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Research Article

Safflower Promotes the Immune Functions of the Common Carp (*Cyprinus carpio* L.) by Producing the Short-Chain Fatty Acids and Regulating the Intestinal Microflora

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To evaluate the functions of safflower on aquatic animal, the effects of dietary safflower supplementation on growth performance, immunity response, short-chain fatty acids, and intestinal microflora of common carp (*Cyprinus carpio* L.) were studied in the present study. Four isonitrogen and isolipid experimental diets were formulated by adding 0, 0.25%, 0.50%, and 1.00% safflower to the basal diet (CON, S-0.25%, S-0.50%, and S-1.00%, respectively). The results showed that safflower supplementation (S-0.50%) significantly improves the growth performance (FBW, WGR, SGR) compared with that of the CON group ($P < 0.05$). Similarly, safflower also promoted the CAT and LZM activities in the midgut, yet the content of MDA in the S-0.50% group was reduced ($P < 0.05$). The content of proinflammatory factors (IL-1β, IL-12) in serum underwent significant reduction ($P < 0.05$); the level of anti-inflammatory factor IL-10 had an upward trend in the S-0.50% group. Moreover, the mRNA expression levels of the *IL-10*, *TGF-β*, *NF-κB*, and *TLR-1* gene in the head kidney were remarkably upregulated ($P < 0.05$) in safflower addition groups, while *IL-6* and *IL-1β* were downregulated significantly in the S-1.00% group ($P < 0.05$). After 96 hours of the *A. hydrophila* challenge test, the survival rate of common carp had been improved greatly with the safflower addition, which was most impressive in the S-1.00% group. Additionally, the levels of acetic acid and butyric acid in intestinal contents were significantly higher in the S-0.50% and S-1.00% groups than in the CON group ($P < 0.05$). Dietary safflower increased the intestinal microbiota abundance of *Fusobacteria* at the phylum level. At the genus level, the abundance of beneficial bacteria (*Lactobacillus* and *Bifidobacterium*) increased significantly in the S-1.00% group; some pathogens such as *Aeromonas*, *Shigella*, and *Streptococcus* were inhibited. Spearman coefficient analysis revealed that the contents of SCFAs were positively correlated with probiotics and negatively correlated with pathogens. In summary, safflower addition at an appropriate dose can promote the immune functions of the common carp by producing the short-chain fatty acids and regulating the intestinal microflora.

1. Introduction

With the expansion and development of aquaculture in China, intensive and large-scale farming gradually replaced the extensive and semiextensive farming model [1–3]. However, the transformation in aquaculture patterns not only brought high yields and profits to the aquaculturist but also brought disaster such as the deterioration of farming environment, the frequent diseases of aquaculture organisms, and other negative problems to the aquaculture during this time [4–6]. Moreover, antibiotics are used for prophylaxis of diseases in intensive aquaculture. Antibiotics can lead to drug residues in aquatic products, and can hazardous human security through bioaccumulation [7–9]. Consequently,

researches have been focused on the nonantibiotic approaches (such as Chinese herbal medicine) to solve these problems which plague large numbers of aquaculturists [10].

Chinese herbal medicines (CHM) play important roles in the prevention and treatment of disease. In addition, it is a widely recognized material heritage [11, 12]. Most CHM grow in the natural environment with many effective ingredients, including polysaccharides, organic acids, alkaloids, saponins, and other active substances, which have the advantages of obvious effect and small side effects [13]. In addition, previous studies have shown that the addition of CHM in aquatic feed can effectively promote growth, enhance immunity, and regulate the intestinal microflora homeostasis [14–18]. Safflower (*Carthamus tinctorius* L.) belongs to the family of Compositae, which is mainly distributed in the Henan, Hunan, and Sichuan provinces of China. And its petals which have a special aroma can be used as traditional Chinese medicine [19, 20]. Similarly, safflower contains many effective ingredients, including flavonoids, pigments, safflower polysaccharide, and organic acids [21, 22]. Furthermore, pharmacological studies have shown that safflower would not only improve blood circulation, inhibit platelet aggregation, lower blood pressure, and regulate immunity but also have the anti-inflammatory and antitumor functions [23–25]. In addition, safflower contains numerous chemical components (secondary metabolites) which can treat gastrointestinal diseases [26].

The gut of fish is colonized by a large number of microorganisms, which has been considered as the second genome of the host [27, 28]. It has been proven that the intestinal flora plays a key role in maintaining and regulating the balance of the intestinal environment, strengthening the host immune defense barrier and resisting the infection of pathogenic bacteria. Short-chain fatty acids, as a metabolite of intestinal flora, play a significant role in maintaining the morphology and function of intestinal epithelial cells [29–31]. In addition, many important physiological pathways associated with gut cells are directly regulated by gut microbes, including the proinflammatory cytokine pathway [32]. Gut microbes and their structural components can also be recognized by the host immune cell's pattern receptors, such as toll-like receptors (TLRs), to stimulate the host immune response [33]. However, the effects of safflower on common carp (*Cyprinus carpio* L.) have not been reported. Therefore, the objective of this present study was to evaluate the effects of safflower as a feed additive to replace antibiotics in aquaculture.

2. Materials and Methods

2.1. Experimental Diets. The experimental safflower was provided free of charge by the Xinfulin safflower planting professional cooperatives in Weihui, Henan province. The processing of safflower was based on the laboratory-owned Chinese herbal medicine treatment methods with some modifications. In brief, sample was washed with sterile water and dried in an oven at a constant temperature (65°C). The dried sample was crushed into powder by a pulveriser and stored at 4°C. The basic diet of common carp was purchased from Henan Tongwei Feed Co., Ltd. The basic diets were crushed into powder and 0% (CON), 0.25% (S-0.25%), 0.5% (S-0.50%), and 1% (S-1.00%) safflower were added to make four kinds of isonitrogen and isolipid diets using a pellet-making machine (F-26 (II), South China University of Technology). Then, the pellets were air-dried at room temperature and stored at 4°C. The ingredients and proximate composition of the experimental diets are presented in Table 1.

2.2. Fish and Feeding Trial. In this study, the experimental common carp were bred in the aquaculture base of the College of Fisheries, Henan Normal University, Xinxiang, China. All the fish were acclimatized for 2 weeks before the trial began in a closed recirculating aquacultural system. At the start of the feeding trial, healthy fish with similar size (average body weight 21.05 ± 1.03 g) were randomly distributed into twelve closed recirculating aquacultural systems with 30 individuals per tank (triplicate tanks per dietary treatment). The fish were fed three times daily (08:30, 13:00, and 17:30) at the same fixed rate of about 3% body weight. The body weight of common carp was recorded every 2 weeks, and the amount of feed was adjusted according to water temperature, feed waste, and death situation. During the 8-week feeding trial, the water quality parameters were recorded as follows: temperature at 26.3 ± 1°C, pH at 6.5-7.5, and dissolved oxygen concentration at 6.0-8.0 mg/L.

2.3. Sample Collection. At the end of the feeding trial, the fish were fasted for 12 h and anaesthetized with MS-222, and the fish from each tank were individually weighed. Sampling procedures were carried out in a clean space using an alcohol burner to create a relatively sterile condition. Three fish per replicate were selected randomly from the caudal vein to draw blood samples. Three fish per replicate were taken out randomly from the intestinal contents for the analysis of short-chain fatty acids and high-throughput sequencing. The contents were collected in sterile condition. The whole intestines were carefully removed, and the mesentery, fat, and other substances were removed with forceps. Then, the contents were aseptically squeezed into sterile tubes. Frozen in liquid nitrogen and store at -80°C. The tissue samples were frozen in liquid nitrogen and removed for storage at −80°C. The head kidney and middle intestine tissue placed in 1.5 mL sterilized tubes of three fish from each barrel were randomly selected for gene expression analysis. The remaining intestine was placed in 5 mL centrifuge tubes for the subsequent intestinal enzyme activity test.

2.4. Biochemical Parameter Analysis

2.4.1. Intestinal Antioxidant-Related Parameters. The midgut samples were homogenized with 10 volumes (w/v) of 0.1 M PBS and centrifuged at 4°C for 5 min (3000 rpm/min). The supernatant was collected and kept frozen at -80°C until analysis. The contents of intestinal malondialdehyde (MDA), total antioxidant enzymes (T-AOC), catalase (CAT), lysozyme (LZM), and superoxide dismutase (SOD) were measured with commercial assay kits (Nanjing Institute of

Table 1: Ingredients and proximate composition (% dry matter) of four experimental diets.

Ingredients	Diet			
	CON	S-0.25%	S-0.50%	S-1.00%
Fish meal	8.00	8.00	8.00	8.00
Soybean meal	34.00	34.00	34.00	34.00
Rapeseed meal	20.00	20.00	20.00	20.00
Rice bran	10.00	10.00	10.00	10.00
Soybean oil	3.00	3.00	3.00	3.00
Fish oil	1.00	1.00	1.00	1.00
Flour	10.00	10.00	10.00	10.00
Wheat bran	10.00	10.00	10.00	10.00
$Ca(H_2PO_4)$	2.00	2.00	2.00	2.00
Vitamin premix[a]	0.40	0.40	0.40	0.40
Mineral premix[b]	0.40	0.40	0.40	0.40
Choline chloride	0.20	0.20	0.20	0.20
Microcrystalline cellulose	1.00	0.75	0.50	0.00
Safflower	0	0.25	0.50	1.00
Total	100	100	100	100
Proximate composition				
Crude protein (%)	40.88	40.53	40.93	40.74
Crude lipid (%)	8.90	8.99	8.62	8.78
Crude ash (%)	10.49	11.01	10.86	10.82

[a]Vitamin premix (mg or IU/kg diet): vitamin A, 6000 IU; vitamin D3, 2000 IU; vitamin E, 50 mg; ascorbic acid, 200 mg; pantothenic acid, 35 mg; nicotinic acid, 30 mg; thiamine, 15 mg; riboflavin, 15 mg; pyridoxine HCl, 6 mg; cyanocobalamin, 0.03 mg; menadione, 5 mg; inositol, 200 mg; folic acid, 3 mg; biotin, 0.2 mg. [b]Mineral premix (mg or g/kg diet): magnesium, 100 mg; iron, 150 mg; zinc, 80 mg; manganese, 20 mg; copper, 4 mg; iodine, 0.4 mg; cobalt, 0.1 mg; selenium, 0.1 mg.

Bioengineering, Nanjing, China) according to the manufacturer's protocol.

2.4.2. Serum Immune Parameters. The concentration of cytokines in the serum was determined by the ELISA method with a polyclonal antibody prepared by our laboratory [34]. In brief, 96-well plates (Corning, USA) were coated with a culture supernatant in 0.05 M carbonate buffer (pH = 9.6) and stored at 4°C overnight. The plates are sealed with 10% skim milk and washed. The first antibodies (TNF-α, IL-1β, IL-6, IL-12, IL-10, TGF-β, 1 : 2000) were added to the plates and incubated at 37°C for 2 h. Next, the plates were washed again, the second antibody (goat anti-rabbit, combined with horseradish peroxide, 1 : 10000) was added. After that, the solution was inclubated at 37°C for 1 h. The signal was produced by the reaction of TMB with 37°C for 30 min. The absorption at 450 nm was measured with a 2 M H_2SO_4 (50 μL) termination reaction using a Microplate Reader (PerkinElmer, USA). The equivalent levels were determined by comparing to reference curves constructed using the corresponding standards. The results were expressed as the concentration of cytokines in the serum (pg/mL).

