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Optimal Dietary Protein Level from *Chlorella vulgaris* Meal Improved the Growth Performance, Serum Antioxidant Capacity, Immune Response, and Muscle Quality of Juvenile Grass Carp (*Ctenopharyngodon idellus*)

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Abstract

The aim of this study was to assess the optimal protein level for grass carp when using Chlorella vulgaris meal (CVM) as a sole protein source. Six diets (CVM35, CVM42, CVM49, CVM55, CVM62, and CVM69) with varying protein levels (21.51%, 24.95%, 29.16%, 32.75%, 36.79%, and 39.74%, respectively) were fed to triplicate tanks with 30 fish each (7.0±0.1 g) for 8 weeks. Results indicated the FBW and SGR increased first and then plateaued as dietary protein level increased, while the FR plateaued first and then decreased. The FE and HSI increased linearly with dietary protein level, while VSI and MFI decreased linearly ($P<0.05$). As dietary protein level increased, serum IgM and GSH-Px first increased and then decreased, and peaked in the CVM55 group, while MDA showed the opposite trend. High protein diets (CVM62 and CVM69) significantly activated mTOR signaling and altered fish proximate composition. Moreover, optimal protein diet enhanced fish hardness and regulated the expression of genes associated with myosin types (myh7, myh1, myh2, and myh4) and muscle development (MRFs, FGF6s and mstn). An optimal dietary protein level of 36.54% was recommended for juvenile grass carp based on the SGR using CVM as protein source.

Introduction

Protein plays a key role in the maintenance, tissue growth, reproduction and energy metabolism of fish. Fish meal is considered the most acceptable and valuable protein source in the fish diet (Jobling, 2012). With the increasing market demand for aquatic products, the shortage and high price of fish meal have become critical issues restricting the rapid development of the aquaculture industry (Bostock et al., 2010). Therefore, finding alternative protein sources to replace fish meal is necessary for the sustainable aquaculture. Therefore, to promote sustainability, recent studies have focused on evaluating alternative protein sources (Gunathilaka et al., 2023; Sahin & Ergün, 2021; Zarantoniello et al., 2023) that may serve as replacements for fish meal, as well as identifying optimal levels (Liu et al., 2022; Wang et al., 2022) for incorporating various protein sources.

Chlorella vulgaris, a globally produced functional green algae, is getting rising attention for various utilization (Alagawany et al., 2021). The mature *C. vulgaris* meal (CVM) shares a high total protein content, which is comparable to soybean protein (Caporgno & Mathys, 2018). CVM is also rich in highly unsaturated fatty acids, chlorophyll, and many primary carotenoids, and these pigments exhibit immunomodulatory and antioxidant properties by significantly reducing the oxidative stress induced by harmful chemicals and increasing the antioxidant activity in the tested animals

(Chen et al., 2022; Galal et al., 2018). CVM also exhibits a balanced amino acid profile, good biosafety and ease of cultivation, making it a potential alternative protein source for fish meal (Safi et al., 2014). It has been reported that diet enriched with CVM ameliorated the toxic effects of diazinon on Nile tilapia by modulating immunity and increasing antioxidant capacity (Abdelhamid et al., 2020). Dietary CVM supplementation also improved the muscle development and growth performance in crucian carp and largemouth bass (Shi et al., 2017; Yu et al., 2022). The above mentioned results indicate the potential value of CVM in fish diets.

