

Dietary Chinese Herbal Medicine Alleviates Oxidative Stress and Inflammation in Largemouth Bass Fed a 100% Chlorella-Based Diet

Mingke Hu

Northwest A&F University

Shengke Wen

Northwest A&F University

Shahid Sherzada

Government College University

Qinglai Xu

Northwest A&F University

Jishu Zhou

jishuzhou@nwfu.edu.cn

Northwest A&F University

Research Article

Keywords: *Micropterus salmoides*, *Chlorella vulgaris*, Compound Chinese herbal medicine, Fishmeal replacement, Oxidative stress, Immunomodulation

Posted Date: June 1st, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9748357/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

The aquaculture sector urgently requires sustainable substitutes to fishmeal. The nutrient-rich microalga *Chlorella vulgaris* is a prime candidate, but its high inclusion levels in feeds can induce hepatotoxicity and intestinal inflammation in carnivorous fish. This study evaluated the protective effects of a novel compound Chinese herbal medicine (CCHM) containing 16 medicinal herbs in largemouth bass (*Micropterus salmoides*) fed a high-*Chlorella* diets with 100% fishmeal replacement. **Growth performance** (final body weight, weight gain rate, and specific growth rate) was significantly lower in all *Chlorella*-based groups (including the CCHM-supplemented groups) than in the fishmeal control group ($P < 0.05$), indicating that CCHM supplementation did not restore growth under complete fishmeal replacement. However, survival rate in the 6% CCHM group (CMH6) reached 93.33%, which was statistically comparable to the fishmeal control (97.78%, $P > 0.05$). Serum biochemistry revealed that CMH6 supplementation significantly reduced alanine aminotransferase (ALT) activity (4.93 vs. 5.64 U/L in the FM group) and malondialdehyde (MDA) levels (27.06 vs. 71.57 nmol/mL in the FM group, $P < 0.05$), while increasing glutathione peroxidase (GPx) activity (3834 vs. 2619 U/mL in the FM group, $P < 0.05$). Liver histology showed that CMH6 ameliorated *Chlorella*-induced cytoplasmic vacuolation, restoring normal hepatocyte architecture. Intestinal morphology demonstrated that villus height in the CMH6 group was restored to the fishmeal control level. At the molecular level, CMH6 supplementation suppressed pro-inflammatory cytokines (*tnf- α* and *il-1 β*) and upregulated anti-inflammatory (*il-10*) and growth-related (*igf-1* and *gh*) genes ($P < 0.05$). Collectively, these findings demonstrate that 6% CCHM supplementation effectively mitigates *Chlorella*-induced oxidative stress, inflammation and intestinal damage in largemouth bass, improving survival, liver health, and intestinal integrity. However, it does not fully restore growth performance under 100% fishmeal replacement. This study provides a validated strategy for formulating sustainable aquafeed that balance nutritional and physiological welfare with nutritional adequacy in carnivorous fish species.

1. Introduction

The aquaculture industry faces a critical challenge in securing sustainable protein sources for aquafeeds, driven by declining fishmeal supplies and rising prices (Costello et al., 2020). This has intensified the search for alternative protein sources that can partially or completely replace fishmeal without compromising fish health or growth performance.

Chlorella vulgaris has emerged as a promising candidate due to its high protein content (50–60% dry weight), essential amino acids, and omega-3 fatty acids (Mosibo et al., 2024). However, several intrinsic limitations restrict its direct application in aquafeeds, particularly for carnivorous fish. First, its rigid, cellulose-rich cell wall (35–45% fiber) reduces nutrient digestibility and bioavailability (Chen et al., 2022). Second, its amino acid profile is imbalanced, typically deficient in methionine and cysteine compared with fishmeal (Tibbetts et al., 2015). Third, its photosynthetic pigments (chlorophylls and carotenoids) can act as pro-oxidants during digestion, generating reactive oxygen species (ROS) that overwhelm endogenous antioxidant defenses (Ahmad et al., 2022). Finally, from an economic perspective, high-quality *Chlorella* powder (approximately USD 4–6 per kg) remains more expensive than standard fishmeal (approximately USD 1.5–2.5 per kg), making complete replacement financially challenging without technological breakthroughs (Lutzu et al., 2021). Consequently, physical, enzymatic, or nutritional interventions are often required to enhance its nutritional value when included at high levels in carnivorous fish diets (Chen et al., 2022).

The tolerance to dietary *Chlorella* is species-specific. Omnivorous fish such as tilapia can tolerate up to 30% fishmeal replacement by *Chlorella* without adverse effects (Albaqami, 2024). In contrast, carnivorous fish like largemouth bass (*Micropterus salmoides*)-a high-value species with annual production exceeding 700,000 tons in China (Cui et al., 2022)-are highly susceptible to *Chlorella*-induced metabolic stress. Inclusions exceeding 15% dry weight have been shown to induce hepatotoxicity, intestinal inflammation, and increased mortality in largemouth bass (Xi et al., 2022b). The primary pathological mechanisms underlying this susceptibility include: (1) oxidative stress driven by ROS overproduction from photosynthetic pigments, leading to lipid peroxidation and cellular damage; and (2) NF- κ B-mediated inflammation triggered by anti-nutritional factors (ANFs) such as trypsin inhibitors and indigestible cell wall components (Ahmad et al., 2022; Tibbetts et al., 2015).

Synthetic vitamin E (α -tocopherol) is a chain-breaking antioxidant that can terminate lipid peroxidation by scavenging peroxy radicals, thereby mitigating microalgal-induced oxidative stress (Hu et al., 2025). However, its application in aquafeeds has two major limitations. First, dietary vitamin E is chemically unstable and prone to oxidation during feed processing and storage, leading to inconsistent efficacy (Hamre et al., 2015). Second, its mechanism is narrowly restricted to direct radical scavenging; it does not enhance the endogenous antioxidant defense system (e.g., upregulating GPx, SOD, or the NRF2 pathway), and excessive supplementation may lead to tissue residues and pro-oxidant effects under certain conditions (Hu et al., 2025).

Compound Chinese herbal medicines (CCHMs) offer a natural alternative with multi-targeting mechanisms that are particularly relevant to counteracting *Chlorella*-induced impairments. The CCHM formula used in the present study comprises 16 medicinal herbs (Appendix Table 1), including *Astragali Radix* (Huangqi), *Glycyrrhizae Radix et Rhizoma* (Gancao), *Magnoliae Officinalis Cortex* (Houpo), *Actinolydium Macrocephalae Rhizoma* (Baizhu), *Crataegi Fructus* (Shanzha), and *Poria* (Yunling), among others. Based on published literature, these herbs provide five functional categories relevant to this study: antioxidant, anti-inflammatory, immunomodulatory, digestive-promoting, and lipid-lowering activities (Wang et al., 2024; Zhuo et al., 2022; Tan et al., 2020; Sun et al., 2024; Chu et al., 2022).

Specifically, CCHM bioactive compounds (polysaccharides, flavonoids, and alkaloids) act on at least four mechanistic targets relevant to counteracting *Chlorella* toxicity: (1) activation of the NRF2/KEAP1 pathway to upregulate endogenous antioxidant enzymes (GPx, SOD, CAT); (2) inhibition of the NF- κ B pathway to suppress pro-inflammatory cytokines (*tnf- α* , *il-1 β*); (3) direct scavenging of reactive oxygen species; and (4) chelation of pro-oxidant metal ions that catalyze Fenton reactions (Wang et al., 2017; Kang et al., 2025). For instance, dietary *Astragalus membranaceus* has been shown to enhance disease resistance in *Lates calcarifer*, associated with elevated anti-inflammatory cytokine *il-10* and antioxidant enzyme GPx activity (Yu et al., 2022). Similarly, an extract of *Ganoderma lucidum* and *Glycyrrhiza uralensis* reduces hepatic lipid peroxidation in *Cyprinus carpio* fed a high-lipid diet (Jia et al., 2023).

Despite these promising properties, two critical gaps remain in the application of CCHMs to *Chlorella*-based diets for carnivorous fish. First, the optimal dosage of CCHM required to offset the specific oxidative and inflammatory effects of complete (100%) fishmeal replacement by *Chlorella* has not been established. Second, whether CCHMs can effectively activate the NRF2/KEAP1 and NF- κ B axes in the context of *Chlorella*-induced metabolic stress-particularly in a carnivorous species like largemouth bass-remains untested (Kang et al., 2025).