2.5. RNA Extraction and Quantitative Real-Time PCR (qRT-PCR). Total RNA was extracted from the midgut and head kidney with RNAiso Plus (Takara, Dalian, China); the concentration and integrity were determined by an Ultramicro spectrophotometer (Thermo Scientific, USA) and 1.0% agarose gels. According to the manufacturer's instructions, the RNA sample was reverse transcribed into cDNA by a commercial kit (Takara, Dalian, China). The qPCR primers (Table 2) were designed using Primer 5.0 software based on the gene sequences in GenBank. The *18S* rRNA was selected as the internal standard. Quantitative real-time PCR was performed using Roche LightCycle480® real-time PCR instrument (Roche, Switzerland). The reaction system included 5 μL SYBR® Premix Ex Taq™ (Takara, Dalian, China), 0.3 μL PCR forward and reverse primer, 1 μL cDNA template, and 3.4 μL sterile water. A two-step method was used for amplification: predenaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 20 s. The relative mRNA expression levels of each gene were calculated using $2^{-\Delta\Delta Ct}$ at 3 replications per sample.

2.6. Challenge with A. hydrophila. After 8 weeks, 10 fish from each tank (30 fish each group) were randomly selected. 1 μL/g body weight of *A. hydrophila* ($LD_{50} = 5 \times 10^6$ CFU/mL) was injected intraperitoneally into the CON and safflower-adding groups. *A. hydrophila* was stored in our lab. After LB plate activation culture, the isolated bacteria were cultured in LB liquid medium at 28°C and 200 rpm/min for 18 h. Bacteria solution was centrifuged in 2 mL aseptic centrifuge tube for 5 min at 12000 g. The pellets were washed twice with 0.1 M PBS, and 1.5 mL of 0.1 M PBS was added to suspend bacteria. Concentration of the bacterial solution was calculated by measuring the absorbance with the ultraviolet photometer. The mortality was continuously monitored for 96 h.

2.7. Short-Chain Fatty Acid in the Intestinal Contents. The SCFAs in intestinal contents was performed as described by Meng et al. [35]. Briefly, 100 μL standard solution acetic acid, propionic acid, isobutyric acid, n-butyric acid, isovaleric acid, and valeric acid (Macklin, China) were mixed with ethyl ether to 10 mL and diluted to different concentration gradients and determined by gas chromatography (Agilent Technologies 7890B, America) with an HP-INNOWAX capillary column (30 m × 0.32 mm × 0.5 μm) (Agilent, America). Nitrogen (purity of 99.99%) was used as a carrier gas with a flow rate of 8 mL/min; a volume of 1 μL of each aliquoted sample was automatically injected into the inlet, which was maintained at a temperature of 240°C. The regression equation was plotted using the gas signal value as the horizontal coordinate (x) and the short-chain fatty acid concentration as the vertical coordinate (y). Samples of intestinal contents in our research were processed and tested for SCFAs in accordance with the method described above. The result of the gas signal value was substituted into the standard equation to calculate the SCFA content in the sample.

2.8. Intestinal Microbiota DNA Extraction and Sequence. A genomic DNA extraction kit (QIAamp, Germany) was used to extract DNA from intestinal contents. The quantity and purity of DNA was detected by NanoDrop 2000 (Thermo,

Table 2: Primer sequences for qPCR detection genes.

Gene	Accession no.	Forward (5′→3′)	Reverse (5′→3′)	Production size (bp)
TNF-α	AJ311800	GTGTCTACAGAAACCCTGGA	AGTAAATGCCGTCAGTAGGA	109
TGF-β	AF136947.1	ACGCTTTATTCCCAACCAAA	GAAATCCTTGCTCTGCCTCA	95
IL-12	AJ621425.1	TCTGTAGAGGTCACATATCCACG	AAGTTCGGTTTGGAGCAGTC	116
IL-10	KX964678.1	CGCCAGCATAAAGAACTCGT	TGCCAAATACTGCTCGATGT	103
IL-6	AY102632	GCAGCGCATCTTGAGTGTTTAC	CTGCTGCTCCATCACTGTCTTC	73
IL-1β	AJ245635	TTACAGTAAGACCAGCCTGA	AGGCTCGTCACTTAGTTTGT	91
NF-κB	AY163837.1	AATGTGGTGCGTCTGTGCTT	TGTTGTCATAGATGGGGTTGGA	100
Occludin	XM042765630.1	TGGGTGAATGATGTGAATGG	ATCCGCTGCTCGGCGGGAC	184
Claudin-3	KF193858	GCCAGATGCAGTGTAAGGTC	CCGTCCAGGAGACAGGGAT	240
TLR-1	LC150761.1	TCGCTTCAATACATCG	AAGGAACTGCGGTCA	190
TLR-4	XM019114012.1	TCATTGTGGTCGTGTCTC	ATGTTGGCATTGCGTTCC	100
18S	FJ710826.1	GAGACTCCGGCTTGCTAAAT	CAGACCTGTTATTGCTCCATCT	107

USA). The resulting qualified total DNA samples were diluted to 10 ng/μL in sterile water and used for the high-throughput sequencing of 16S rDNA by Zhongke Lanhai Technology Co., Ltd. (Tianjin, China). The V3-V4 region of the 16S rDNA gene was amplified with the barcoded primers V338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and V806R (5′-GGACTACHVGGGTWTCTAAT-3′). All samples were amplified with the same number of cycles as far as possible to ensure the accuracy and reliability of subsequent data analysis. The PCR products were quantitatively detected by the QuantiFluor™-ST (Promega, China) blue fluorescence quantitative system. Then, the construction of the MiSeq Library and the high-throughput sequencing of Illumina PE 300 were performed. Using USEARCH (version 7.0, http://drive5.com/uparse/) to cluster the sequence, the steps were as follows: the nonrepeated sequence in the optimized sequence was extracted to reduce the redundant computation in the analysis process (http://drive5.com/usearch/manual/dereplication.html). OTU clustering was carried out for nonrepeating sequences (excluding single sequences) according to 97% similarity. Chimeras were removed during the clustering process to obtain representative sequences of OTU. QIIME 2 was used for taxonomic analysis of OTU representative sequences at the level of 97% sequence similarity.

2.9. Calculations and Date Statistical Analysis.

$$\text{Survival rate}\,(\%) = \left(\frac{N_f}{N_i}\right) \times 100,$$

$$\text{Weight gain rate}\,(\text{WGR}, \%) = 100 \times \frac{W_f - W_i}{W_i},$$

$$\text{Feed conversion rate}\,(\text{FCR}) = \frac{\text{FI}}{W_g},$$

$$\text{Specific growth rate}\,(\text{SGR}, \%) = \frac{\ln W_f - \ln W_i}{d} \times 100,$$

$$\text{Condition factor}\left(\text{CF}, \text{g/cm}^3\right) = \frac{W_f}{L_b^3} \times 100,$$

$$\text{Hepatosomatic index}\,(\text{HSI}, \%) = \frac{W_h}{W_f} \times 100,$$

$$\text{Viscerasomatic index}\,(\text{VSI}, \%) = \frac{W_v}{W_f} \times 100, \quad (1)$$

where N_f and N_i are the number of final and initial surviving fish, respectively; W_f and W_i are the final and initial body weights (g), respectively; W_g is $W_f - W_i$; FI is the feed intake; d is the feeding days; W_h and W_v are the fish liver and viscera weight (g), respectively; and L_b is the fish body length (cm).

All data are shown as the mean ± standard deviation (SD). All data were analyzed using one-way analysis of variance (ANOVA) after confirmation of normality and homogeneity of variance, followed by Duncan's multiple range test. Difference was considered significant at $P < 0.05$. SPSS 22.0 (SPSS Inc., Chicago, IL, USA) was employed to perform statistical calculations.

3. Results

3.1. Growth Performance. After 8 weeks of the feeding trial, the FBW, WGR, and SGR increased with safflower up to 0.50% supplement and then decreased, which was significantly higher in the S-0.50% group compared to the CON and S-1.00% groups (Table 3) ($P < 0.05$). There was no significant difference in FCR among the different groups ($P > 0.05$). The VSI in the S-0.50% group was significantly higher ($P < 0.05$) than that in the other groups. No significant differences were observed on the CF and HSI among the different groups.

3.2. Intestinal Antioxidant-Related Parameters. The effects of dietary supplement with safflower on the intestinal antioxidant-related parameters are presented in Table 4. Compared with the CON group, the activity of CAT in the safflower addition groups increased significantly ($P < 0.05$). Similarly, the activity of LZM was lower in the CON group compared with the S-0.50% and S-1.00% groups. The content of MDA was higher in the S-0.50% group compared with the

Table 3: Effects of dietary safflower on growth performance and somatic indexes of common carp.

Index	Group				P value
	CON	S-0.25%	S-0.50%	S-1.00%	
IBW (g)	21.04 ± 1.08	21.06 ± 1.05	21.00 ± 1.04	21.08 ± 0.95	0.472
FBW (g)	89.85 ± 9.36^{a}	93.01 ± 11.01^{ab}	97.89 ± 14.63^{b}	88.87 ± 8.63^{a}	0.013
WGR (%)	327.04 ± 44.49^{a}	341.59 ± 52.28^{ab}	366.09 ± 69.65^{b}	318.63 ± 42.27^{a}	0.006
SGR (%/d)	2.58 ± 0.19^{a}	2.64 ± 0.21^{ab}	2.73 ± 0.27^{b}	2.54 ± 0.17^{a}	0.038
FCR	1.05 ± 0.05	1.10 ± 0.06	1.04 ± 0.03	1.10 ± 0.03	0.260
CF (g/cm^{3})	2.66 ± 0.17	2.69 ± 0.28	2.64 ± 0.36	2.68 ± 0.22	0.916
HSI (%)	1.88 ± 0.30	1.95 ± 0.34	2.21 ± 0.27	2.09 ± 0.35	0.195
VSI (%)	8.66 ± 0.62^{b}	7.57 ± 0.88^{a}	9.90 ± 1.13^{c}	8.65 ± 0.94^{b}	<0.001

Note: values (mean ± SD, $n = 9$) in the same row with different superscripts are significantly different ($P < 0.05$).

Table 4: Effects of dietary safflower on intestinal antioxidant-related parameters of common carp.

Index	Group				P value
	CON	S-0.25%	S-0.50%	S-1.00%	
T-AOC (U/mg prot)	0.87 ± 0.13	0.81 ± 0.11	0.83 ± 0.11	0.77 ± 0.13	0.403
SOD (U/mg prot)	1.04 ± 0.22	1.02 ± 0.26	0.98 ± 0.19	1.22 ± 0.33	0.208
CAT (U/mg prot)	2.72 ± 1.05^{a}	4.53 ± 1.30^{b}	4.25 ± 0.52^{b}	4.71 ± 0.97^{b}	0.001
LZM (U/mg prot)	11.08 ± 2.00^{a}	11.70 ± 0.93^{ab}	14.88 ± 1.25^{b}	12.76 ± 1.32^{b}	<0.001
MDA (nmol/mg prot)	3.26 ± 0.53^{b}	3.39 ± 0.64^{b}	2.56 ± 0.64^{a}	3.29 ± 0.56^{b}	0.022

Note: values (mean ± SD, $n = 9$) in the same row with different superscripts are significantly different ($P < 0.05$).

Table 5: Effects of dietary safflower on expression of cytokine content in serum of common carp.