The muscles of teleost fish constitute a significant part of human consumption. Muscle growth including myofiber hyperplasia and hypertrophy (Silva et al., 2009), is regulated by various genes, such as myogenic regulatory factors (MRFs), myostatin (*mstn*), fibroblast growth factor-6 (*fgf6*), etc. (Ganassi et al., 2020; Hernández-Hernández et al., 2017; McPherron et al., 1997). Muscle fiber, profoundly influencing the biochemical characteristics and meat quality, can be classified as types I, IIa, IIx, and IIb based on the contents of the heavy chain myosins, which were encoded by *myh7*, *myh2*, *myh1* and *myh4*, respectively (Valente et al., 2013). When the proportion of type I and IIa fibers is higher, intramuscular fat is higher, and meat is more tender and juicy (Essén-Gustavsson et al., 1994). Currently, there is limited research on the effects of plant protein in fish feed on the meat quality of fish. It was reported that an optimal dietary protein level of 35.98% provided by soybean meal improved the expression of *myh1* and *myh4*, while inhibited the expression of *myh2* and *myh7*, and altered the grass carp's metabolism and antioxidant capacity, thus enhancing the meat quality (Wang et al., 2022). Different dietary protein contents from cottonseed protein concentrate (CPC) also changed the *mrfs* expression to induce the transformation of muscle protein subtypes and improve fish quality (Liu et al., 2022).

Grass carp is one of China's most merchant and important freshwater culture species due to its wide distribution, herbivore food habits and delicious meat (Xie et al., 2018). Although its global production was more than 50 million tons (FAO, 2020), grass carp's meat quality and economic profit have been severely limited by the intensive culture (Zhao et al., 2018). Nutritional regulation on fish growth and meat quality has been investigated in grass carp. Appropriate levels of dietary protein can promote the growth and muscle stiffness of juvenile grass carp by promoting the growth and differentiation of muscle fibers, improving collagen synthesis, and inhibiting collagen degradation (Dong et al., 2022). In previous studies, we explored the effects of soybean meal and CPC as a single feed protein on the growth and meat quality of grass carp, which confirmed their positive nutritional value in grass carp diet (Liu et al., 2022; Wang et al., 2022).

Considering the nutritional features of CVM, the aim of this study was to determine the suitable dietary CVM protein level for grass carp diet and to evaluate the effects of different CVM protein levels on growth performance, immune antioxidant capacity and transcription levels of genes related to protein metabolism and meat quality.

Materials and Methods

Preparation of Experimental Diets

The CVM (containing 57.5% crude protein) used for protein source in this study was provided by Wuhan Demote Biotechnology Co., Ltd. (Wuhan, China). Six experimental diets, namely, CVM35, CVM42, CVM49, CVM55, CVM62 and CVM69, with graded protein contents (21%, 25%, 29%, 33%, 37% and 41%) were formulated according to Wang et al. (2022) with CVM level at 35.40%, 42.20%, 49.00%, 55.70%, 62.50%, and 69.40% respectively. Soya oil + fish oil (1:1) were used as lipid source and corn starch as the carbohydrate source. The ingredients and proximate composition of the experimental diets were shown in Table 1. The raw materials were totally smashed (through an 80-mesh sieve), thoroughly mixed and then extruded into 2 mm diameter spherical-like particles using a thermal feed extruder (the moisture of meals: 21%, the extruder barrel temperature: 130°C) and then dried in an electric oven at 60°C for 30 min. The pellets were then stored at -20°C until use.

Experimental Fish and Feeding

Juvenile grass carps used were obtained from Li Wenhua Aquaculture Co., Ltd, and were adapted to the experiment conditions for two weeks. Before the feeding trial, fish were fasted for 24h and then weighed collectively. 30 fish (initial body weight: 7.0±0.1g) per tank (water volume: 300 L) were randomly distributed into 18 tanks (3 tanks for each diet) in a flow-through aquaculture system equipped with temperature control system and constant aeration. Fish were fed by hand with experimental diets to apparent satiation twice a day for 8 weeks. The feed intake was recorded daily. During the feeding period, the culture conditions in the tank were as follows: the water exchange was 0.5 L/min, average water temperature was at 28.0±1.5°C, the dissolved oxygen level was above 5.0 mg/L, the pH was 7.5~7.7, the ammonium nitrogen content was less than 0.5 mg/L.