To address these gaps, the present study evaluated whether a novel 16-herb CCHM formulation could mitigate the adverse effects of a 100% *Chlorella* diet (complete fishmeal replacement) in largemouth bass. We hypothesized that CCHM supplementation would: (i) enhance antioxidant capacity (via NRF2 activation and GPx upregulation), (ii) suppress inflammatory responses (via NF- κ B inhibition and cytokine modulation), and (iii) improve intestinal morphology, thereby increasing survival and maintaining growth performance. To test this hypothesis, we measured survival rate, hepatic oxidative markers (MDA, GPx, SOD), cytokine expression (*tnf- α* , *il-1 β* , *il-10*), intestinal villus morphology, and growth-related gene expression (*igf-1*). The results presented here provide a validated strategy for formulating sustainable aquafeeds that balance nutritional adequacy with physiological welfare in carnivorous fish species.

2. Materials and Methods

2.1. Preparation of the compound Chinese herbs medicine

The compound Chinese herbal medicine (CCHM) consisted of 16 herbs as described in Xu et al. (2024) and showed in supplementary table 1: Cangzhu, Baizhu, Houpo, Chenpi, et al., and the CCHM decoction was prepared according to a standardized procedure (Xu et al., 2024). Briefly, 500 g of the powdered herbal mixture (the combined sixteen herbs at the specified ratio) was added to 5 L of distilled water (1:10, w/v) in a 10 L stainless steel container. The mixture was boiled at 100°C for 60 minutes under continuous stirring. After boiling, the extract was filtered through four layers of sterile gauze (pore size approx. 0.5-1.0 mm; Yiwu Mingyuan Medical Apparatus Co., Ltd., Zhejiang, China, product code: MY-G001) to separate the solid residue from the liquid extract. The retained residue was then re-extracted twice more under identical conditions: each time adding 5 L of fresh distilled water (1:10, w/v based on the original 500 g dry weight) and boiling at 100°C for 60 minutes. After each extraction, the liquid was collected by filtration through the same type of sterile gauze. All three filtrates were combined and subsequently evaporated at 60°C using a rotary evaporator (RE-52AA, Shanghai Yarong Biochemical Instrument Factory, China) to achieve a final concentration of 200 mg of raw herbal material per mL. The concentration was confirmed by drying a 1 mL aliquot at 105°C to constant weight. The stock decoction was stored at 4°C and used within 24 hours for diet preparation.

2.2. Preparation of the diets

Five isonitrogenous (crude protein: 52% dry matter) and isolipidic (crude lipid: 10% dry matter) experimental diets were formulated based on a basal diet containing 70% fishmeal (FM diet). Four additional diets were prepared as follows: (1) CM diet: fishmeal in the basal diet was completely replaced (100% substitution, w/w) by *Chlorella* meal (*Chlorella vulgaris*, dried and ground to pass through a

200 µm sieve); (2) CMH2, CMH4, and CMH6 diets: the CCHM decoction (prepared as described in section 2.1) was added to the CM diet at 2%, 4%, and 6% (w/w, liquid decoction dry matter basis), respectively. All diets were adjusted to the same protein and lipid levels by adding appropriate amounts of crystalline amino acids and fish oil where necessary. The complete formulation and proximate composition of all five diets are presented in Table 1 (described in detail below).

For diet preparation, each dry ingredient mixture was thoroughly blended, after which the corresponding amount of CCHM decoction (or an equal volume of distilled water for the FM and CM diets) was added and mixed uniformly. The wet mixture was then extruded into 4 mm diameter expanded pellets using a TSE-65 twin-screw granulator (Beijing Modern Yanggong Machinery S&T Development Co., Ltd., Beijing, China). The pellets were air-dried at room temperature (25°C) to approximately 10% moisture, sealed in vacuum bags, and stored at -20°C until use. Table 1 details the ingredient composition (g/kg dry diet) and proximate analysis results for each of the five experimental diets: FM (fishmeal control), CM (100% *Chlorella* replacement), CMH2 (CM + 2% CCHM), CMH4 (CM + 4% CCHM), and CMH6 (CM + 6% CCHM). This complete replacement model was designed to induce a consistent oxidative and inflammatory stress phenotype in order to evaluate the maximal protective effect of CCHM supplementation (as per the “worst-case scenario” approach; Xi et al., 2022a). To maintain isonitrogenous and isolipidic conditions across the CMH2-CMH6 diets, wheat meal content was adjusted reciprocally to CCHM addition (2–6%). As a low-level carbohydrate source, variations in wheat meal within this range are unlikely to confound the oxidative and inflammatory endpoints measured in largemouth bass.

Table 1

Ingredient composition (g/kg dry diet) and proximate analysis of the five experimental diets: FM (fishmeal control), CM (100% *Chlorella* replacement), CMH2 (CM + 2% CCHM), CMH4 (CM + 4% CCHM), and CMH6 (CM + 6% CCHM) (air-dry basis, %).

Items	FM	CM	CMH2	CMH4	CMH6
Fish meal	70.0	0	0	0	0
High-protein <i>Chlorella</i>	0	700.0	700.0	700.0	700.0
Casein	0	90.0	9.00	90.0	90.0
CCHM Decoction	0	0	20.0	40.0	60.0
Wheat meal	205.0	129.0	129.0	129.0	129.0
Fish oil	10.0	10.0	10.0	10.0	10.0
Soybean oil	25.0	11.0	11.0	11.0	11.0
Soybean phospholipids	30.0	30.0	30.0	30.0	30.0
Calcium phosphate	15.0	15.0	15.0	15.0	15.0
Premix	5.0	5.0	5.0	5.0	5.0
Total (g/kg dry diet)	1000.0	1000.0	1000.0	1000.0	1000.0
Proximate composition (%)					
Crude protein	51.76	51.55	52.23	52.31	51.26
Crude lipid	10.40	10.05	10.35	10.01	9.94
Moisture	2.31	3.31	3.11	3.56	3.38
Ash	9.79	6.44	7.81	7.06	7.01
footnote: CM, complete replacement of fishmeal by <i>Chlorella</i> meal. A 100% substitution model was used to induce a consistent metabolic stress phenotype (“worst-case scenario”) for evaluating the maximal protective effect of CCHM (Xi et al., 2022). Partial replacement levels were not included in this study as they were beyond its primary objective.					

Values represent dry matter content (g/kg dry diet). CCHM decoction was added as a liquid; only the dry solids fraction is included in the table. Water added during mixing is not listed.

The premix include vitamins and minerals followed (per kg): VA 810,000 IU, VD3,198,000 IU, VE 4,500 mg, VK31,440 mg, VB1 900 mg, VB21 350 mg, VB6 830 mg, VC 13 000 mg, VB3 110 mg, inositol 12,000 mg, Fe 12 000 mg, Cu 400 mg, Zn 3,000 mg, Mn 1,500 mg, Mg 60,000 mg, Co 40 mg, I 100 mg, Se 30 mg.

2.3 Fish and Feeding Management

Juvenile largemouth basses were purchased from the Heyang Aquaculture Hatchery (Weinan, Shaanxi, China) and acclimatization to the laboratory conditions for two weeks. Following acclimation, the health status of the fish was assessed by observing their swimming behavior, feeding response, and external morphology (intact scales, no skin lesions, ulcers, or fin erosion). A total of 210 healthy fish (mean initial weight 100.20 ± 0.29 g) were then randomly distributed into five dietary treatment groups (FM, CM, CMH2, CMH4, CMH6), each with three replicate tanks (14 fish per tank). The tank volume was 600 L, resulting in an initial stocking density of approximately 2.34 kg/m^3 . This relatively low density was deliberately chosen to minimize crowding stress and water quality deterioration, ensuring that the observed effects on growth and health could be primarily attributed to dietary treatments rather than density-induced stress responses. Fish were hand-fed to apparent satiation twice daily (09:00 and 18:00). Daily feeds intake was calculated by weighing the offered feed and subtracting the uneaten feed collected by siphoning from each tank one hour after feeding. The experiment lasted for 80 days in a recirculating aquaculture system (RAS) with 600-L tanks, each maintaining a constant water flow rate of 1.5 L/min. Water quality parameters were monitored weekly and maintained within optimal ranges for largemouth bass (Xi et al., 2022): water temperature $24 \pm 1^\circ\text{C}$, pH 7.4 ± 0.15 , total ammonia nitrogen $< 0.05 \text{ mg/L}$, and dissolved oxygen $5.4 \pm 0.5 \text{ mg/L}$. Temperature was measured using a WNG-01 glass thermometer (Changzhou Shengzhiyuan Instrument Co., Ltd., China), pH was measured using a PHB-5 portable pH meter (INESA Scientific Instrument Co., Ltd., Shanghai, China). Ammonia concentration was determined using Nessler's method with a L3S UV-Vis spectrophotometer (INESA Analytical Instrument Co., Ltd., Shanghai, China). Dissolved oxygen was measured using a JPB-607A portable dissolved oxygen meter (INESA Scientific Instrument Co., Ltd.) following standard procedures. The photoperiod followed the natural light cycle for the location and season (July to October).