Index (pg/mL)	Group				P value
	CON	S-0.25%	S-0.50%	S-1.00%	
IL-10	19.48 ± 2.92	21.71 ± 3.94	23.58 ± 5.84	20.29 ± 3.03	0.226
TGF-β	15.74 ± 3.17	16.71 ± 3.14	14.06 ± 1.55	14.32 ± 0.73	0.410
TNF-α	11.83 ± 2.62	11.35 ± 2.13	9.70 ± 1.27	11.16 ± 1.39	0.169
IL-12	83.84 ± 15.10^{b}	51.34 ± 6.61^{a}	51.06 ± 5.36^{a}	53.13 ± 7.08^{a}	0.010
IL-6	70.19 ± 12.53	58.12 ± 6.57	48.86 ± 7.54	62.20 ± 8.13	0.246
IL-1β	639.38 ± 164.66^{b}	513.23 ± 32.55^{ab}	348.46 ± 24.23^{a}	494.26 ± 29.86^{ab}	0.113

Note: values (mean ± SD, $n = 9$) in the same row with different superscripts are significantly different ($P < 0.05$).

other groups. There was no significant difference in the activity of T-AOC and SOD among the different groups ($P > 0.05$).

3.3. Serum Immune Parameters. The effects of dietary supplement with safflower on the proinflammatory cytokines (TNF-α, IL-1β, IL-6, IL-12) and anti-inflammatory cytokines (IL-10, TGF-β) in the serum are presented in Table 5. The contents of proinflammatory cytokines (IL-1β and IL-12) in the S-0.50% group were significantly lower than those in the CON group ($P < 0.05$).

3.4. Survival Rate of Common Carp in A. hydrophila Challenge Test. After 96 h of the *A. hydrophila* challenge test, we calculated the cumulative death to obtain the survival rate. Final survival rates (%) were 20, 45, 45, and 60 in the 0%, 0.25%, 0.50%, and 1.00% safflower-supplemented groups, respectively. Compared to the CON group, the survival rate of fish was significantly increased in all groups treated with safflower (Figure 1).

3.5. Expression Levels of Head Kidney Immune-Related and Intestinal Mucosal Barrier-Related Genes. The mRNA expression levels of immune responses in the head kidney are shown in Figures 2(a)–2(i). The mRNA levels of the anti-inflammatory cytokines *IL-10* and *TGF-β* showed a significant increase ($P < 0.05$) following the addition of the safflower (Figures 2(a) and 2(b)). As shown in Figure 2(c), the mRNA levels of the proinflammatory immune factor *IL-1β* were downregulated in the S-1.00% group ($P < 0.05$), but there was no significant difference in the expression levels of *IL-6*, *TNF-α*, and *IL-12* (Figures 2(d)–2(f)) ($P > 0.05$). The expression of *NF-κB* and *TLR-1* had a

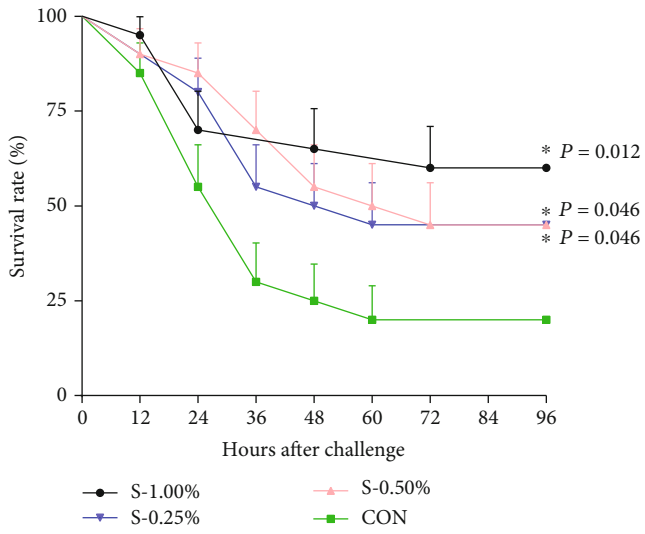

Figure 1: Survival rate (%) of common carp in each group after fish were challenged with *A. hydrophila*. Bars represent the mean values ± SD. "∗" represents a significant difference from CON group ($P < 0.05$).

remarkable increase in the safflower addition groups compared with the CON group ($P < 0.05$) (Figures 2(g) and 2(h)), but *TLR-4* had no significant difference between all groups ($P > 0.05$) (Figure 2(i)).

The mRNA expression levels of intestinal tight junction proteins in the midgut are shown in Figures 3(a)–3(c). Compared to the CON group, the expression level of *Claudin* was significantly increased in the S-0.50% and S-1.00% groups ($P < 0.05$); the expression level of *NF-κB* and *Occludin* had no significant difference between all groups ($P > 0.05$).

3.6. Levels of SCFAs in Intestinal Contents. The result showed that dietary supplementation with safflower (S-0.50% and S-1.00%) remarkably increased the contents of acetic acid (Figure 4(a)) and 4n-butyric acid (Figure 4(b)) ($P < 0.05$). Additionally, the total SCFAs in the S-0.50% and S-1.00% groups were significantly higher than that in the CON group (Figure 4(c)) ($P < 0.05$).

3.7. Intestinal Microbiota Analyses. To evaluate the effect of different concentrations of safflower on the intestinal microbiota of the common carp, high-throughput sequencing of the bacterial 16S rDNA was used to investigate the structural changes. A total of 1,965,804 effective sequences with an average length of 419.83 bp were obtained from the samples. In the four experimental groups, the 496 OTUs were shared between each group (Figure 5(a)). It also presents the group with the maximum number of unique OTUs in the S-1.00% group and the group with the minimum number of unique OTUs in the CON group. The Sobs index (Figure 5(b)) and Shannon indexes (Figure 5(c)), which represent species richness, have a rising tendency in the three safflower-adding groups. The comparison with the CON group showed that the diversity of the intestinal microbiota was remarkably enhanced by adding safflower ($P < 0.05$). In addition, principal coordinate analysis (PCoA) based on OUT level and Bray-Curtis dissimilarity showed that the bacterial flora of the safflower-treated groups was isolated from that of the CON group (Figures 6(a) and 6(b)).

At the phylum level, the average intestinal microbiota community of different groups was dominated by *Proteobacteria*, *Fusobacteria*, *Deinococcus-Thermus*, and *Bacteroidetes* (Figure 7(a)). There was no obvious change in the abundance of *Proteobacteria* among the different groups (Figure 7(b)), the abundance of *Fusobacteria* increased significantly ($P < 0.05$), and the abundance of *Deinococcus-Thermus* decreased remarkably ($P < 0.05$) in the safflower-treated groups. Moreover, the ratio of *Firmicutes*/*Bacteroidetes* decreased in the S-0.50% group (Figure 7(c)).

To further understand the differences in bacterial communities at the genus level, a heat map of 25 abundant bacteria at the genus level is shown in Figure 8. After being fed diets containing safflower, the abundance of *Cetobacterium*, *Romboutsia*, and *Enterococcus* had a significant increase ($P < 0.05$). Some widely recognized probiotics such as *Bifidobacterium* and *Lactobacillus* increased significantly in the S-1.00% group ($P < 0.05$). The abundance of some opportunistic pathogens such as *Aeromonas*, *Shigella*, and *Streptococcus* decreased significantly in the S-1.00% group ($P < 0.05$). These results indicated that there was a significant structural difference in terms of beta diversity between the safflower supplemental group and the CON group.

3.8. Correlation Analysis. Spearman's correlation analysis was used to calculate the association between gut microbial communities and SCFAs and immune and intestinal barrier-related genes. The drawing method was based on the published paper in our laboratory [35]. As shown in Figure 9, the contents of SCFAs and the mRNA level of *Claudin* in the midgut were positively correlated with *p_Fusobacteria*, *g_Cetobacterium*, *g_Lactobacillus*, and *g_Bacteroides* but negatively correlated with *p_Deinococcus-Thermus*, *g_Meiothermus*, *g_Acinetobacter*, and *g_Aeromonas*. The mRNA level of anti-inflammatory cytokines *TGF-β* was positively correlated with *p_Fusobacteria* and negatively correlated with *g_Aeromonas* ($P < 0.01$). The mRNA level of proinflammatory immune factors *IL-6* and *IL-1β* was positively correlated with *p_Deinococcus-Thermus*, *g_Meiothermus*, and *g_Acinetobacter* ($P < 0.01$).

4. Discussion

The common carp is one of the most important freshwater fish in China, in which the amount in China reached 2.885 million tons in 2019, accounting for 11.32% of the total freshwater fish production in China. At present, intensive rearing and abuse of antibiotics have caused many problems such as the development of a drug-resistant bacteria, environmental pollution, and residues in fish. Therefore, it is important of practical significance to search for antibiotic substitutes and develop nontoxic and nonresidual green feed additives [4, 6]. The objective of this study was to investigate the effects of safflower as a feed additive on growth performance, reinforcing immunity, disease resistance, and microbial community of the common carp.

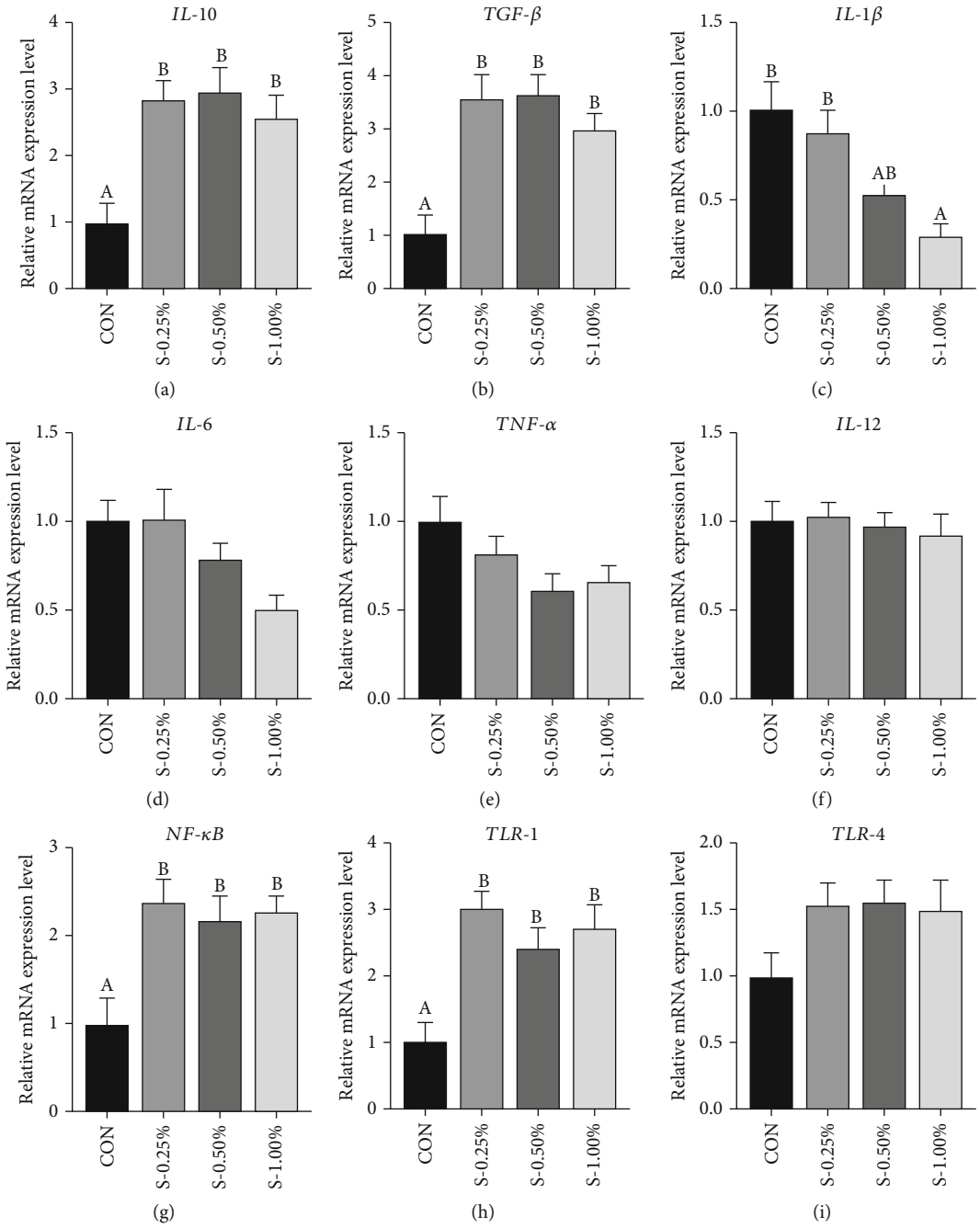

Figure 2: Relative mRNA expression levels of immune responses in the head kidney of common carp fed diets with different safflower contents: (a) *IL-10*; (b) *TGF-β*; (c) *IL-1β*; (d) *IL-6*; (e) *TNF-α*; (f) *IL-12*; (g) *NF-κB*; (h) *TLR-1*; (i) *TLR-4*. Values are given as the mean ± SD ($n = 9$). The mRNA expression level values were normalized to those of *18S* and are expressed as a ratio with the control. Different superscript letters in the values showed significant difference ($P < 0.05$), and the same or unmarked letters showed no significant difference ($P > 0.05$).