Sample Collection

At the end of the feeding trial, fish were fasted for 24h. After the rapid anesthesia with MS-222 (100 mg/L), fish in each tank were counted and collectively weighed to calculate the specific growth rate (SGR), feeding rate (FR), and feed efficiency (FE). Three fish in each tank

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

Ingredients	Diet treatment (% in dry matter)					
	CVM35	CVM42	CVM49	CVM55	CVM62	CVM69
High protein CVM[1]	35.40	42.20	49.00	55.70	62.50	69.40
Soya oil + Fish oil (1:1)	3.08	2.37	1.65	0.95	0.25	0.00
Corn starch	22.70	22.20	21.70	21.20	20.60	20.10
Microcrystalline cellulose	35.42	29.83	24.25	18.75	13.25	7.10
1% premix[2]	1.00	1.00	1.00	1.00	1.00	1.00
Monocalcium phosphate	1.80	1.80	1.80	1.80	1.80	1.80
Choline chloride (50%)	0.50	0.50	0.50	0.50	0.50	0.50
Yttrium oxide	0.10	0.10	0.10	0.10	0.10	0.10
Total	100.00	100.00	100.00	100.00	100.00	100.00
Proximate composition						
Moisture (%)	5.08	5.04	5.07	5.53	5.16	4.39
Crude protein (%)	21.51	24.95	29.16	32.75	36.79	39.74
Crude lipid (%)	6.82	6.97	7.07	7.26	7.21	6.95
Total ash (%)	4.14	4.57	5.27	5.78	6.64	7.63

[1]CVM, *C. vulgaris* meal, containing 2.7% moisture, 57.5% protein, 10.2% crude lipid, 5.9% ash, 16.2% crude fiber and 7.5% nitrogen free extraction; [2]1% Premix provided (/Kg diet): vitamin C phosphate (35%), 900 mg; Vitamin E 450 mg; Inositol, 225 mg; Nicotinamide, 120 mg; Calcium pantothenate, 60 mg; Vitamin A, 30 mg; Vitamin K3, 3 mg; Vitamin B12, 12 μg; Biotin, 3 mg; Ferric sulfate monohydrate, 300 mg; Zinc sulfate monohydrate, 200 mg; Sodium chloride, 100 mg; Manganese sulfate monohydrate, 25 mg; Cupric sulfate pentahydrate, 30 mg; Cobalt chloride (10% cobalt), 5 mg; Sodium selenite (10% selenium), 5 mg; Potassium iodate (2.9%), 3 mg; Magnesium sulfate, 900 mg.

were then randomly sampled and frozen at -80°C for subsequent proximate analysis of whole fish. Another five fish from each tank were randomly sampled, individually weighed and measured (body length) to calculate the condition factor (CF), and then, blood samples were collected from the caudal vein, serum was subsequently obtained by centrifugation (3000 ×g, 10min, 4°C) and then frozen at -80°C for various assays (Liu et al., 2022). After that, the viscera, hepatopancreas, mesenteric fat were dissected from the five fish and weighed to calculate viscerosomatic index (VSI), hepatosomatic index (HSI) and mesenteric fat index (MFI). The muscle samples for enzyme activity assay and quantitative real time polymerase chain reaction (qRT-PCR) were flash frozen in liquid nitrogen and then stored at -80°C. In addition, muscle samples was collected and steeped in 4% buffered formalin for histopathological analysis (Wang et al., 2022). Fresh muscle was sampled from two fish for texture analysis.

Analytical Method

Determination of Muscle Texture Analysis

The dorsal white muscle (about 2-3 g) was weighed, wrapped in gauze and then cooked in boiling deionized water (100°C) for 5 min. After cooking, the muscle was taken out and weighed after surface water removal using an absorbent paper. The cooked muscle was subsequently cut into a 1 × 1 × 1cm cube to perform the texture profile analysis (TPA) by a TA. XT Plus texture analyzer (Stable Micro Systems, Godalming, UK) equipped with a flat-bottomed cylindrical probe P/36R (20 mm diameter). During the test, samples were compressed twice at a constant depression speed of 1 mm/s to 35% of the original height, with a holding time of 5 s. In addition, the pre-test speed was 2 mm/s, the post-test speed was 5 mm/s, and the type of loadbearing probe was Auto-5 g. The data collection rate was 200 points per second (pps). Textural parameters were calculated as described by Bourne (1978).