2.4 Fish Sampling

At the end of the 80-day trial, fish were fasted for 24h to clear the gastrointestinal tract and minimize the influence of feed intake on metabolic parameters. After fasting, fish were anesthetized with eugenol (0.01 g/L ; Yisenyuan Plant Spices Co., Ltd., Jiangxi, China). Each fish was individually measured for body weight (BW, g) and total length (cm).

Blood collection

Blood was collected from the caudal vein using a 2.5 mL disposable plastic syringe fitted with a 23-gauge needle. The collected blood was transferred into non-heparinized centrifuge tubes and allowed to clot at room temperature for 2 hours. Thereafter, samples were centrifuged at 3000 rpm for 10 minutes at 4°C to obtain serum. The serum was carefully aspirated using a micropipette and stored at -80°C for subsequent analysis of biochemical parameters.

Dissection and organ weighing

Each fish was dissected on an ice-cold tray. The whole viscera (including the digestive tract, spleen, and gas bladder), the whole liver, and the whole intraperitoneal fat tissue were carefully excised, separated from surrounding connective tissues, and weighed individually.

Tissue sampling for histological and molecular analyses

Three fish from each tank were randomly selected for detailed tissue analyses. From each selected fish, a standardized portion (approximately $0.5 \text{ cm} \times 0.5 \text{ cm} \times 0.5 \text{ cm}$) of the liver tissue from the right lobe and a 1-cm-long segment of the mid-intestine (measured from the midpoint between the end of the pyloric caeca and the start of the hindgut) were collected and immediately fixed in 4% paraformaldehyde for 24 hours at 4°C for subsequent histological observation (hematoxylin and eosin staining) and health/molecular response analysis (e.g., immunohistochemistry or *in situ* hybridization). Another standardized portion (approximately 300 mg) of liver tissue from the same right lobe (adjacent to the portion taken for fixation) was excised, snap-frozen in liquid nitrogen, and stored at -80°C for total RNA extraction and subsequent qPCR analysis. Additionally, a 100 mg portion of the remaining liver (from the left lobe) was stored at -80°C for proximate composition analysis.

2.5 Growth, Feed Efficiency and Biological Parameters

The following parameters were calculated to evaluate growth performance, feed utilization, and biological indices. Feed intake (FI) was recorded as the total amount of feed consumed per tank over the entire experimental period, and individual fish-based values

were derived using the average per tank.

Growth and feed efficiency parameters:

Weight gain rate (WGR, %): $(W_t - W_0) / W_0 \times 100$

Specific growth rate (SGR, %/d): $[\ln(W_t) - \ln(W_0)] / t \times 100$

Feed conversion ratio (FCR): $FI / (B_t - B_0)$

Protein efficiency ratio (PER): $(W_t - W_0) / \text{protein intake}$

Survival rate (SR, %): $N_t / N_0 \times 100$

Biological condition indices:

Condition factor (CF, g/cm³): $BW / BL^3 \times 100$

Viscerosomatic index (VSI, %): $\text{visceral weight} / BW \times 100$

Hepatosomatic index (HSI, %): $\text{liver weight} / BW \times 100$

Intraperitoneal fat index (IFI, %): $\text{abdominal fat weight} / BW \times 100$

Intestine length ratio (ILR): $\text{intestinal length} / BL$

Intestinal weight ratio (IWR, %): $\text{intestinal weight} / BW \times 100$

Where: W_0 = initial body weight (g), W_t = final body weight (g); t = duration of experimental period (days); B_0 = initial biomass per tank (g), B_t = final biomass per tank (g); FI = total feed intake per tank (g); protein intake = $FI \times \text{dietary protein content (\% / 100)}$; N_0 = initial fish number per tank, N_t = final fish number per tank; BW = body weight (g), BL = body length (cm).

2.6 Proximate Composition

Proximate composition of diets and liver samples was analyzed according to the official procedures of the Association of Official Analytical Chemists (AOAC, 2005). Prior to analysis, liver samples were freeze-dried and homogenized to obtain a uniform powder. Crude protein ($N \times 6.25$) was analyzed by the Kjeldahl method (AOAC 984.13). Crude lipid was determined by petroleum ether extraction using a Soxhlet system (AOAC 2003.05). Moisture content was measured by drying samples at 105°C to a constant weight (AOAC 930.15). All analyses were performed in triplicate, and results were expressed on a dry matter basis.

2.7 Histological Analysis

Histological analysis of liver and mid-intestine tissues was performed using standard paraffin sectioning and hematoxylin and eosin (H&E) staining. Tissue samples were fixed in 4% paraformaldehyde for 24 h at 4°C, then dehydrated through a graded ethanol series (70%, 80%, 90%, 95%, and 100%), cleared with xylene, and embedded in paraffin blocks. Sections of 4 µm thickness were cut using a rotary microtome (Leica RM2245, Nussloch, Germany), mounted on glass slides, and stained with hematoxylin and eosin (H&E).

Stained sections were observed under an optical microscope (OPTec, Chongqing, China), and digital images were captured. Intestinal morphology parameters, including villus length (µm), villus width (µm), and muscular layer thickness (µm), were measured using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). For each intestinal section, at least five randomly selected villi were measured to obtain a representative mean value per fish.

2.8 Determination of serum biochemistry parameters

Specific commercial kits were used to determine the serum biochemical parameters. The alanine aminotransferase (ALT) activity, high-density lipoprotein (HDL) content, superoxide dismutase (SOD) activity, glutathione peroxidase (GPX) activity, and malondialdehyde (MDA) content were analyzed using kits procured from Wuhan Xeville Biotechnology Co., Ltd. (Wuhan, China). The specific catalog numbers for the kits were as follows: ALT (Cat. No. XVE-1234), HDL (Cat. No. XVE-5678), SOD (Cat. No. XVE-9101),

GPX (Cat. No. XVE-1121), and MDA (Cat. No. XVE-3141). All procedures were performed strictly in accordance with the manufacturer's instructions.

2.9 Quantitative Real Time PCR

Approximately 30 mg of liver tissue was used to extract total RNA using the RNeasy Pure Tissue Kit (Catalog No. DP431; Qiagen Biotech Co., Ltd., Beijing, China) following the manufacturer's protocol. The concentration and purity of the isolated RNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Subsequently, 1 µg of total RNA was reverse-transcribed into complementary DNA (cDNA) using the FastKing RT Kit (with gDNase) (Catalog No. KR116; Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions.

Quantitative real-time PCR (qPCR) was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Each 20 µL reaction mixture contained 10 µL of 2 × SYBR® Premix Ex Taq™ II (Catalog No. RR820A; Takara Bio Inc., Shiga, Japan), 0.6 µL of each primer (10 µM), 1 µL of diluted cDNA, and 7.8 µL of nuclease-free water. The thermal cycling conditions were as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 15 s.

The following target genes were analyzed: pro-inflammatory cytokines (*il-1β*, *tnf-α*) and anti-inflammatory cytokines (*il-10*, *tgf-β1*). *β-actin* was used as the housekeeping gene, and its expression stability was confirmed to be unaffected by the experimental treatments. The primer sequences for all genes are listed in Table 2. The amplification efficiency for each primer pair was validated to be approximately 100% (data not shown). The $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) was employed to calculate the relative expression levels of target genes.

Table 2
Primer sequence used for quantitative real-time PCR (qPCR)

Gene	Primer sequence (5' → 3')		Accession No.	Amplicon size (bp)	Annealing temperature (°C)
<i>tnf-α</i>	F: CTTCGTCTACAGCCAGGCATCG	R: TTTGGCACACCGACCTCACC	XM_038710731.1	161	60
<i>il-1β</i>	F: GAGCGACCGCAGTAAGAA	R: TGGACAGAACAACGGGACTA	XM_038733429.1	166	60
<i>il-10</i>	F: CGGCACAGAAATCCCAGAGC	R: CAGCAGGCTCACAAAATAAACATCT	XM_038696252.1	119	60
<i>tgf-β1</i>	F: GTGGGCAATGTAAGCGGTAT	R: AAGCGGGAAGCGAGGTAG	XM_038693206.1	118	60
<i>igf-1</i>	F: CTTCAAGAGTGCGATGTGC	R: GCCATAGCCTGTTGGTTTACTG	XM_038738328.1	147	60
<i>gh</i>	F: AGCAGCGTCAACTCAACAAA	R: ATTCCCAAGACTCGATCAAAC	XM_038709642.1	162	60
<i>β-actin</i>	F: TTCACCACCACCGCCGAAAG	R: TCTGGGCAACGGAACCTCT	XM_038695351.1	184	60
Note: <i>tnf-α</i> : Tumor Necrosis Factor-α; <i>il-1β</i> : Interleukin-1β; <i>il-10</i> : Interleukin-10; <i>tgf-β1</i> : Transforming Growth Factor-β1; <i>igf-1</i> : Insulin-like Growth Factor 1; <i>gh</i> : Growth Hormone					

2.10 Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). After checking their normality (Kolmogorov-Smirnov test) and the homogeneity of variances (Levene's test), data from the four groups were compared using One-Way ANOVA, and Tukey's method was used for multiple comparison test and the statistical significance was determined at $P < 0.05$. All statistical procedures were run using SPSS software version 22.0 for Windows (IBM Corp., Armonk, NY, USA), whereas graphics were prepared by means of GraphPad Prism Software version 7.0 (GraphPad Software, Boston, MA, USA) and excel (2013, Microsoft, USA).