In our study, the FBW, WGR, and SGR of the S-0.50% group were significantly higher than those of the CON group. CHM is an excellent source of polysaccharides, which can significantly promote the growth of aquatic animals. Studies have shown that the feed conversion rate of largemouth bass (*Micropterus salmoides*), tilapia (*Oreochromis niloticus*), African catfish (*Clarias gariepinus*), and the common carp (*Cyprinus carpio*) was reduced and the growth performance was improved markedly by adding Chinese herbal medicine polysaccharides such as Astragalus polysaccharides [36], Lentinan polysaccharides [37], aloe vera polysaccharides [38], and Chinese yam polysaccharides [17]. Furthermore, growth enhancement may be related to the safflower polysaccharide (SPS), which is one of the effective components of safflower. The basic components of SPS were glucose, xylose, arabinose, and galactose. Moreover, the

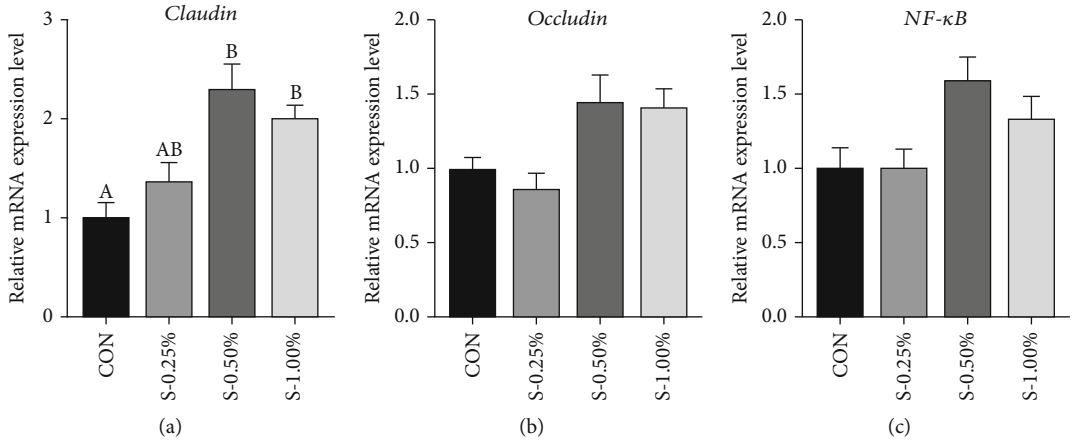

Figure 3: Relative mRNA expression levels of tight binding proteins in the midgut of common carp-fed diets with different safflower contents: (a) *Claudin*; (b) *Occludin*; (c) *NF-κB*. Values are given as the mean ± SD ($n = 9$). The mRNA expression level values were normalized to those of *18S* and are expressed as a ratio with the control. Different superscript letters in the values showed significant difference ($P < 0.05$), and the same or unmarked letters showed no significant difference ($P > 0.05$).

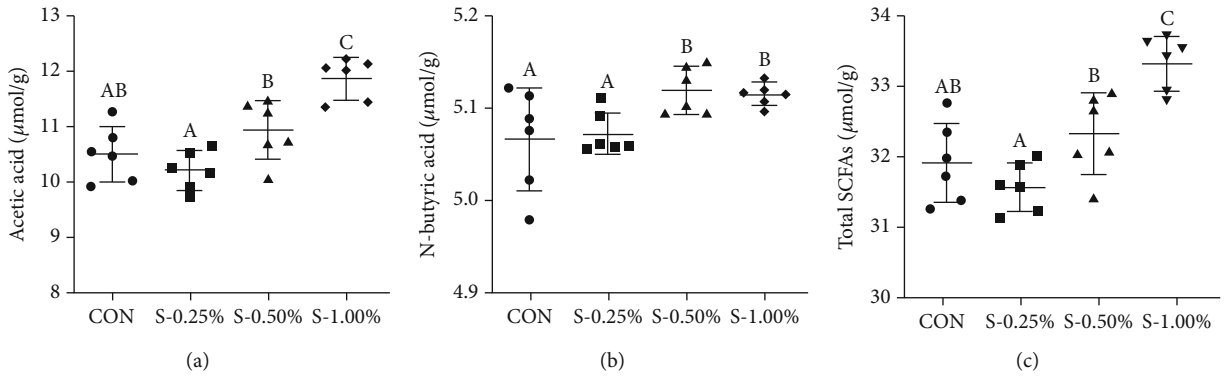

Figure 4: Effect of safflower on acetic acid (a), N-butyric acid (b), and total SCFAs (c). Values are given as the mean ± SD ($n = 6$). Different superscript letters in the values showed significant difference ($P < 0.05$), and the same or unmarked letters showed no significant difference ($P > 0.05$).

study of SPS mainly focused on the regulation of immune function and antitumor effect [39–41]. However, the reasons of growth promotion due to SPS did not have definitive explanation in aquatic animals. Studies on other animals showed that the regulation of safflower on growth performance may be related to the intestinal microbial promoting the decomposition of SPS. And its beneficial metabolites promoted the protection of intestinal epithelial cells, enhancing the intestinal absorption and utilization of nutrients [42]. These results indicate that a suitable inclusion of dietary safflower could improve the growth rate of common carp, but that was depressed by higher inclusion of safflower; the underlying mechanism will be discussed later.

The immune system of animals is mainly composed of specific and nonspecific immunity, and the aquatic animals depend on antioxidant function, which is an important part of nonspecific immunity [43]. Antioxidant enzymes which include CAT and SOD are often used as functional parameters to assess immune potential, prevent or reduce oxidative damage, and protect the body from disease by eliminating oxygen free radicals and reducing oxidative stress [44, 45]. LZM has a notable antagonistic effect on pathogenic bacteria [46]. MDA is the end-product of lipid peroxidation, which causes toxic stress in cells and is used as a biomarker to measure the level of oxidative stress [47]. In this study, the CAT and LZM activities gradually increased at the S-0.50% group, whereas the MDA content was depressed. According to previous researches, the antioxidant activity of safflower is the result of synergistic action of several compounds [48]. And SPS can effectively stimulate the production of NO in macrophages [49]. Therefore, adding safflower to the diet may reduce lipid peroxidation by scavenging oxygen free radicals and enhance the body's nonspecific immunity ability.

The importance of gut health and the development of a stable, healthy gut structure on feed efficiency, overall performance, and productivity have drawn huge attention lately in aquaculture. It is known that the intestinal has tract not only the function of digesting and absorbing nutrients but also the major function of preventing bacteria, endotoxins, and other harmful substances from invading the body (barrier function) [50, 51]. Furthermore, the tight junction protein of the intestine is an important part of the intestinal

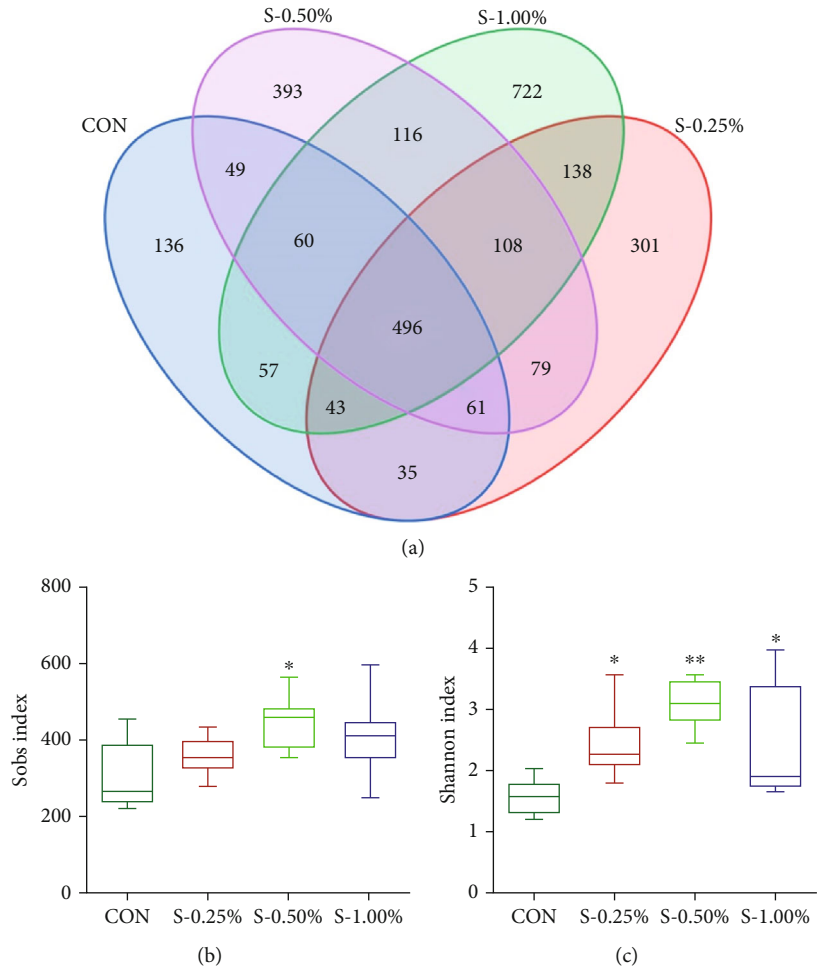

Figure 5: Analysis of intestinal microbial diversity in each group. Venn analysis at OUT level of each group of samples (a). Sobs index of the diversity of the intestinal microbiota (b). Shannon index of the diversity of the intestinal microbiota (c). Different superscript letters in the values showed significant difference ($P < 0.05$), and the same or unmarked letters showed no significant difference ($P > 0.05$).

mucosa barrier. The tight junction protein *Occludin* and *Claudin* can form a stable connection, which is of great significance to the integrity of the barrier of the intestinal mucosa [52, 53]. The promotion of *Astragalus* polysaccharide and *Bletilla striata* polysaccharide on the mRNA expression of *Occludin* and *Claudin* has been reported [54, 55]. In this study, the safflower addition increased the mRNA expression levels of *Occludin* and *Claudin* in the middle gut, suggesting that safflower enhances the protection of the gut barrier, consistent with increased levels of short-chain fatty acids (SCFAs).