Measurements of Serum Biochemical Indexes

The serum total protein (TP), blood urea nitrogen (BUN), glucose (GLU), total cholesterol (TC), triglyceride (TG), and aspartate aminotransferase (AST) activities were measured by the commercially available kits using an automated biochemical analyzer (Abbott Aeroset®, Abbott Laboratories). The serum activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), total antioxidant capacity (T-AOC), and malondialdehyde (MDA) content were spectrophotometer measured by the commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum activities of lysozyme (LYZ) (Product number ml036413), complement 3 (C3) (Product number ml092636), and the content of immunoglobulin M (IgM) (Product number ml092683) were determined by commercially available kits (Shanghai Enzyme-linked Biotechnology Co., Ltd, Shanghai, China) according to the manufacturer's instructions.

Proximate Analysis of Fish and Experimental Diets

The proximate composition was analyzed according the AOAC official method (AOAC, 1995). Briefly, by air drying to a stable weight at 105°C, moisture was measured. Crude protein was determined by measuring nitrogen (N × 6.25) using the Kjeldahl method. Crude lipid was measured by a Soxhlet extract apparatus with ether exaction. Moreover, ash was determined by incinerating at 550°C for 5-6 h in a muffle furnace.

Muscle Histological Analysis

Muscle histology analysis was conducted according to the protocol as described by Wang et al. (2022). Briefly, dorsal muscle was fixed in 4% paraformaldehyde solution, then dehydrated by several levels of ethyl alcohol, and implanted into paraffins. The histological slides (7 μm) were prepared using a microtome then stained with hematoxylin-eosin (HE) for histological observation by light microscope. Diameter of muscles fibers were measured by M Shot Image Analysis (Micro-shot, Guangzhou, China).

Exaction of RNA and Real-time PCR Analysis

Primer premier 6.0 software was used to design specific primer pairs for each target gene using known sequences in the NCBI database (Table 2). Total RNA was extracted from muscle samples by RNAiso Plus Kit (Takara, Dalian, China) and then resuspended in 50 μL RNase-free water. And then RNA quality and quantity were tested by electrophoresis on 1% agarose gel and by NanoDrop® ND-1000 UV–vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 260 and 280 nm. 1μg of total RNA was used for reverse transcription through PrimeScript™ RT reagent Kit (Takara, Dalian, China) according to the instruction from the manufacturers. Quantitative RT-PCR was performed under the conditions: pre-incubation at 95°C for 5 min, forty cycles at 95°C for 10 s, annealing temperature (corresponding specific primer pairs) for 20 s and 72°C for 20 s. Melting curves were systematically monitored (temperature gradient at 0.5°C/s from 60 to 95°C) to confirm that only one fragment was amplified. The expression of the genes was quantified relative to *β-actin* and *ef1α* as reference genes using the $2^{-\Delta\Delta Ct}$ value method as described by Pfaffl (2001).

Statistical Analysis

All data were analyzed by one-way ANOVA and statistical Duncan's multiple comparison in the SPSS 26.0 (IBM, Armonk, NY, USA) and presented as mean ± SEM (*n*=3). Before carrying out data analysis, normality and homogeneity of variance were tested first. When significant treatment effect was observed, the acquired data were also subjected to orthogonal polynomial contrasts (linear, quadratic, or cubic) and further analyzed for regression analysis to determine the optimal model. The R square (R^2) was used to determine model fit for optimal regression selection and differences were considered significant at P<0.05.

Results

Effects of Dietary CVM on Growth Performance and Morphology

The growth performance and morphological parameters were shown in Table 3. The FBW and SGR increased linearly and then stabilized with the increase of dietary protein level (P<0.05), and broken-line analysis based on SGR suggested an optimal dietary protein level of 36.54% using CVM as protein source (Figure 1). FR increased and then decreased with dietary protein level, peaked in the CVM42 group. FE demonstrated a linear increase with dietary protein level (P<0.05). Furthermore, as the dietary protein level increased, VSI and MFI decreased linearly (P<0.05, R^2=0.781 and 0.845, respectively), while HSI increased linearly (P<0.05, R^2=0.764). However, dietary protein level did not significantly affect the survival rate (SR) (P>0.05).