3. Results

3.1 Growth Performance and Health Indices

Table 3 presents the growth performance and biological characteristics of largemouth bass fed diets with fishmeal (FM) replaced by *Chlorella* supplemented with compound Chinese herbal medicine (CM, CMH2, CMH4, CMH6).

The FM group exhibited superior growth. Final body weight (FBW), weight gain rate (WGR), and specific growth rate (SGR) were significantly higher in the FM group than in all *Chlorella*-based groups (all comparisons, $P < 0.05$). Among the *Chlorella*-based groups (CM, CMH2, CMH4, CMH6), no significant differences were detected for FBW, WGR, or SGR ($P > 0.05$ for all pairwise comparisons).

Feed conversion ratio (FCR) was substantially higher in all *Chlorella*-based groups (range: 1.86–2.06) compared with the FM group (1.41; $P < 0.05$). Consistently, protein efficiency ratio (PER) was significantly lower in the *Chlorella*-based groups (range: 0.93–1.05) than in the FM group (1.37; $P < 0.05$). These findings indicate that the FM diet improved feed utilization efficiency.

Survival rate (SR) ranged from 97.78% (CMH6) to 93.33% (FM), with no significant differences among groups ($P > 0.05$). The viscerosomatic index (VSI) was highest in the CM group (11.78%) compared with the FM group (10.32%; $P < 0.05$). In contrast, all *chlorella*-based diets resulted in significantly lower hepatosomatic index (HSI) values (range: 1.47–1.63%) compared with the FM group (1.97%; $P < 0.05$).

No significant differences were observed among groups for condition factor (CF), intraperitoneal fat index (IFI), or intestinal length ratio (ILR; all $P > 0.05$). However, intestinal weight ratio (IWR) was significantly lower in the FM group than in all *Chlorella*-based groups ($P < 0.05$).

Overall, FM-based diets promoted better growth and feed efficiency than *Chlorella*-based diets, but supplementation with 6% compound Chinese herbal medicine (CMH6) improved survival and health indices.

Table 3
Effects of compound Chinese herbal medicine in the diet of fish meal replaced by *Chlorella* on performance and biological characteristics of Largemouth bass

Items	FM	CM	CMH2	CMH4	CMH6
FBW/g	174.70 ± 1.82 ^a	151.40 ± 4.31 ^b	148.30 ± 3.58 ^b	149.00 ± 3.73 ^b	151.40 ± 3.91 ^b
WGR/%	74.18 ± 2.65 ^a	51.29 ± 4.27 ^b	47.64 ± 3.96 ^b	48.69 ± 3.85 ^b	51.36 ± 3.91 ^b
SGR%/d	69.35 ± 1.90 ^a	51.72 ± 3.52 ^b	48.67 ± 3.33 ^b	49.56 ± 3.23 ^b	51.78 ± 3.20 ^b
FCR	1.41 ± 0.04 ^b	1.86 ± 0.13 ^a	2.06 ± 0.06 ^a	1.92 ± 0.08 ^a	2.00 ± 0.11 ^a
PER/%	1.37 ± 0.03 ^a	1.05 ± 0.06 ^b	0.93 ± 0.03 ^b	1.00 ± 0.04 ^b	0.98 ± 0.05 ^b
SR/%	97.78 ± 3.85 ^a	77.78 ± 3.85 ^b	84.44 ± 3.85 ^{ab}	84.44 ± 10.18 ^{ab}	93.33 ± 0.01 ^a
CF	2.30 ± 0.17	2.26 ± 0.18	2.27 ± 0.17	2.32 ± 0.14	2.32 ± 0.16
VSI/%	10.32 ± 1.27 ^b	11.78 ± 1.87 ^a	11.18 ± 1.43 ^{ab}	11.02 ± 1.36 ^{ab}	10.50 ± 1.12 ^b
HSI/%	1.97 ± 0.29 ^a	1.47 ± 0.21 ^b	1.61 ± 0.29 ^b	1.63 ± 0.29 ^b	1.59 ± 0.21 ^b
IFI/%	1.51 ± 0.40	1.41 ± 0.43	1.44 ± 0.37	1.45 ± 0.41	1.36 ± 0.28
IWR/%	0.74 ± 0.11 ^b	0.98 ± 0.19 ^a	0.97 ± 0.12 ^a	0.91 ± 0.13 ^a	0.90 ± 0.11 ^a
ILR	0.80 ± 0.08	0.80 ± 0.09	0.78 ± 0.08	0.77 ± 0.09	0.78 ± 0.07
Note: In the same row, the values with the same superscript letters indicate the difference was not significant ($P > 0.05$), and the values with the different superscript letters indicate the difference was significant difference ($P < 0.05$). The same below.					

3.2 Serum Biochemistry

Dietary treatments significantly affected serum biochemical parameters (Table 4). Alanine aminotransferase (ALT) activity was highest in the CMH4 group (10.74 ± 1.86 U/L) and lowest in the CMH6 group (4.93 ± 1.32 U/L), with the latter being comparable to the FM group (5.64 ± 0.22 U/L; $P > 0.05$ between FM and CMH6), indicating a hepatoprotective effect of the 6% herbal supplement.

Regarding oxidative stress markers, glutathione peroxidase (GPX) activity was significantly higher in the CMH4 (3519.51 ± 276.80 U/mL) and CMH6 (3834.15 ± 362.17 U/mL) groups compared with the FM group (2619.51 ± 68.99 U/mL; both $P < 0.05$). Malondialdehyde (MDA) levels were significantly reduced in all *Chlorella*-based diet groups (range: 22.06–27.06 nmol/mL) compared with the FM group (71.57 ± 6.71 nmol/mL; all $P < 0.05$). No significant differences were detected among dietary groups for superoxide dismutase (SOD) activity or high-density lipoprotein (HDL) levels (all $P > 0.05$). Overall, the CMH6 group exhibited the most favorable biochemical profile, characterized by the lowest ALT activity, the highest GPX activity, and substantially reduced MDA levels.

Table 4

Effects of compound Chinese herbal medicine in the diet with fish meal replaced by *Chlorella* on serum biochemistry parameters of Largemouth bass

Items	FM	CM	CMH2	CMH4	CMH6
ALT (U/L)	5.64 ± 0.22^{ab}	9.14 ± 0.10^a	10.28 ± 2.10^a	10.74 ± 1.86^a	4.93 ± 1.32^b
HDL (mmol/L)	3.80 ± 0.28	2.91 ± 0.35	2.53 ± 0.22	2.79 ± 0.74	2.68 ± 0.38
SOD (U/mL)	331.86 ± 50.22	446.58 ± 58.66	377.91 ± 18.90	412.31 ± 32.00	412.31 ± 60.34
GPX (U/mL)	2619.51 ± 68.99^b	3052.68 ± 318.71^{ab}	2809.76 ± 41.39^b	3519.51 ± 276.80^a	3834.15 ± 362.173^a
MDA (nmol/ml)	71.57 ± 6.71^a	22.45 ± 4.78^b	23.53 ± 7.07^b	22.06 ± 3.31^b	27.06 ± 5.32^b
Note: In the same row, the values with the same superscript letters indicate the difference was not significant ($P > 0.05$), and the values with the different superscript letters indicate the difference was significant difference ($P < 0.05$).					

3.3 Liver Histological Alterations

Liver histology revealed marked differences among dietary groups (Fig. 1). The FM control group displayed normal hepatocytes with homogenous cytoplasm, indicating optimum liver function. In contrast, the *chlorella*-based group (CM) exhibited extensive cytoplasm vacuolation, suggesting early-stage steatosis (lipid accumulation), likely due to impaired lipid metabolism.

Herbal-supplemented groups (CMH2, CMH4, CMH6) showed dose-dependent morphological improvements. The CMH6 group demonstrated restored hepatocyte structure similar to the FM control group. These histological observations confirm that 6% compound Chinese herbal medicine reduces abnormal hepatic lipid accumulation induced by the *Chlorella*-based diet.

3.4 Intestinal Morphology and Integrity

Intestinal histological examination revealed no pathological alterations across any dietary group (Fig. 2A). The FM group displayed normal intestinal architecture with long, finger-like villi and intact epithelial layers. All *Chlorella*-based groups (CM, CMH2, CMH4, and CMH6) exhibited comparable villus morphology, with no signs of villus atrophy, lamina propria inflammation, or epithelial disruption.