SCFAs are carboxylic acids with carbon atom numbers less than 6, which are important metabolites of the gut microbiome [56]. It participates in many physiological regulation processes in the intestine, including protecting the integrity of the intestinal barrier, preventing intestinal inflammation, participating in immune regulation, and improving the homeostasis of intestinal flora [57–59]. The results of this study showed that safflower could regulate the contents of SCFAs, especially acetic acid, butyric acid, and total acid in the intestine contents of the common carp. The interactions between microorganisms and dietary polysaccharides and the resulting SCFAs are important sources of energy and signaling molecules. The SPS in safflower, which can be metabolized by the intestinal flora to produce SCFAs, provide energy for intestinal epithelial cells and enhance intestinal digestion and absorption [60, 61]. Research showed that the increase of SCFAs can decrease the intestinal pH value, inhibit the proliferation of pathogenic microorganisms, and promote the proliferation of probiotics such as *Lactobacillus* and *Bacillus* under acid condition. Eventually, the probiotic metabolites can promote immune function and maintain intestinal health [62, 63].

The inflammatory response is a key element in the innate immune response system and is primarily mediated by cytokines [64]. Cytokines produced by activated macrophages play important roles in the immune system. When induced, they trigger the disease-resistant response of the immune system and enhance the immune response [65–67]. In the present study, it was observed that the expression of proinflammatory cytokines (IL-1β and IL-12) in the S-0.50% group was significantly lower than that in the CON group. There was an upward trend in the anti-inflammatory cytokine (IL-10), but there were was no significant difference.

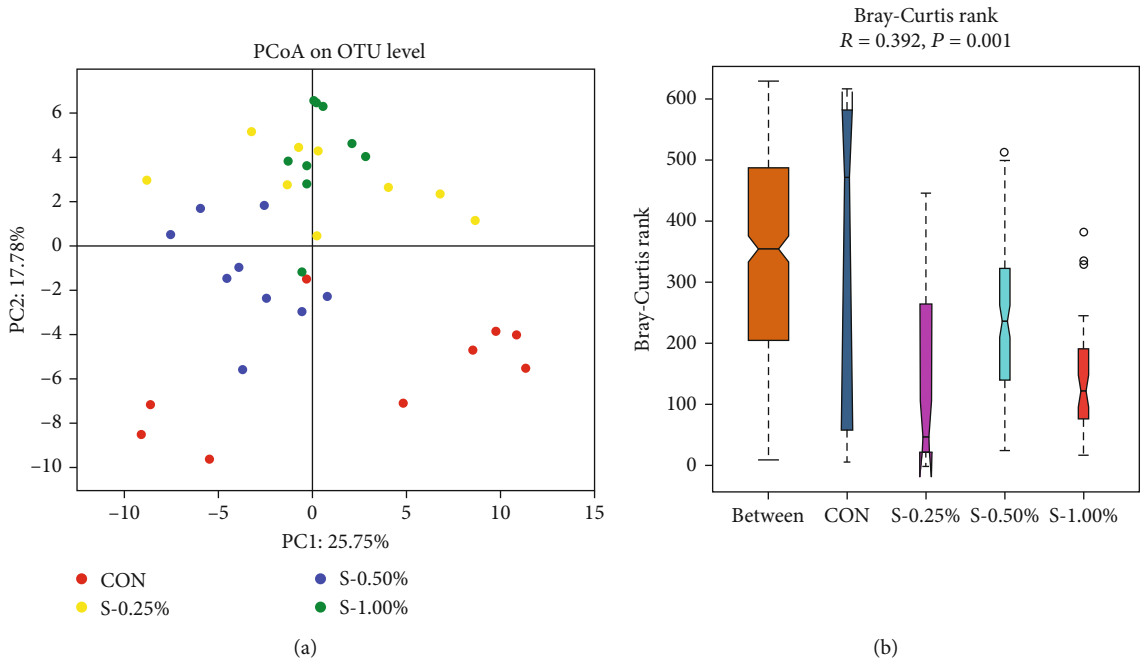

Figure 6: Analysis of intestinal microbial beta diversity in each group. Principal coordinate analysis (PCoA) of the unweighted UniFrac scores of the microbial communities (a). Bray-Curtis rank analysis of the microbial communities (b).

Similar results showed that SPS can induce the production of IL-12, TNF-α, and IFN-g by peritoneal macrophages in a dose-dependent manner [40]. The head kidney is an important immune organ of teleost, in which inflammatory cytokines are highly expressed [68, 69]. Toll-like receptors (TLRs) are discovered as crucial bridge pattern recognition receptors (PRRs) between specific and nonspecific mediators, and they can regulate inflammatory responses through increased regulation of cytokine synthesis. Among them, *TLR4* recognizes and binds to the lipopolysaccharide on the Gram-negative, activating *NF-κB* signal transduction pathway, thus inducing the expression of inflammatory cytokines [70–72]. In the present study, the mRNA expression levels of *NF-κB*, *TLR4*, and anti-inflammatory cytokines *IL-10* and *TGF-β* in the safflower-supplemented group were significantly higher than those in the CON group. In addition, the mRNA expression levels of proinflammatory cytokines *IL-6* and *IL-1β* in the S-1.00% group were significantly decreased than those in the CON group. These results suggest that safflower addition can stimulate the immune reaction of the common carp. It may be attributed to the SPS in safflower; evidences had clearly demonstrated that SPS mainly stimulate *TLR4* and induce *NF-κB* activation by peritoneal macrophages [40]. In addition, previous studies have shown that the polysaccharide as an effective immune stimulus also can participate in the activation of macrophages. Specifically, the receptor binding of *TLR4*/*NF-κB* to target cells promotes the production of inflammatory cytokines and stimulates the immune response [73–75]. Meanwhile, according to the challenge test of *A. hydrophila*, safflower addition significantly increased the survival rate of the common carp, and this result also directly reflected the improvement of immunity and disease resistance of the common carp [76]. However, the molecular basis of these signal transduction pathways activated by plant polysaccharides remains to be studied due to the lack of identification of a specific cell surface receptor.

The intestinal microbiota is important for dynamic balance which guarantees a certain homeostasis in the immune system, which prevents the invasion and colonization of pathogens in many ways [77]. Meanwhile, many studies have shown that the addition of prebiotics, such as the polysaccharides from plant, to aquaculture feed instead of antibiotics, can enhance the immune system by regulating the intestinal flora [78]. The yam polysaccharide and the polysaccharide from fermented wheat bran reshaped the intestinal microflora of the common carp [17, 79]. In this study, the increase of the α diversity parameter and the results of the PCoA cluster analysis indicated that safflower addition changed the intestinal microflora structure of the common carp. At the phylum level, it can be seen that the intestinal microflora of the common carp is mainly composed of *Proteobacteria*, *Fusobacteria*, *Deinococcus-Thermus*, and *Bacteroides*. All *Proteobacteria* are Gram-negative, including many pathogens such as *E. coli*, *Salmonella*, *Vibrio cholerae*, and *Helicobacter pylori*, and have been proposed as the potential signature of dysbiosis and risk of disease [80]. Furthermore, studies have shown that the presence of *Fusobacteria* can activate the host's inflammatory response, thereby protecting the host from pathogens that promote tumor growth [81]. *Deinococcus-Thermus* (such as *Thermus*) are known for their resistance to extreme stresses including oxidation and high temperature [82]. The ratio of *Firmicutes*/*Bacteroidetes* is widely considered to have an important effect on the maintenance of normal intestinal homeostasis. The increase of the proportion is usually associated with obesity. And the

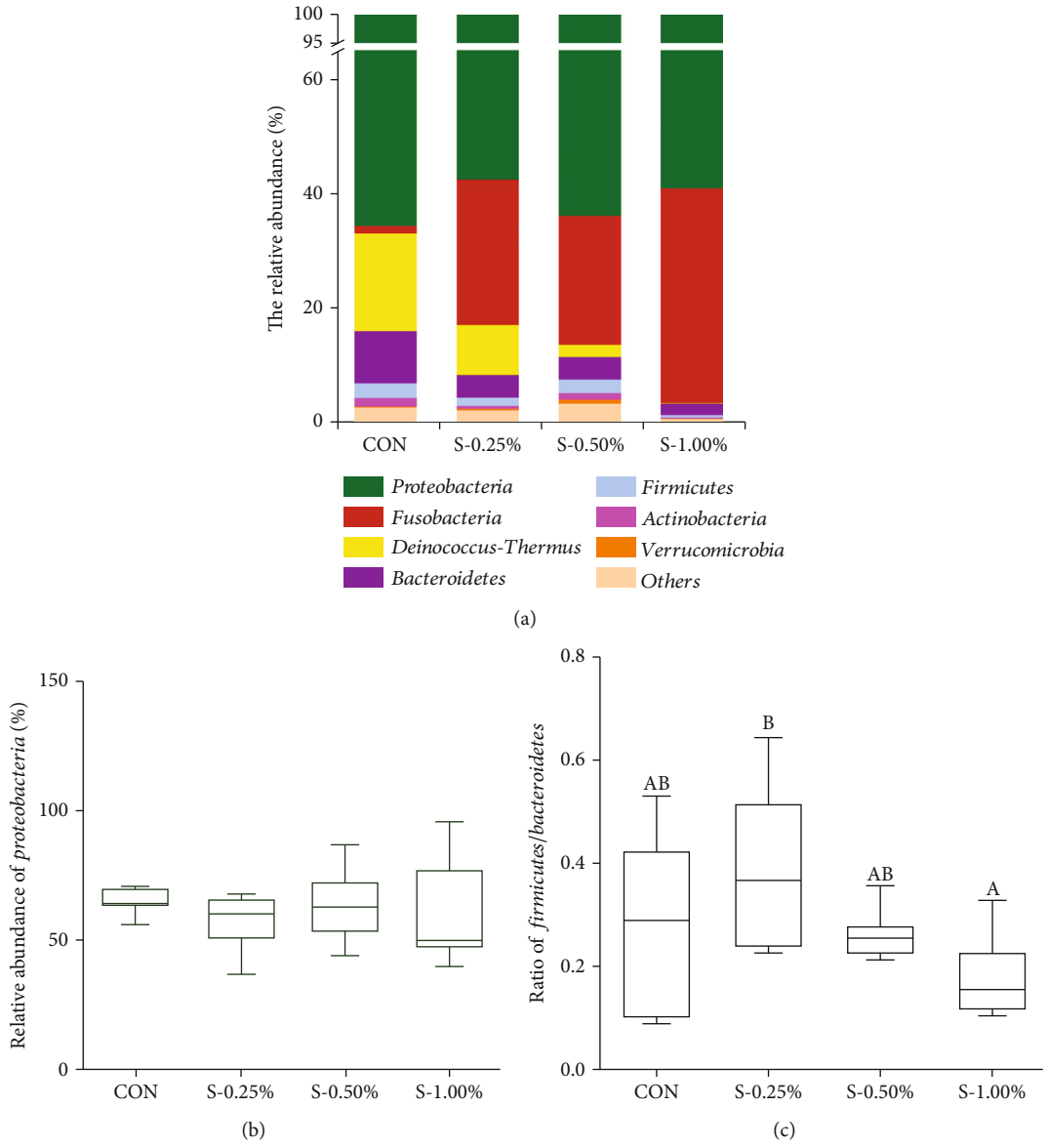

Figure 7: The bacterial composition of the different communities at the phylum level. Relative abundances of major phyla (a). Relative abundance of *Proteobacteria* (b). Ratio of *Firmicutes* to *Bacteroidetes* (c). Different superscript letters in the values showed significant difference ($P < 0.05$), and the same or unmarked letters showed no significant difference ($P > 0.05$).

decrease of the proportion is associated with inflammatory bowel disease [83]. In the present study, the abundance of *Proteobacteria* and *Deinococcus-Thermus* had a decreasing trend, and the ratio of *Firmicutes*/*Bacteroidetes* was reduced in the S-0.50%- and S-1.00%-treated groups. These results indicated that safflower altered the homeostasis and community composition of the intestinal microflora.