Effects of Dietary CVM on Serum Biochemical, Immune Response and Antioxidation Indices

As shown in Table 4, GLU and TP first rose and then declined when dietary protein level increased, while TG showed an opposite trend (P<0.05). All these changes were described by the quadratic model (R^2=0.759, 0.852, and 0.517, respectively). As dietary protein level increased, the levels of serum AST and TC significantly decreased (R^2=0.695 and 0.504, respectively), while BUN did not vary significantly (P>0.05).

Table 2. Sequences of primers employed in this study

Genes	Forward primer(5'-3')	Reverse primer(5'-3')	Accession number
fgf6a	CGCATACGAGTCTTCCAT	CCTACGAGAACATCCAACA	MK050993
fgf6b	TCCAGTCCGCTTCCGAGTA	AGATGAAACCCGATGCCTACA	MK050992
myf5	GTGCCTGTGCCTCATCTCCT	AATGCGTGGTTCACCTTCTTCA	GU290227
mrf4	TCGCTCCTGTATTGATGTTGATGA	GCTCCTGTCTCGCATTCGTT	KT899334
myog	TTACGAAGGCGGCGATAACTT	TGGTGAGGAGACATGGACAGA	JQ793897
myod	ATGGAGTTGTCGGATATTCCCTTC	GCGGTCAGCGTTGGTTGTT	MG544985
mstn	CTGACGCCAAGTTCCACATACA	CGACTCTGCTTCAAGTTCTTCTCT	KP719016
myh7	AACTGCGCTGTAACGGTGTA	AGTGTGCCCAAACCTGTACT	MW113233
myh2	ACAGTGGCCAGCATTGATGA	TGTCCAGCACACCAATGAAGA	MW113235
myh1	TTCCGTTGTTGTGTCAGGCT	TACTGGATGACGCGTTTGGT	MW113234
myh4	TACTGGATGACGCGTTTGGT	TGTCCAGCACACCAATGAAGA	MW113236
tor	TCCCACTTTCCACCAACT	ACACCTCCACCTTCTCCA	JX854449
4ebp1	GCTGGCTGAGTTTGTGGTTG	CGAGTCGTGCTAAAAAGGGTC	KT757305
s6k1	ACATAAAGCAGCCTGACG	TGGAGGAGGTAATGGACG	EF373673
β-actin	TATGTTGGTGACGAGGCTCA	GCAGCTCGTTGTAGAAGGTG	M25013
ef1α	TGACTGTGCCGTGCTGAT	CGCTGACTTCCTTGGTGATT	GQ266394

Table 3. Effects of dietary protein level from *C. vulgaris* meal on growth performance and morphological parameters of juvenile grass carp

