activity, the relative expression of intestinal immune-related genes and resistance of the gill to ammonia stress. The results showed that dietary supplementation with curcumin could have a positive effects on survival rate, intestinal structure and immune capacity, gill resistance to ammonia stress and stress recovery. We believe that our research may contribute to reducing the use of chemical drugs and antibiotics for healthy fish farming, and improving the production efficiency of practical aquaculture farming. However, the assessment of intestinal health is still not comprehensive enough, so further studies are needed such as the effect on the resistance to pathogenic bacteria, the effect on antioxidant capacity and intestinal microbial diversity. This will provide a better reference for practical application.

Ethics statement

No protected species were used during the experiment. This study was conducted in strict accordance with the recommendation in the Animal Welfare Committee of Chinese Academy of Fishery Sciences (approval code: 2020TD55).

Declarations

Author contribution statement

Yuhang He and Zhenhua Ma - Conceived and Designed the experiments; Wrote the paper.
Shiming Dai - Performed the experiments.
Zhengyi Fu - Analyzed and interpreted the data.
Gang Yu - Contributed reagents, materials, analysis tools.
Xiaomei Wang - Wrote the paper.