Analysis of the microbial composition of the gut at the genus level revealed that the abundance of *Cetobacterium* increased in the safflower addition group. Cetobacterium is a dominant indigenous bacterium in the mucus of fish gut. It colonizes effectively in the gut, and helps the gut to form a microbial barrier [84]. What is more, *Cetobacterium* has many beneficial effects which can efficiently produce vitamin B12, metabolize carbohydrates that produce mainly acetic acid, and produce trace amounts of butyric, propionic, lactic, and succinic acids [85]. Probiotics, such as *Lactobacillus* and *Bacillus*, participate in the process of feed digestion by releasing a large number of digestible enzymes and nutrients, reduce the content of antinutritional factors, and absorb the nutrients in the feed effectively, thus improving the health of the host [86]. A study showed that *Lactobacillus* could effectively improve the disease resistance, antioxidant capacity, and growth performance of *Cyprinus carpio* Huanghe var [87]. In the present study, the abundance of *Lactobacillus* and *Bacillus* was also significantly increased following safflower addition. The use of these host-related probiotics (from the environment in which the aquatic animal lives or from the host animal itself) has attracted attention; its benefits include improving growth performance,

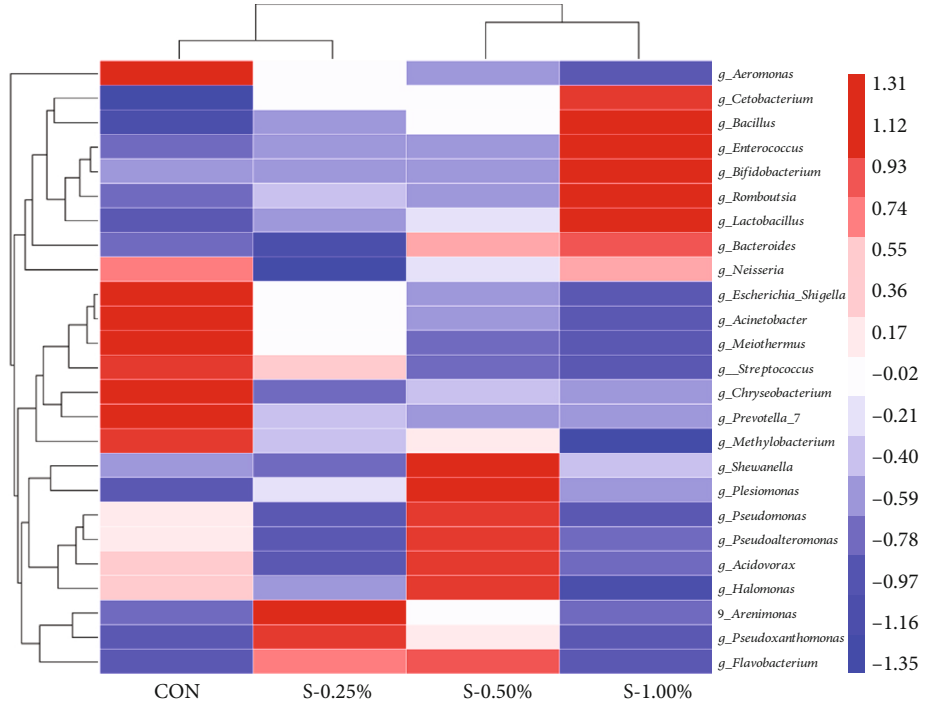

Figure 8: Changes in abundance of intestinal microbiota at the genus level. The heat maps of the specimens show the relative abundances of the main identified bacteria at the genus taxonomic level. Red indicates a higher relative abundance, whereas blue indicates a lower relative abundance.

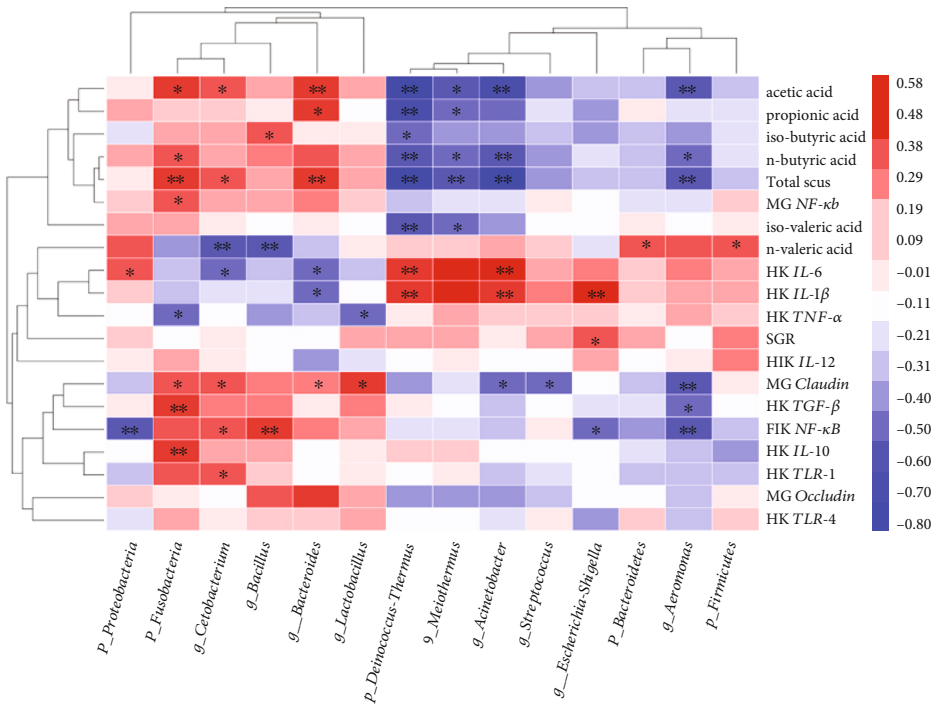

Figure 9: Correlations between the representative microbial genera, SCFAs, and immune- related genes in the head kidney and tight binding proteins in the midgut. The drawing method was based on the published paper in our laboratory [35]. Heat map constructed according to Spearman correlation coefficients. Red represents a positive correlation, and blue represents a negative correlation. "∗" represents $P < 0.05$; "∗∗" represents $P < 0.01$.

feed value, and digestion [88]; inhibiting the adhesion and colonization of pathogenic microorganisms in the gastrointestinal tract [89]; and increasing blood parameters and immune response [90]. Correspondingly, a large number of pathogenic bacteria are presented in the intestines of fish; in our search, the abundance of *Aeromonas*, *Streptococcus*, and *Prevotella* in the safflower-treated group were lower than that in the CON group. *Aeromonas* is not only the primary pathogen of various aquatic animals but also a typical human-animal-fish coexisting pathogen [91]. The ability of fish to resist disease is tested by observing the survival rate by intraperitoneal *Aeromonas* [92]. The decrease of pathogenic bacteria revealed the protective effect of safflower on the intestine of the common carp, which was consistent with the enhancement of immunity and anti-*Aeromonas* of the common carp mentioned above. Similar results were also reported in the previous studies [93]. The results showed that safflower could inhibit the growth of harmful bacteria by increasing the number of probiotics and reshape the intestinal flora to enhance the immunity of the body. However, some recent studies suggest that strains belonging to *Aeromonas* and *Shewanella* may also play an important role in the gut as potential probiotics in aquaculture at the species level [94, 95]. Therefore, the relationship between intestinal microbiota changes and body health remains an area for further study.

In this research, Spearman coefficient analysis revealed that the contents of acetic acid and total SCFAs were positively correlated with *p_Fusobacteria*, *g_Cetobacterium*, *g_Bacillus*, and *g_Bacteroides* and were negatively correlated with *p_Deinococcus-Thermus*, *g_Meiothermus*, *g_Acinetobacter*, and *g_Aeromonas*. The expression results of the tight junction protein-related genes (*Claudin* and *Occludin*) were similar. Polysaccharides from safflower are the primary energy source for *g_Bacteroides* and can induce multiple glucosidase activities from it, which regulate the diversity of the gut microbiome, promote the interaction of the gut microbiome, and ultimately contribute to immunity. However, the mechanisms of correlation between these factors are important for future study and need to be further explored.

In summary, adding safflower at an appropriate dose (0.50%) can promote growth performance and improve immunity function by regulating the abundance of beneficial bacteria and pathogenic bacteria of common carp. In addition, adding safflower can increase the contents of SCFAs in the intestinal contents of common carp. This study provided a theoretical basis for the application of safflower as an immunostimulant in aquatic feed.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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References

[1] P. da Costa, L. Loureiro, and A. Matos, "Transfer of multidrug-resistant bacteria between intermingled ecological niches: the interface between humans, animals and the environment," *International Journal of Environmental Research and Public Health*, vol. 10, no. 1, pp. 278–294, 2013.

[2] C. D. Golden, K. L. Seto, M. M. Dey et al., "Does aquaculture support the needs of nutritionally vulnerable nations?," *Frontiers in Marine Science*, vol. 4, p. 159, 2017.

[3] P. Wang, J. Ji, and Y. Zhang, "Aquaculture extension system in China: development, challenges, and prospects," *Aquaculture Reports*, vol. 17, article 100339, 2020.

[4] F. C. Cabello, "Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment," *Environmental Microbiology*, vol. 8, no. 7, pp. 1137–1144, 2006.

[5] S. M. Limbu, L. Zhou, S. Sun, M. Zhang, and Z. Y. du, "Chronic exposure to low environmental concentrations and legal aquaculture doses of antibiotics cause systemic adverse effects in Nile tilapia and provoke differential human health risk," *Environment International*, vol. 115, pp. 205–219, 2018.

[6] P. Arechavala-Lopez, C. Diaz-Gil, J. L. Saraiva et al., "Effects of structural environmental enrichment on welfare of juvenile seabream (*Sparus aurata*)," *Aquaculture Reports*, vol. 15, p. 100224, 2019.

[7] M. Biošić, M. Mitrevski, and S. Babić, "Environmental behavior of sulfadiazine, sulfamethazine, and their metabolites," *Environmental Science and Pollution Research International*, vol. 24, no. 10, pp. 9802–9812, 2017.

[8] E. Wang, Z. Yuan, K. Wang, D. Gao, Z. Liu, and M. R. Liles, "Consumption of florfenicol-medicated feed alters the composition of the channel catfish intestinal microbiota including enriching the relative abundance of opportunistic pathogens," *Aquaculture*, vol. 501, pp. 111–118, 2019.

[9] G. Zhang, Y. Zhang, H. Wang, L. Xu, and L. Lv, "Comparative genomic analysis of five high drug-resistance *Aeromonas hydrophila* strains induced by doxycycline in laboratory and nine reference strains in Genbank," *Aquaculture Research*, vol. 49, no. 7, pp. 2553–2559, 2018.

[10] J. Galina, G. Yin, L. Ardó, and Z. Jeney, "The use of immunostimulating herbs in fish. An overview of research," *Fish Physiology and Biochemistry*, vol. 35, pp. 669–676, 2009.

[11] C. Lai, J. Chiang, J. Lin, H. Yen, C. Tu, and Y. Chen, "Chinese herbal medicine reduced the risk of stroke in patients with Parkinson's disease: a population-based retrospective cohort study from Taiwan," *PLoS One*, vol. 13, no. 9, article e0203473, 2018.

[12] S. Lyu, G. Guo, L. Mo et al., "Research progress in prevention and treatment of bacterial diseases by traditional Chinese medicine," *Agricultural Biotechnology*, vol. 5, no. 4, pp. 38–41, 2016.

[13] H. Pu, X. Li, Q. Du, H. Cui, and Y. Xu, "Research progress in the application of Chinese herbal medicines in aquaculture: a review," *Engineering*, vol. 3, no. 5, pp. 731–737, 2017.