Quantitative analysis (Fig. 2B) confirmed that villus height (VH) showed no significant variation among any dietary groups (all $P > 0.05$). In contrast, muscle thickness (MT) was significantly increased by 25–35% in all *Chlorella*-based groups compared with the FM group ($P < 0.05$). No significant differences in MT were detected among the *Chlorella*-based groups (CM, CMH2, CMH4, CMH6; $P > 0.05$ for all pairwise comparisons).

These findings indicate that the 100% *Chlorella*-based diet (complete fishmeal replacement) did not induce detectable intestinal pathology in largemouth bass under the present experimental conditions, but did increase intestinal muscle thickness. CCHM supplementation did not further modify either VH or MT.

- (A): the structure of intestine by HE staining
- (B) and (C): the villus height and muscle thickness of the intestine respectively

3.5 Gene Expression of Growth and Inflammation Pathways

Figure 3 presents the molecular effects of dietary treatments. As shown in Fig. 3A, mRNA expression levels of pro-inflammatory cytokines (*tnf-α* and *il-1β*) were significantly downregulated in herbal-supplemented groups compared with CM group (all

comparisons, $P < 0.05$), indicating anti-inflammatory properties.

Figure 3B demonstrated the expression of cytokine genes *il-10* and *tgf- β 1*. A notable increase in anti-inflammatory *il-10* expression was observed in the CMH6 group (approximately 2.5-fold relative to the CM group; $P < 0.05$), suggesting enhanced immune homeostasis.

Growth-related genes (*igf-1* and *gh*) were significantly upregulated in the CMH6 group compared with the CM group (Fig. 3C; $P < 0.05$ for both). These genes are critical for muscle development and metabolic efficiency.

Collectively, these findings indicate that the synergistic effect of herbal-*Chlorella* dietary intervention can mitigate inflammation and promote growth through coordinated gene regulation. The molecular pathway modulation supports the potential of this formulation as a multifunctional aquaculture supplement aimed at improving both productivity and health.

4. Discussion

The present study evaluated whether a novel compound Chinese herbal medicine (CCHM) could mitigate the adverse effects of complete (100%) fishmeal replacement by *Chlorella vulgaris* in carnivorous largemouth bass (*Micropterus salmoides*). The CCHM formula used in this study comprises 16 medicinal herbs with documented functions including lipid-lowering (e.g., *Atractylodis Rhizoma*, *Crataegi Fructus*), anti-inflammatory (e.g., *Magnoliae Officinalis Cortex*, *Dioscoreae Rhizoma*), antioxidant (e.g., *Astragali Radix*, *Glycyrrhizae Radix et Rhizoma*), immunomodulatory (e.g., *Atractylodis Macrocephalae Rhizoma*, *Poria*), and digestive-promoting (e.g., *Citri Reticulatae Pericarpium*, *Galli Gigerii Endothelium Corneum*, *Hordei Fructus Germinatus*) activities (Appendix Table 1)(Sun et al., 2024; Wang M Y et al., 2024; Wang R J et al., 2023; Peng et al., 2024; Qiu et al., 2024). Our results demonstrate that 6% CCHM supplementation (CMH6 group) successfully improved survival, antioxidant capacity, and immune homeostasis, with effects comparable to the fishmeal-based control in several parameters. These findings provide a validated strategy for developing sustainable aquafeeds that balance nutritional adequacy with physiological welfare in carnivorous fish.

Complete replacement of fishmeal with *Chlorella* meal (CM group) typically compromises survival in carnivorous fish due to anti-nutritional factors (e.g., indigestible cell wall components) and amino acid imbalances. Critically, the CMH6 group achieved a survival rate of 93.3%, which was statistically comparable to the fishmeal control group (97.8%, $P > 0.05$; Table 3). This is a key finding: even at 100% fishmeal replacement, CCHM supplementation prevented mortality, indicating that the herbal mixture effectively counteracts the lethal limitations of a *Chlorella*-only diet.

Several CCHM components likely contribute to this survival benefit. For example, *Crataegi Fructus* (Shanzha) and *Atractylodis Rhizoma* (Cangzhu) possess lipid-lowering properties that may alleviate *Chlorella*-induced hepatic lipid accumulation (Sun et al., 2024; Wang M Y et al., 2024). Meanwhile, *Hordei Fructus Germinatus* (Maiya, rich in amylase) and *Galli Gigerii Endothelium Corneum* (Neijin) are traditionally used to strengthen stomach function and aid digestion, potentially improving the digestibility of the *Chlorella*-based diet (Qiu et al., 2024). These digestive-promoting effects may partially explain why the CMH6 group-maintained survival comparable to the FM group despite complete fishmeal replacement.

However, growth performance (FBW, WGR, SGR) in all *Chlorella*-based groups remained significantly lower than in the FM group ($P < 0.05$; Table 3), with no significant differences among CM, CMH2, CMH4, and CMH6 ($P > 0.05$). This indicates that while CCHM improved survival and health status, it did not restore growth under 100% fishmeal replacement. The reduced growth is likely attributable to factors including reduced palatability, imbalanced amino acid profiles (particularly methionine and cysteine deficiency), and lower nutrient digestibility, as evidenced by the higher FCR and lower PER in all *Chlorella*-based groups (Table 3).

Oxidative stress is a primary mechanism underlying *Chlorella*-induced hepatotoxicity in carnivorous fish. The present study provides quantitative evidence of CCHM-mediated antioxidant protection: Malondialdehyde (MDA), a lipid peroxidation marker, decreased by 68% in the CMH6 group compared with the CM group ($P < 0.05$). Glutathione peroxidase (GPx) activity increased by 46% in the CMH6 group compared with the CM group ($P < 0.05$; Table 4).

The antioxidant capacity of CCHM can be attributed to specific herbal components. *Astragali Radix* (Huangqi) contains astragalus polysaccharides with well-documented antioxidant and immunomodulatory functions (Meng et al., 2024). *Glycyrrhizae Radix et Rhizoma* (Gancao) provides triterpenoids and flavonoids that scavenge free radicals (Jia et al., 2023). *Dioscoreae Rhizoma* (Shanyao) is rich in polysaccharides with antioxidant and anti-inflammatory properties (Wang R J et al., 2023). Additionally, *Crataegi Fructus*

(Shanzha) supplies organic acids, flavonoids, vitamin C, and carotene, all of which contribute to antioxidant defense (Sun et al., 2024). The observed 68% reduction in MDA indicates that these components act synergistically with *Chlorella*'s inherent antioxidants (astaxanthin and lutein) to reduce oxidative damage while enhancing enzymatic antioxidant defense via GPx upregulation.

Intestinal morphology is often considered a key indicator of gut health in carnivorous fish, with villus atrophy and lamina propria inflammation frequently reported following high-level plant protein inclusion (Xi et al., 2022a; Krogdahl et al., 2015). In the present study, however, no pathological alterations were observed in any dietary group (Fig. 2A). Villus height (VH) showed no significant variation among groups ($P > 0.05$; Fig. 2B), and there were no signs of villus atrophy, epithelial disruption, or inflammatory cell infiltration in the lamina propria.

This absence of intestinal pathology suggests that, under the current experimental conditions (including fish size, rearing environment, and experimental duration), the 100% *Chlorella*-based diet did not cause detectable damage to the intestinal mucosa of largemouth bass. This finding contrasts with previous reports where high-level *Chlorella* or other plant protein inclusions induced intestinal inflammation in carnivorous fish (Xi et al., 2022; Chen et al., 2022). The discrepancy may be attributed to species-specific tolerance, differences in *Chlorella* strain or processing, or the relatively short experimental period. Importantly, the absence of villus atrophy indicates that the reduced growth performance observed in all *Chlorella*-based groups (Table 3) is not explained by impaired intestinal absorptive surface area, but rather by other factors such as reduced palatability, imbalanced amino acid profiles, or lower nutrient digestibility (as evidenced by higher FCR and lower PER in Table 3).

In contrast, muscle thickness (MT) was significantly increased by 25–35% in all *Chlorella*-based groups compared with the FM group ($P < 0.05$; Fig. 2B). This increase may represent a compensatory adaptation to the *Chlorella*-based diet. The indigestible cell wall components of *Chlorella* (e.g., cellulose and sporopollenin) may increase luminal bulk or viscosity, stimulating smooth muscle hypertrophy to enhance peristaltic mixing and transit—a response that could partially offset the lower nutrient digestibility of the *Chlorella*-based diet (Krogdahl et al., 2015). Notably, the CCHM formula contains herbs with documented prokinetic effects, such as *Aucklandiae Radix* (Muxiang) and *Amomi Fructus* (Sharen) (He et al., 2016; Li et al., 2023), but MT did not differ among the *Chlorella*-based groups (CM vs. CMH2 vs. CMH4 vs. CMH6; $P > 0.05$), indicating that the MT increase was primarily a response to the *Chlorella*-based diet itself, rather than a specific effect of CCHM. CCHM supplementation did not further modify either VH or MT.