Diet treatments[2]	IBW (g fish^{-1})	FBW (g fish^{-1})	SGR (% d^{-1})	SR (%)	FR (%BW d^{-1})	FE (%)	CF (%)	HSI (%)	VSI (%)	MFI (%)
CVM35	7.04±0.01	14.05±1.28^{a}	1.22±0.16^{a}	90.00±5.09	3.12±0.08^{bc}	41.38±1.92^{a}	1.79±0.02	1.33±0.02^{a}	8.67±0.18^{d}	2.80±0.10^{e}
CVM42	7.03±0.02	15.02±0.76^{ab}	1.35±0.10^{ab}	86.67±5.09	3.21±0.07^{c}	43.22±1.87^{a}	1.80±0.01	1.39±0.01^{ab}	8.28±0.14^{cd}	2.72±0.01^{de}
CVM49	7.01±0.03	17.06±0.28^{abc}	1.59±0.03^{bc}	95.00±0.96	3.17±0.14^{bc}	50.56±0.56^{b}	1.79±0.01	1.46±0.01^{bc}	8.16±0.27^{cd}	2.49±0.01^{cd}
CVM55	6.96±0.01	17.48±0.48^{bc}	1.64±0.05^{bc}	84.45±4.01	3.09±0.04^{bc}	52.75±0.88^{b}	1.78±0.00	1.48±0.01^{bc}	7.93±0.04^{bc}	2.40±0.08^{bc}
CVM62	6.98±0.03	21.52±1.36^{d}	1.86±0.16^{c}	87.78±4.84	2.87±0.15^{ab}	62.74±3.19^{c}	1.80±0.01	1.49±0.03^{c}	7.47±0.14^{ab}	2.17±0.11^{b}
CVM69	7.01±0.02	19.92±1.05^{cd}	1.86±0.10^{c}	94.45±2.22	2.75±0.07^{a}	64.36±3.83^{c}	1.80±0.01	1.51±0.01^{c}	7.36±0.08^{a}	1.82±0.11^{a}
PSE [3]	0.141	1.657	0.195	6.973	0.173	4.069	0.000	0.032	0.277	0.141
Orthogonal contrast (Pr>F) [4]										
Linear	0.064	0.000	0.000	0.675	0.004	0.000	0.557	0.000	0.000	0.000
Quadratic	0.106	0.791	0.568	0.492	0.065	0.497	0.471	0.021	0.843	0.079
Cubic	0.262	0.288	0.813	0.311	0.699	0.541	0.299	0.538	0.789	0.359
Regression										
Model [5]	NOS	Broken line	Broken line	NOS	Quadratic	Linear	NOS	Linear	Linear	Linear
R^2		0.910	0.963		0.579	0.969		0.764	0.781	0.845
Pr>F [4]		0.000	0.000		0.002	0.000		0.000	0.000	0.000
OPTI INCL [6]		39.59%	36.54%							

[1]Values are means of three replicates (n = 3). [2] IBW: initial body weight; FBW: final body weight; SGR (specific growth rate, %/d) = 100 × [ln (FBW) − ln (IBW)] / [experimental period (d)]; SR (survival rate, %) = 100 × fish number at the end of the trial/fish number at the start of the trial; FR (feeding rate, % BW/d) = [dry feed intake (g) / [days× [(FBW (g) + IBW (g)) /2] × 100; FE (feed efficiency, %) = 100 × [FBW (g) − IBW (g)]/dry feed intake (g); CF (condition factor, %) = 100×fish weight (g)/ [fish length (cm)]^3; HSI (hepatosomatic index, %) = 100 × [final liver weight (g) / final body weight (g)]; VSI (viscerosomatic index, %) = 100 × [final visceral weight (g) / final body weight (g)]; MFI (mesenteric fat index, %) = 100 × [mesenteric fat weight (g) / final body weight (g)]; [3] PSE: pooled standard error (n=3). [4] If statistical significance (P<0.05) was detected, the model that fits best the data was selected. [5] NOS: no structure. [6] OPTI INCL

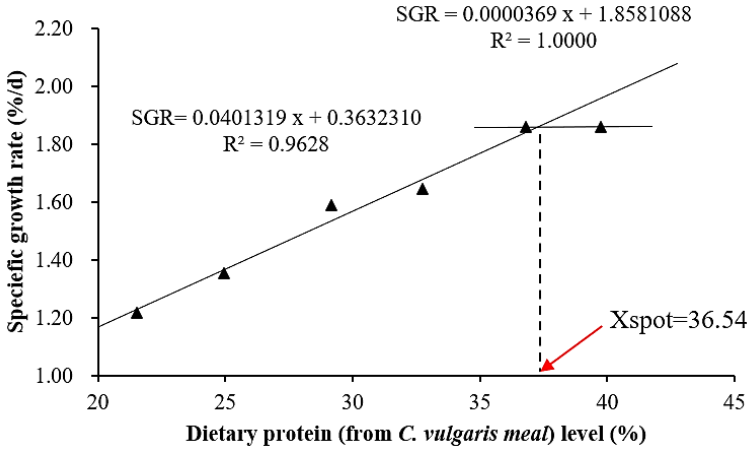

Figure 1. Broken-line analysis of the relationship between the dietary protein level and growth performance of grass carp.