[14] B. Gao, R. Wang, Y. Peng, and X. Li, "Effects of a homogeneous polysaccharide from Sijunzi decoction on human intestinal microbes and short chain fatty acids *in vitro*," *Journal of Ethnopharmacology*, vol. 224, pp. 465–473, 2018.

[15] B. Hu, L. Song, S. Mao, and P. Xu, "Effects of four Chinese herbal preparations on growth performance and antioxidant activity in juvenile *Micropterus salmoides*," *Journal of Guangdong Ocean University*, vol. 39, no. 4, pp. 101–107, 2019.

[16] Y. Liu, Y. Miao, N. Xu et al., "Effects of dietary *Astragalus* polysaccharides (APS) on survival, growth performance, activities of digestive enzyme, antioxidant responses and intestinal development of large yellow croaker (*Larimichthys crocea*) larvae," *Aquaculture*, vol. 517, article 734752, 2020.

[17] X. Meng, W. Hu, S. Wu et al., "Chinese yam peel enhances the immunity of the common carp (*Cyprinus carpio* L.) by improving the gut defence barrier and modulating the intestinal microflora," *Fish and Shellfish Immunology*, vol. 95, pp. 528–537, 2019.

[18] Q. Zhai and J. Li, "Effectiveness of traditional Chinese herbal medicine, San-Huang-San, in combination with enrofloxacin to treat AHPND-causing strain of *Vibrio parahaemolyticus* infection in *Litopenaeus vannamei*," *Fish and Shellfish Immunology*, vol. 87, pp. 360–370, 2019.

[19] X. Yang, Y. Li, H. Zhang et al., "Safflower yellow regulates microglial polarization and inhibits inflammatory response in LPS-stimulated Bv2 cells," *International Journal of Immunopathology and Pharmacology*, vol. 29, no. 1, pp. 54–64, 2016.

[20] X. Zhou, L. Tang, Y. Xu, G. Zhou, and Z. Wang, "Towards a better understanding of medicinal uses of *Carthamus tinctorius* L. in traditional Chinese medicine: a phytochemical and pharmacological review," *Journal of Ethnopharmacology*, vol. 151, no. 1, pp. 27–43, 2014.

[21] Y. Tu, Y. Xue, D. Guo, L. Sun, and M. Guo, "*Carthami flos*: a review of its ethnopharmacology, pharmacology and clinical applications," *Revista Brasileira de Farmacognosia*, vol. 25, no. 5, pp. 553–566, 2015.

[22] S. Yue, Y. Tang, C. Xu, S. Li, Y. Zhu, and J. Duan, "Two new quinochalcone C-glycosides from the florets of Carthamus tinctorius," *International Journal of Molecular Sciences*, vol. 15, no. 9, pp. 16760–16771, 2014.

[23] S. H. Lee, H. S. Lillehoj, R. A. Heckert et al., "Immune enhancing properties of safflower leaf (*Carthamus tinctorius*) on chicken lymphocytes and macrophages," *The Journal of Poultry Science*, vol. 45, no. 2, pp. 147–151, 2008.

[24] H. Yin, Z. He, and Y. Ye, "Studies on chemical constituents of Carthamus tinctorius," *Chinese Traditional and Herbal Drugs*, vol. 32, no. 9, pp. 11–13, 2001.

[25] D. Zhou, Z. Qu, H. Wang et al., "The effect of hydroxy safflower yellow A on coronary heart disease through Bcl-2/Bax and PPAR-γ," *Experimental and Therapeutic Medicine*, vol. 15, no. 1, pp. 520–526, 2018.

[26] S. S. Gomashe, K. P. Ingle, Y. A. Sarap, D. Chand, and S. Rajkumar, "Safflower (*Carthamus tinctorius* L.): an underutilized crop with potential medicinal values," *Annals of Phytomedicine*, vol. 10, no. 1, pp. 242–248, 2021.

[27] L. V. Hooper and J. I. Gordon, "Commensal host-bacterial relationships in the gut," *Science*, vol. 292, no. 5519, pp. 1115–1118, 2001.

[28] J. K. Nicholson, E. Holmes, J. Kinross et al., "Host-gut microbiota metabolic interactions," *Science*, vol. 336, no. 6086, pp. 1262–1267, 2012.

[29] W. S. Garrett, J. I. Gordon, and L. H. Glimcher, "Homeostasis and inflammation in the intestine," *Cell*, vol. 140, no. 6, pp. 859–870, 2010.

[30] J. Wong, R. de Souza, C. Kendall, A. Emam, and D. Jenkins, "Colonic health: fermentation and short chain fatty acids," *Journal of Clinical Gastroenterology*, vol. 40, no. 3, pp. 235–243, 2006.

[31] Y. Bhattarai, J. Si, M. Pu et al., "Role of gut microbiota in regulating gastrointestinal dysfunction and motor symptoms in a mouse model of Parkinson's disease," *Gut Microbes*, vol. 13, no. 1, p. 1, 2021.

[32] Y. Xia, Y. Chen, G. Wang et al., "*Lactobacillus plantarum* AR113 alleviates DSS-induced colitis by regulating the TLR4/MyD88/NF- κB pathway and gut microbiota composition," *Journal of Functional Foods*, vol. 67, article 103854, 2020.

[33] A. Iwasaki and R. Medzhitov, "Toll-like receptor control of the adaptive immune responses," *Nature Immunology*, vol. 5, no. 10, pp. 987–995, 2004.

[34] J. Feng, X. Chang, Y. Zhang, X. Yan, J. Zhang, and G. Nie, "Effects of *Lactococcus lactis* from *Cyprinus carpio* L. as probiotics on growth performance, innate immune response and disease resistance against *Aeromonas hydrophila*," *Fish & Shellfish Immunology*, vol. 93, pp. 73–81, 2019.

[35] X. Meng, S. Wu, W. Hu et al., "Clostridium *butyricum* improves immune responses and remodels the intestinal microbiota of common carp (*Cyprinus carpio* L.)," *Aquaculture*, vol. 530, pp. 735–753, 2021.

[36] S. Lin, Y. Jiang, Y. Chen, L. Luo, S. Doolgindachbaporn, and B. Yuangsoi, "Effects of *Astragalus* polysaccharides (APS) and chitooligosaccharides (COS) on growth, immune response and disease resistance of juvenile largemouth bass, *Micropterus salmoides*," *Fish & Shellfish Immunology*, vol. 70, pp. 40–47, 2017.

[37] M. Ahmed, N. Abdullah, A. S. Shuib, and S. Abdul Razak, "Influence of raw polysaccharide extract from mushroom stalk waste on growth and pH perturbation induced-stress in Nile tilapia, *Oreochromis niloticus*," *Oreochromis niloticus. Aquaculture*, vol. 468, no. 1, pp. 60–70, 2017.

[38] N. N. Gabriel, M. R. Wilhelm, H.-M. Habte-Tsion et al., "Effect of dietary *Aloe vera* polysaccharides supplementation on growth performance, feed utilization, hemato-biochemical parameters, and survival at low pH in African catfish (*Clarias gariepinus*) fingerlings," *International Aquatic Research*, vol. 11, pp. 57–72, 2019.

[39] D. Cui, D. Zhao, and S. Huang, "Structural characterization of a safflower polysaccharide and its promotion effect on steroid-induced osteonecrosis in vivo," *Carbohydrate Polymers*, vol. 233, article 115856, 2020.

[40] I. Ando, Y. Tsukumo, T. Wakabayashi et al., "Safflower polysaccharides activate the transcription factor NF-κB via Toll- like receptor 4 and induce cytokine production by macrophages," *International Immunopharmacology*, vol. 2, no. 8, pp. 1155–1162, 2002.

[41] A. Ren, Y. Lu, Y. Zou, and H. Shen, "Separation, purification and preliminary structure analysis of acidic polysaccharides from safflower," *European Food Research and Technology*, vol. 237, no. 3, pp. 449–455, 2013.

[42] Z. Zuo, B. Shang, Y. Shao, W. Li, and J. Sun, "Screening of intestinal probiotics and the effects of feeding probiotics on the growth, immune, digestive enzyme activity and intestinal flora of *Litopenaeus vannamei*," *Fish & Shellfish Immunology*, vol. 86, pp. 160–168, 2018.

[43] F. Zhu, "A review on the application of herbal medicines in the disease control of aquatic animals," *Aquaculture*, vol. 526, article 735422, 2020.

[44] M. Ghelichpour, A. T. Mirghaed, S. M. Hoseini, and A. P. Jimenez, "Plasma antioxidant and hepatic enzymes activity, thyroid hormones alterations and health status of liver tissue in common carp (*Cyprinus carpio*) exposed to lufenuron," *Aquaculture*, vol. 516, article 734634, 2019.

[45] A. Moure, J. M. Cruz, D. Franco et al., "Natural antioxidants from residual sources," *Food Chemistry*, vol. 72, no. 2, pp. 145–171, 2001.

[46] Y. Kong, Y. Kang, J. X. Tian et al., "Oral immunization with recombinant *Lactobacillus casei* expressing flaB confers protection against *Aeromonas veronii* challenge in common carp, *Cyprinus carpio*," *Fish and Shellfish Immunology*, vol. 87, pp. 627–637, 2019.

[47] M. Yousefi, S. V. Shabunin, Y. A. Vatnikov et al., "Effects of lavender (*Lavandula angustifolia*) extract inclusion in diet on growth performance, innate immunity, immune-related gene expression, and stress response of common carp, *Cyprinus carpio*," *Aquaculture*, vol. 515, article 734588, 2020.

[48] M. Jin, J. R. Li, Y. X. Cai, J. Liu, and L. P. Qin, "Antioxidative effect of aqueous components of flower *Carthamus tinctorius* L," *Journal of Cardiovascular and Pulmonary Diseases*, vol. 17, no. 4, pp. 277–279, 1998.

[49] T. Wakabayashi, S. Hirokawa, N. Yamauchi, T. Kataoka, J. T. Woo, and K. Nagai, "Immunomodulating activities of polysaccharide fractions from dried safflower petals," *Cytotechnology*, vol. 25, no. 1-3, p. 205, 1997.

[50] Z. Mujagic, P. de Vos, M. V. Boekschoten et al., "The effects of *Lactobacillus plantarum* on small intestinal barrier function and mucosal gene transcription; a randomized double-blind placebo controlled trial," *Scientific Reports*, vol. 7, no. 1, article 40128, 2017.

[51] W. W. Xiao, W. D. Jiang, L. Feng et al., "Effect of dietary enzyme-treated soy protein on the immunity and antioxidant status in the intestine of juvenile jian carp (*Cyprinus carpio* var. jian)," *Aquaculture Research*, vol. 50, no. 5, pp. 1411–1421, 2019.

[52] L. González-Mariscal, A. Betanzos, P. Nava, and B. E. Jaramillo, "Tight junction proteins," *Progress in Biophysics and Molecular Biology*, vol. 81, no. 1, pp. 1–44, 2003.

[53] P. Ugalde-Silva, O. Gonzalez-Lugo, and F. Navarro-Garcia, "Tight junction disruption induced by type 3 secretion system effectors injected by Enteropathogenic and Enterohemorrhagic Escherichia coli," *Frontiers in Cellular and Infection Microbiology*, vol. 6, p. 87, 2016.

[54] L. Lei, Z. Zhang, J. Xue et al., "Bletilla striata polysaccharide has a protective effect on intestinal epithelial barrier disruption in TAA-induced cirrhotic rats," *Experimental and Therapeutic Medicine*, vol. 16, no. 3, pp. 1715–1722, 2018.