At the molecular level, the CMH6 group exhibited a clear anti-inflammatory gene expression profile: Downregulation of pro-inflammatory cytokines: *tnf- α* and *il-1 β* were significantly suppressed compared with the CM group ($P < 0.05$); Upregulation of anti-inflammatory cytokine: *il-10* was significantly increased ($P < 0.05$); Upregulation of growth-related gene: *igf-1* was significantly increased ($P < 0.05$; Fig. 3). The immunomodulatory effects of CCHM are supported by multiple herbal components. *Codonopsis Radix* (Dangshen) contains various saccharides and terpenoids that promote digestion, absorption, and enhance immunity (Liu et al., 2020). *Astragali Radix* (Huangqi) is well-known for its immunomodulatory properties (Meng et al., 2024). *Poria* (Yunling) enhances immunity and exhibits antiviral and anti-inflammatory activities (Chu et al., 2022). *Magnoliae Officinalis Cortex* (Houpo) provides magnolol and honokiol with anti-inflammatory effects (Tan et al., 2020).

This pattern suggests that CCHM may inhibit the NLRP3 inflammasome pathway, a mechanism recently demonstrated in other fish species fed herbal supplements (Trivedi et al., 2023). The concurrent upregulation of *il-10* indicates activation of regulatory T-cell pathways, consistent with findings in Atlantic salmon (Tawfik et al., 2024). This effect probably indicates that the anti-inflammatory effects of CCHM occur at the molecular level without detectable macroscopic tissue damage, suggesting that CCHM modulates immune homeostasis proactively rather than repairing existing pathology.

The multi-target protection of CCHM can be attributed to its 16 constituent herbs (Supplementary table 1). Based on their known bioactivities, these herbs provide complementary functions: antioxidant (e.g., *Astragalus*, *Glycyrrhiza*), anti-inflammatory (e.g., *Magnolia*, *Poria*), digestive-promoting (e.g., *Citrus*, *Hordeum*), immunomodulatory (e.g., *Codonopsis*, *Astragalus*), and lipid-lowering (e.g., *Atractylodes*, *Crataegus*) effects (Meng et al., 2024; Jia et al., 2023; Tan et al., 2020; Chu et al., 2022; Liu et al., 2020; Sun et al., 2024). Importantly, the absence of intestinal pathology suggests that CCHM acts primarily through biochemical and molecular pathways—reducing oxidative stress, suppressing pro-inflammatory cytokines, and upregulating growth-related genes—rather than through macroscopic changes in intestinal morphology.

5. Conclusion

In conclusion, dietary supplementation with 6% Chinese herbal compound (CCHM) effectively alleviates multiple adverse effects induced by complete (100%) fishmeal replacement with *Chlorella vulgaris* in largemouth bass, including improvements in survival, antioxidant capacity, immune homeostasis, and hepatic lipid metabolism, although growth performance was not restored to the level of the fishmeal control. These benefits are attributed to the synergistic multifunctional properties of the 16-component CCHM formula. The increased muscle thickness observed across all *Chlorella*-based diets reflects a compensatory adaptation rather than a CCHM-specific effect, and no intestinal pathology was detected. Overall, integrating microalgae with herbal compounds represents a promising functional strategy to support fish health under high-level fishmeal substitution. Future studies should focus on identifying bioactive constituents, evaluating long-term reproductive effects, and assessing commercial feasibility.

Declarations

Author Contributions: Conceptualization, investigation, resources, project administration, funding acquisition, **Jishu Zhou**; methodology, validation, formal analysis, **Qinglai Xu**; data curation, **Shengke Wen** and **Qinglai Xu**; writing—original draft preparation, **Shengke Wen**, **Mingke Hu**; writing—review and editing, **Jishu Zhou** and **Shahid Sherzada**; visualization, **Shengke Wen**, **Mingke Hu**, **Shahid Sherzada**. All authors have read and agreed to the published version of the manuscript.

Funding: This study was financially supported by the National Key Research and Development Program of China (2019YFD0900200), Shaanxi Special plan project of technological innovation guidance (2022QFY12-03) and the Foreign Expert Project (H20240497).

Institutional Review Board Statement: The present experimental procedures were strictly abiding by the guidelines of the National Council for Control of Animal Experimentation (CONCEA) for the caring and use of the laboratory animals in Northwest A&F University of Animal Experimentation Ethics Committee, Yangling, China (No. DK2022007).

Data Availability Statement: Data are available upon request to the corresponding author.

Conflicts of Interest: Authors declare that there are no known financial conflicts of interest or personal relationships that could have impacted the research presented in this paper.

References

1. Ahmad, A., W. Hassan, S., & Banat, F. (2022). An overview of microalgae biomass as a sustainable aquaculture feed ingredient: Food security and circular economy. *Bioengineered*, 13(4), 9521–9547.
2. Ahmadifar, E., Pourmohammadi Fallah, H., Yousefi, M., Dawood, M. A., Hoseinifar, S. H., Adineh, H., ... Doan, H. V. (2021). The gene regulatory roles of herbal extracts on the growth, immune system, and reproduction of fish. *Animals*, 11(8), 2167.
3. Albaqami, N. M. (2024). *Chlorella vulgaris* as unconventional protein source in fish feed: A review. *Aquaculture*, 741404.
4. AOAC. Official Methods of Analysis, 17th ed.; AOAC: Gaithersburg, MD, USA, 2005.
5. Cai D S, Jiang G Z, Zhang D D, et al. Effects of dietary glycyrrhetic acid supplementation on intestinal digestive enzyme activities and carcass composition of juvenile blunt snout bream (*Megalobrama amblycephala*) [J]. *Jiangsu Agricultural Sciences*, 2015, 43(6): 212–214. (in Chinese)
6. Chen, F., Qian, J., He, Y., Leng, Y., & Zhou, W. (2022). Could *Chlorella pyrenoidosa* be exploited as an alternative nutrition source in aquaculture feed? A study on the nutritional values and anti-nutritional factors. *Frontiers in Nutrition*, 9, 1069760.
7. Chu Y X, Zhang Z, Zhang F D, et al. Effects of *Poria cocos* polysaccharide on antioxidant capacity, liver function, non-specific immunity and digestive enzymes of blood parrot fish (*Vieja synspila* × *Vieja melanurus*) [J]. *Tianjin Agricultural Sciences*, 2022, 28(9): 39–45. (in Chinese)
8. Costello, C., Cao, L., Gelcich, S., Cisneros-Mata, M. Á., Free, C. M., Froehlich, H. E., ... Lubchenco, J. (2020). The future of food from the sea. *Nature*, 588(7836), 95–100.
9. Cui, Z., Zhang, J., Ren, X., & Wang, Y. (2022). Replacing dietary fish meal improves ecosystem services of largemouth bass (*Micropterus salmoides*) farming. *Aquaculture*, 550, 737830.
10. Gressler L T, Centenaro R J, Braz H P, et al. Influence of dietary bitter orange peel powder on growth, body composition, blood parameters, gut morphometry, and thermal tolerance of Nile tilapia (*Oreochromis niloticus*) [J]. *Fish Physiology and Biochemistry*, 2024, (prepublish): 1–12.