As shown in Table 5, the serum LZM activity decreased linearly with dietary protein level (P<0.05), while IgM activity increased first and then declined, peaked at CVM49 and CVM55 groups (P<0.05). As dietary protein level increased, GSH-Px activity also increased first and then gradually decreased while MDA worked the opposite, which both were expressed by significant quadratic regression models (P<0.05, R^2=0.772 and 0.548, respectively). Moreover, the T-AOC activity linearly increased with dietary protein level (P<0.05, R^2=0.416). However, no significant differences in C3, SOD and CAT activities were observed among groups (P>0.05).

Effect of Dietary CVM Protein Level on Body Proximate Composition

As shown in Table 6, body moisture and ash contents significantly improved linearly with dietary protein level (P<0.05), while the lipid content linearly decreased (P<0.05). In contrast, the body crude protein increased first and then decreased (P<0.05) responding to the increase of dietary protein level in a quadratic model.

Effects of Dietary CVM Protein Level on Muscle Texture of Grass Carp

As presented in Table 7, muscle hardness, springiness, chewiness and resilience increased gradually and then decreased (P<0.05), while gumminess decreased first and then increased (P<0.05), which showed quadratic models in response to increasing dietary protein level (R^2=0.795, 0.567, 0.595 0.534 and 0.733, respectively). Meanwhile, no statistical difference among groups was observed in cooking loss and cohesiveness (P>0.05).

Histology of Skeletal Muscle

Table 8 and Figure 2 demonstrated the significant impact of dietary CVM protein level on white muscle microstructure of grass carp. The muscle fiber diameter and area clearly exhibited a firstly decreasing and then rising trend with the increase of dietary protein content, while the muscle density increased first and then declined, which all can be fitted with a quadratic regression model ($P<0.05$, R^2=0.829, 0.682, 0.771).

Gene Expression Associated with Muscle Protein Synthesis and Myogenesis

Figure 3 shows the relative expression of the genes involved in protein synthesis. The mRNA of *tor* and *s6k1* increased first and then decreased significantly in response to the increasing level of dietary CVM protein level in quadratic regression models ($P<0.05$, R^2=0.841, 0.661), while the mRNA for *4ebp1* was linearly reduced ($P<0.05$, R^2=0.888).

As presented in Figure 4, the mRNA levels of *myh2*, *myod* and *mstn* significantly decreased first and then increased thereafter ($P<0.05$, R^2=0.879, 0.684, 0.870), while the *myh1, myh4, myf5, fgf6a, fgf6b* mRNA levels gradually increased and then decreased with increasing dietary CVM protein level, which were explained by quadratic regression models ($P<0.05$, R^2=0.785, 0.773, 0.711, 0.760, 0.749). Moreover, expression of *myh7* and *myog* linearly increased with the increasing dietary protein level, while *mrf4* linearly decrease ($P<0.05$, R^2=0.759, 0.544, 0.740).

Discussion

Growth Performance and Serum Biochemical Indices

Protein requirement of grass carp varied largely due to temperature, fish life stage and protein sources (Wang et al., 2022). As a novel protein source, the nutritional value of CVM is unclear in this fish, so the dietary protein level was designed from 21% to 41%. In

Table 4. Effects of dietary protein level from *C. vulgaris* meal on serum physiological and biochemical indices of juvenile grass carp[1]