[55] X. Wang, Y. Li, X. Yang, and J. Yao, "Astragalus polysaccharide reduces inflammatory response by decreasing permeability of LPS-infected Caco2 cells," *International Journal of Biological Macromolecules*, vol. 61, pp. 347–352, 2013.

[56] Y. Yao, X. Cai, W. Fei, Y. Ye, M. Zhao, and C. Zheng, "The role of short-chain fatty acids in immunity, inflammation and metabolism," *Critical Reviews in Food Science and Nutrition*, vol. 1-12, pp. 1–12, 2020.

[57] R. Corrêa-Oliveira, J. L. Fachi, A. Vieira, F. T. Sato, and M. A. R. Vinolo, "Regulation of immune cell function by short-chain fatty acids," *Clinical & Translational Immunology*, vol. 5, no. 4, article e73, 2016.

[58] Z. Li, N. T. Tran, P. Ji, Z. Sun, X. Wen, and S. Li, "Effects of prebiotic mixtures on growth performance, intestinal microbiota and immune response in juvenile chu's croaker, *Nibea coibor*," *Fish & Shellfish Immunology*, vol. 89, pp. 564–573, 2019.

[59] P. Liu, Y. Wang, G. Yang et al., "The role of short-chain fatty acids in intestinal barrier function, inflammation, oxidative stress, and colonic carcinogenesis," *Pharmacological Research*, vol. 165, pp. 105420–105420, 2021.

[60] A. Tornero-Martínez, R. Cruz-Ortiz, M. E. Jaramillo-Flores et al., "In vitro Fermentation of Polysaccharides from *Aloe vera* and the Evaluation of Antioxidant Activity and Production of Short Chain Fatty Acids," *Molecules*, vol. 24, no. 19, pp. 3605–3624, 2019.

[61] S. Macfarlane and G. T. Macfarlane, "Regulation of short-chain fatty acid production," *Proceedings of the Nutrition Society*, vol. 62, no. 1, pp. 67–72, 2003.

[62] M. Asaduzzaman, S. Iehata, S. Akter et al., "Effects of host gut-derived probiotic bacteria on gut morphology, microbiota composition and volatile short chain fatty acids production of Malaysian Mahseer *Tor tambroides*," *Aquaculture Reports*, vol. 9, pp. 53–61, 2018.

[63] H. Shen, Z. Xu, Z. Shen, and Z. Lu, "The regulation of ruminal short-chain fatty acids on the functions of rumen barriers," *Frontiers in Physiology*, vol. 10, p. 1305, 2019.

[64] J. Han and R. J. Ulevitch, "Limiting inflammatory responses during activation of innate immunity," *Nature Immunology*, vol. 6, no. 12, pp. 1198–1205, 2006.

[65] O. O. Abiodun, A. Rodríguez-Nogales, F. Algieri et al., "Anti-inflammatory and immunomodulatory activity of an ethanolic extract from the stem bark of *Terminalia catappa* L. (Combretaceae): In *vitro* and in *vivo* evidences," *Journal of Ethnopharmacology*, vol. 192, pp. 309–319, 2016.

[66] J. Habijanic, M. Berovic, B. Boh, M. Plankl, and B. Wraber, "Submerged cultivation of *Ganoderma lucidum* and the effects of its polysaccharides on the production of human cytokines TNF-α, IL-12, IFN-γ, IL-2, IL-4, IL-10 and IL-17," *New Biotechnology*, vol. 32, no. 1, pp. 85–95, 2015.

[67] Z. Hu, B. Wu, F. Meng, Z. Zhou, H. Lu, and H. Zhao, "Impact of molecular hydrogen treatments on the innate immune activity and survival of zebrafish (*Danio rerio*) challenged with *Aeromonas hydrophila*," *Fish & Shellfish Immunology*, vol. 67, pp. 554–560, 2017.

[68] Z. Ren, S. Wang, Y. Cai et al., "Antioxidant capacity, non-specific immunity, histopathological analysis and immune-related genes expression in Nile tilapia *Oreochromis niloticus* infected with *Aeromonas schubertii*," *Aquaculture*, vol. 529, article 735642, 2020.

[69] C. Xu, E. Li, Y. Suo et al., "Histological and transcriptomic responses of two immune organs, the spleen and head kidney, in Nile tilapia (*Oreochromis niloticus*) to long-term hypersaline stress," *Fish & Shellfish Immunology*, vol. 76, pp. 48–57, 2018.

[70] G. E. Elshopakey, E. F. Risha, O. A. Abdalla et al., "Efficacy of dietary fermented vegetable product on immune response, up-regulation of immune-related genes and protection of kuruma shrimp (*Marsupenaeus japonicus*) against *Vibrio parahaemolyticus*," *Aquaculture*, vol. 497, pp. 431–439, 2018.

[71] W. Xu, H. Li, L. Wu et al., "Dietary *Scenedesmus ovalternus* improves disease resistance of overwintering gibel carp

(*Carassius gibelio*) by alleviating toll-like receptor signaling activation," *Fish & Shellfish Immunology*, vol. 97, pp. 351–358, 2020.

[72] Z. Yan, "Regulation of TLR4 expression is a tale about tail," *Arteriosclerosis Thrombosis & Vascular Biology*, vol. 26, no. 12, pp. 2582–2584, 2006.

[73] X. Li, Z. Zhao, P. Kuang, X. Shi, Z. Wang, and L. Guo, "Regulation of lipid metabolism in diabetic rats by *Arctium lappa* L. polysaccharide through the PKC/NF-κB pathway," *International Journal of Biological Macromolecules*, vol. 136, pp. 115–122, 2019.

[74] I. A. Schepetkin and M. T. Quinn, "Botanical polysaccharides: macrophage immunomodulation and therapeutic potential," *International Immunopharmacology*, vol. 6, no. 3, pp. 317–333, 2006.

[75] Manabu Suzuki, Fumihiko Takatsuki, Y. Y. Maeda, Junji Hamuro, and Goro Chihara, "Antitumor and immunological activity of lentinan in comparison with LPS," *International Journal of Immunopharmacology*, vol. 16, no. 5–6, pp. 463–468, 1994.

[76] J. Feng, Z. Cai, X. Zhang et al., "The Effects of Oral *Rehmannia glutinosa* Polysaccharide Administration on Immune Responses, Antioxidant Activity and Resistance Against *Aeromonas hydrophila* in the Common Carp, *Cyprinus carpio* L," *Frontiers in Immunology*, vol. 11, pp. 904–915, 2020.

[77] M. J. Paterson, S. Oh, and D. M. Underhill, "Host-microbe interactions: commensal fungi in the gut," *Current Opinion in Microbiology*, vol. 40, pp. 131–137, 2017.

[78] Q. Song, Y. Wang, L. Huang et al., "Review of the relationships among polysaccharides, gut microbiota, and human health," *Food Research International*, vol. 140, article 109858, 2021.

[79] R. Wang, X. An, Y. Wang et al., "Effects of polysaccharide from fermented wheat bran on growth performance, muscle composition, digestive enzyme activities and intestinal microbiota in juvenile common carp," *Aquaculture Nutrition*, vol. 26, no. 4, pp. 1096–1107, 2020.

[80] N. R. Shin, T. W. Whon, and J. W. Bae, "Proteobacteria: microbial signature of dysbiosis in gut microbiota," *Trends in Biotechnology*, vol. 33, no. 9, pp. 496–503, 2015.

[81] D. Kelly, L. Yang, and Z. Pei, "Gut microbiota, fusobacteria, and colorectal cancer," *Diseases*, vol. 6, no. 4, p. 109, 2018.

[82] Y. Liu, Z. Chen, J. Dai et al., "Sodium butyrate supplementation in high-soybean meal diets for turbot (*Scophthalmus maximus* L.): effects on inflammatory status, mucosal barriers and microbiota in the intestine," *Fish & Shellfish Immunology*, vol. 88, pp. 65–75, 2019.

[83] S. Stojanov, A. Berlec, and B. Trukelj, "The influence of probiotics on the Firmicutes/Bacteroidetes ratio in the treatment of obesity and inflammatory bowel disease," *Microorganisms*, vol. 8, no. 11, pp. 1715–1730, 2020.

[84] C. Ramírez, J. Coronado, A. Silva, and J. Romero, "Cetobacterium is a major component of the microbiome of giant amazonian fish (Arapaima gigas) in Ecuador," *Animals*, vol. 8, no. 11, pp. 189–195, 2018.

[85] S. M. Finegold, M.-L. Vaisanen, D. R. Molitoris et al., "*Cetobacterium somerae* sp. nov. from human feces and emended description of the genus *Cetobacterium*," *Systematic and Applied Microbiology*, vol. 26, no. 2, pp. 177–181, 2003.

[86] E. Ringø, H. van Doan, S. H. Lee et al., "Probiotics, lactic acid bacteria and bacilli: interesting supplementation for aquaculture," *Journal of Applied Microbiology*, vol. 129, no. 1, pp. 116–136, 2020.

[87] C. Zhang, J. Zhang, W. Guan et al., "Effects of *Lactobacillus delbrueckii* on immune response, disease resistance against *Aeromonas hydrophila*, antioxidant capability and growth performance of *Cyprinus carpio* Huanghe var," *Fish & Shellfish Immunology*, vol. 68, pp. 84–91, 2017.

[88] H. V. Doan, S. H. Hoseinifar, E. Ringø et al., "Host-associated probiotics: a key factor in sustainable aquaculture," *Reviews in Fisheries Science & Aquaculture*, vol. 28, no. 1, pp. 16–42, 2020.

[89] X. Li, E. Ringø, S. H. Hoseinifar, H. L. Lauzon, H. Birkbeck, and D. Yang, "The adherence and colonization of microorganisms in fish gastrointestinal tract," *Reviews in Aquaculture*, vol. 11, no. 3, pp. 603–618, 2019.

[90] C. C. Lazado, C. M. A. Caipang, and E. G. Estante, "Prospects of host-associated microorganisms in fish and penaeids as probiotics with immunomodulatory functions," *Fish & Shellfish Immunology*, vol. 45, no. 1, pp. 2–12, 2015.

[91] T. Defoirdt, P. Sorgeloos, and P. Bossier, "Alternatives to antibiotics for the control of bacterial disease in aquaculture," *Current Opinion in Microbiology*, vol. 14, no. 3, pp. 251–258, 2011.

[92] K. Govindaraj, V. Samayanpaulraj, V. Narayanadoss, V. Velu, and R. Uthandakalaipandian, "Effects of dietary supplementation of *Lactobacillus fermentum* URLP18 on growth, innate immunity and survival against *Aeromonas hydrophila* ATCC 7966 challenge in freshwater fish *Cyprinus carpi*o (common carp)," *Aquaculture Research*, vol. 52, no. 3, pp. 1160–1176, 2020.

[93] L. Feng, W. He, J. Jiang, Y. Liu, and X. Q. Zhou, "Effects of dietary pyridoxine on disease resistance, immune responses and intestinal microflora in juvenile Jian carp (*Cyprinus carpio* var. Jian)," *Aquaculture Nutrition*, vol. 16, no. 3, pp. 254–261, 2010.

[94] C. Li, B. Zhang, X. Wang et al., "Improved utilization of soybean meal through fermentation with commensal *Shewanella sp*. MR-7 in turbot (*Scophthalmus maximus L.*)," *Microbial Cell Factories*, vol. 18, no. 1, pp. 1262–1267, 2019.

[95] H. Li, X. Tian, and S. Dong, "Growth performance, nonspecific immunity, intestinal histology and disease resistance of *Litopenaeus vannamei* fed on a diet supplemented with live cells of *Clostridium butyricum*," *Aquaculture*, vol. 498, pp. 470–481, 2019.