11. He B X, Yang Y T, He Y L, et al. Preliminary study on developmental toxicity of essential oil from *Aucklandia lappa* Decne. on zebrafish embryos [J]. *China Journal of Traditional Chinese Medicine and Pharmacy*, 2016, 31(11): 4714–4716. (in Chinese)
12. Hu, X., Ma, W., Zhang, D., Tian, Z., Yang, Y., Huang, Y., & Hong, Y. (2025). Application of Natural Antioxidants as Feed Additives in Aquaculture: A Review. *Biology*, 14(1), 87.
13. Jia, D., Tang, Y., Qin, F., Liu, B., Hu, T., & Chen, W. (2023). Ganoderma lucidum polysaccharide alleviates Cd toxicity in common carp (*Cyprinus carpio*): Neuropeptide, growth performance and lipid accumulation. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 271, 109663.
14. Kang, J., Fei, S., Zhang, J., Liu, H., Luo, Q., Ou, M., ... Zhao, J. (2025). Effects of Chinese Herbal Medicines on Growth Performance, Antioxidant Capacity, and Liver and Intestinal Health of Hybrid Snakehead (*Channa maculata* × *Channa argus*). *Fishes*, 10(1), 33.
15. Khazaei, M., & Ardeshtir, R. A. (2024). Protective effects of sulfated polysaccharides from *Enteromorpha intestinalis* on oxidative stress, liver iron overload and Ferroptosis in Zebra fish exposed to ethanol. *Biomedicine & Pharmacotherapy*, 181, 117715.
16. Krogdahl, Å., Sundby, A., & Holm, H. (2015). Characteristics of digestive processes in Atlantic salmon (*Salmo salar*). Enzyme pH optima, chyme pH, and enzyme activities. *Aquaculture*, 449, 27–36.
17. Li C H, Dong H B, Zheng X T, et al. Effects of essential oil of *Amomum villosum* on growth, digestion, antioxidant capacity and serum biochemical indices of juvenile Nile tilapia (*Oreochromis niloticus*) [J]. *South China Fisheries Science*, 2023, 19(6): 51–59. (in Chinese)
18. Liu F, Geng C, Qu Y K, et al. The feeding of dietary *Codonopsis pilosula* polysaccharide enhances the immune responses, the expression of immune-related genes and the growth performance of red swamp crayfish (*Procambarus clarkii*) [J]. *Fish & Shellfish Immunology*, 2020, 103: 321–331.
19. Liu X D, Huang Y, Liu Y F, et al. Effect of compound Chinese herbal medicine additive on production performance of livestock and poultry [J]. *Feed Industry*, 2025, 46(3): 14–19. (in Chinese)
20. Livak, K.J., Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)). *Method. Methods*, 25, 402–408.
21. Lutz, G. A., Ciurli, A., Chiellini, C., Di Caprio, F., Concas, A., & Dunford, N. T. (2021). Latest developments in wastewater treatment and biopolymer production by microalgae. *Journal of Environmental Chemical Engineering*, 9(1), 104926.
22. Meng M H, Ma L, You H Z, et al. Research progress on application of *Astragalus* and its polysaccharides in aquaculture [J]. *Modern Journal of Animal Husbandry and Veterinary Medicine*, 2024(12): 77–81. (in Chinese)
23. Mosibo, O. K., Ferrentino, G., & Udenigwe, C. C. (2024). Microalgae proteins as sustainable ingredients in novel foods: recent developments and challenges. *Foods*, 13(5), 733.
24. Peng R Z, Su R B, Li J J, et al. Study on medication rules of Chinese herbal compound containing *Citri Reticulatae Pericarpium* in the treatment of functional dyspepsia based on data mining [J]. *Journal of Guangdong Pharmaceutical University*, 2024, 40(4): 77–83. (in Chinese)
25. Qiu Y J, Tong P Z, Wu M L, et al. Identification and quality study of *Galli Gigerii Endothelium Corneum* and its adulterants [J]. *China Pharmaceuticals*, 2024, 33(20): 57–62. (in Chinese)
26. Sun H Q, Wang Y, Li J X, et al. Effects of *Crataegus pinnatifida* compound Chinese herbal medicine on growth, digestion and health of largemouth bass (*Micropterus salmoides*) [J]. *China Feed*, 2024(3): 89–96. (in Chinese)
27. Tan Z Y, Deng J G, Zhang T, et al. Research progress on modern pharmacology of *Magnolia officinalis* in traditional Chinese medicine [J]. *Chinese Journal of Experimental Traditional Medical Formulae*, 2020, 26(22): 228–234. in Chinese
28. Tawfik, M. M., Betancor, M. B., McMillan, S., Norambuena, F., Tocher, D. R., Douglas, A., & Martin, S. A. (2024). Modulation of metabolic and immunoregulatory pathways in the gut transcriptome of Atlantic salmon (*Salmo salar* L.) after early nutritional programming during first feeding with plant-based diet. *Frontiers in Immunology*, 15, 1412821.
29. Tibbetts, S. M., Milley, J. E., & Lall, S. P. (2015). Chemical composition and nutritional properties of freshwater and marine microalgal biomass cultured in photobioreactors. *Journal of Applied Phycology*, 27, 1109–1119.
30. Trivedi, S. P., Dwivedi, S., Singh, S., Khan, A. A., Kumar, M., Shukla, A., ... Tiwari, V. (2023). Evaluation of immunostimulatory attributes of *Asparagus racemosus* and *Withania somnifera* supplemented diets in fish, *Channa punctatus* (Bloch, 1793). *Veterinary Immunology and Immunopathology*, 258, 110561.

31. Wang M Y, Shen J, Liu J, et al. Lipid-lowering effect of atractylone on obese zebrafish [J]. *Chinese Traditional Patent Medicine*, 2024, 46(4): 1360–1364. (in Chinese)
32. Wang R J, Liu W, Liu L, et al. Characterization, in vitro digestibility, antioxidant activity and intestinal peristalsis in zebrafish of *Dioscorea opposita* polysaccharides [J]. *International Journal of Biological Macromolecules*, 2023, 250: 126155.
33. Wang, W., Sun, J., Liu, C., & Xue, Z. (2017). Application of immunostimulants in aquaculture: current knowledge and future perspectives. *Aquaculture Research*, 48 (1), 1–23.
34. Wang, X., Qu, P., Huangfu, Y., Liu, D., Wu, Y., Chen, P., ... Zhang, W. (2024). A compound of herbs improves the growth performance, intestinal and liver histology, antioxidative capacity and immunity of juvenile large yellow croaker *Larimichthys crocea*. *Aquaculture Reports*, 36, 102086.
35. Xi, L., Lu, Q., Liu, Y., Su, J., Chen, W., Gong, Y., ... Xie, S. (2022a). Effects of fish meal replacement with Chlorella meal on growth performance, pigmentation, and liver health of largemouth bass (*Micropterus salmoides*). *Animal Nutrition*, 10, 26–40.
36. Xu Q.L., Zhou J.S., Zhu Z. L., Wang A., Zhang N. K., He H. (2024). Compound Chinese herbal medicine on flesh quality of largemouth bass (*Micropterus salmoides*) in the diet of fish meal replaced by Chlorella meal. (2024). *Acta Hydrobiologica Sinica*, 48(7), 1245–1257.
37. Yu, W., Yang, Y., Zhou, Q., Huang, X., Huang, Z., Li, T., ... Lin, H. (2022). Effects of dietary Astragalus polysaccharides on growth, health and resistance to *Vibrio harveyi* of Lates calcarifer. *International Journal of Biological Macromolecules*, 207, 850–858.
38. Zhuo W W, Ren S J, Wang X, et al. Effect of *Atractylodes macrocephala* polysaccharide on growth and health maintenance of yellow catfish (*Pelteobagrus fulvidraco*) [J]. *Feed Industry*, 2022, 43(24): 32–38. (in Chinese)

Figures

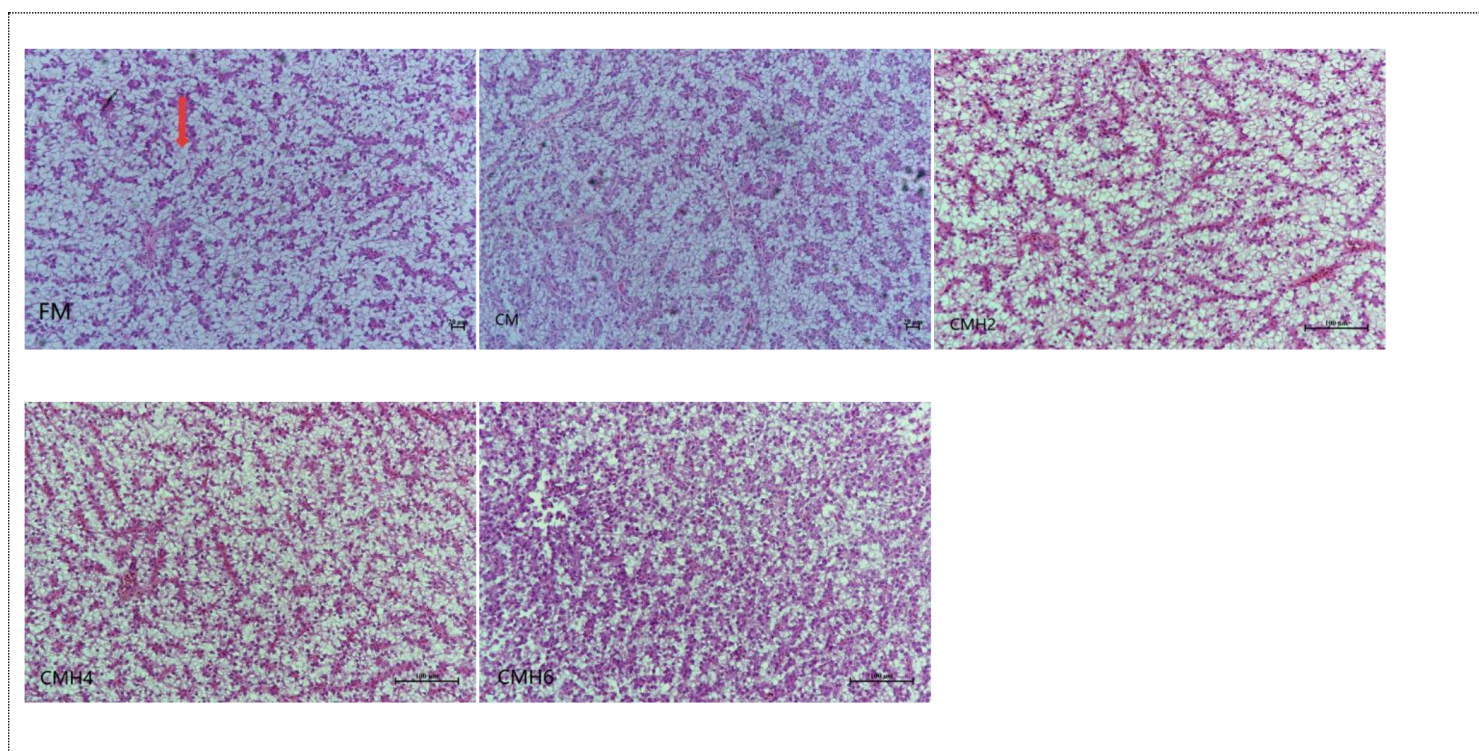
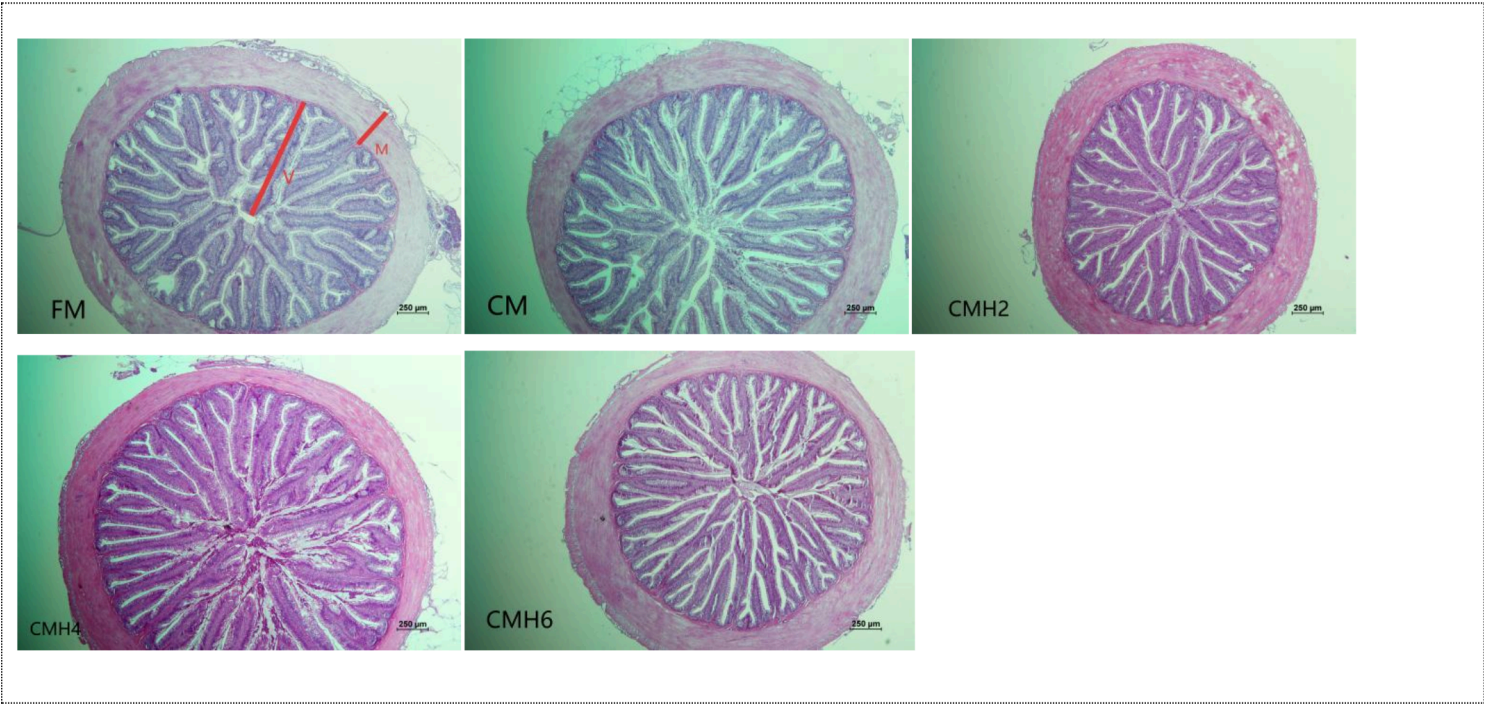


Figure 1

The effects of compound Chinese herbal medicine in the diet of fish meal replaced by *Chlorella* meal on the liver of Largemouth bass (*Micropterus salmoides*). Red arrows indicate the vacuoles of the liver cell.

(A): the structure of intestine by HE staining



(B) and (C): the villus height and muscle thickness of the intestine respectively

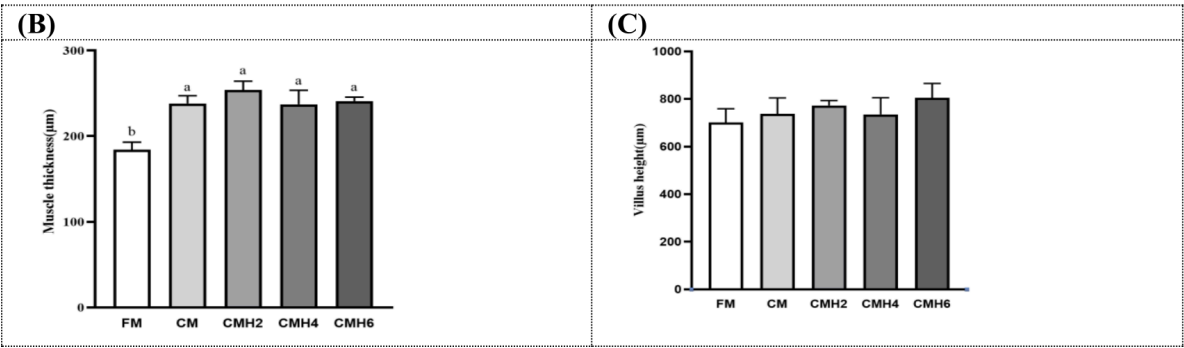


Figure 2

The effects of compound Chinese herbal medicine in the diet of fish meal replaced by *Chlorella* meal on the structure of intestine of Largemouth bass. A shows the structure of intestine by HE staining and B shows the villus height and muscle thickness of the intestine, where VH means the villus height of the intestine and the MT means the muscle thickness of the intestine in the figure.

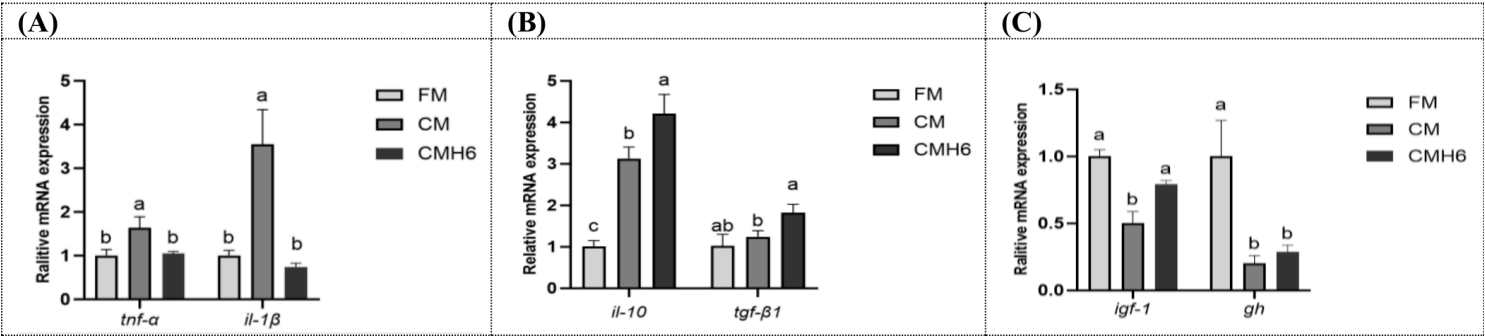


Figure 3

Effects of compound Chinese herbal medicine in the diet with fish meal replaced by *Chlorella* meal on the relative expression of inflammatory genes (A), cytokines (B), and growth-related genes (C) in Largemouth bass

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [AppendixTable1.docx](#)