Diet treatments [2]	AST (U/L)	TP (g/L)	GLU (U/L)	BUN (mmol/L)	TC (mmol/L)	TG (mmol/L)
CVM35	84.67±2.40^{d}	30.03±0.84^{b}	5.31±0.05^{b}	1.41±0.05	7.38±0.16^{b}	1.69±0.05^{d}
CVM42	69.67±1.45^{c}	32.65±0.03^{bc}	5.46±0.02^{b}	1.48±0.02	6.36±0.22^{a}	1.35±0.01^{c}
CVM49	59.33±0.88^{ab}	32.87±0.88^{c}	5.34±0.19^{b}	1.15±0.06	6.35±0.20^{a}	1.28±0.04^{bc}
CVM55	64.33±0.33^{bc}	32.87±0.09^{c}	5.28±0.10^{b}	1.30±0.09	6.32±0.07^{a}	1.23±0.03^{b}
CVM62	63.00±2.89^{bc}	30.40±0.52^{bc}	4.62±0.11^{a}	1.47±0.11	6.30±0.00^{a}	1.06±0.02^{a}
CVM69	51.67±0.88^{a}	27.37±0.35^{a}	4.58±0.12^{a}	1.27±0.03	6.04±0.08^{a}	1.25±0.01^{b}
PSE [3]	2.991	0.969	0.195	0.118	0.251	0.118
Orthogonal contrast (Pr >F) [4]						
Linear	0.000	0.000	0.000	0.285	0.001	0.000
Quadratic	0.004	0.000	0.006	0.213	0.018	0.000
Cubic	0.000	0.906	0.249	0.261	0.007	0.780
Regression						
Model [5]	Linear	Quadratic	Linear	NOS	Linear	Quadratic
R^2	0.695	0.852	0.605		0.504	0.854
Pr>F [4]	0.000	0.000	0.000		0.001	0.000

[1] Values are means of three replicates (*n*=3). [2] AST: aspartate transferase; TP: total protein; GLU: glucose; BUN: blood urine nitrogen; TC: total cholesterol; TG: triglyceride. [3] PSE: pooled standard error (*n*=3). [4] If statistical significance (P<0.05) was detected, the model that fits best the data was selected. [5] NOS: no structure

Table 5. Effects of dietary protein level on immune and antioxidation indices of grass carp

Diet treatments[2]	LZM (mmol/L)	IgM (mmol/L)	C3 (mmol/L)	T-AOC (U/mg)	MDA (mmol/mg)	GSH-Px (U/mg)	SOD (U/mg)	CAT (mol/L)
CVM35	40.03±2.28^{b}	7.72±0.10^{b}	31.19±0.68	0.11±0.02^{ab}	14.95±0.27^{ab}	418.80±11.61^{a}	143.78±0.86	39.18±1.98
CVM42	36.95±3.22^{b}	7.88±0.04^{b}	28.15±1.08	0.14±0.01^{abc}	14.81±0.81^{ab}	496.72±2.84^{b}	130.61±5.28	34.86±2.66
CVM49	35.45±1.34^{b}	8.28±0.14^{c}	27.91±2.73	0.09±0.01^{a}	12.61±0.87^{a}	557.38±18.93^{b}	144.17±3.35	34.17±7.56
CVM55	35.76±0.07^{b}	8.30±0.09^{c}	28.77±1.73	0.19±0.02^{c}	13.24±0.55^{a}	654.10±19.67^{c}	150.07±0.68	31.49±3.96
CVM62	34.26±1.23^{b}	7.74±0.14^{ab}	28.50±1.74	0.19±0.05^{c}	15.00±0.56^{ab}	501.09±31.11^{b}	114.69±0.68	30.31±4.59
CVM69	28.05±0.47^{a}	7.41±0.13^{a}	29.37±2.94	0.21±0.00^{c}	16.67±0.96^{b}	384.70±25.14^{a}	134.15±2.72	35.02±1.25
PSE [3]	3.093	0.192	3.445	0.045	1.228	35.278	4.912	7.299
Orthogonal contrast (Pr >F) [4]								
Linear	0.001	0.067	0.750	0.004	0.150	0.889	0.002	0.299
Quadratic	0.365	0.000	0.454	0.437	0.002	0.000	0.066	0.318
Cubic	0.080	0.471	0.544	0.537	0.441	0.018	0.802	0.718
Regression								
Model [5]	Linear	Quadratic	NOS	Linear	Quadratic	Quadratic	NOS	NOS
R^2	0.541	0.730		0.416	0.548	0.772		
Pr>F [4]	0.001	0.000		0.004	0.003	0.000		

[1] Values are means of three replicates (*n*=3). [2] LZM: lysozyme; IgM: immunoglobulin M; C3: complement 3; T-AOC: total antioxidant capacity; MDA: malondialdehyde; GSH-Px: Glutathione peroxidase; SOD: Superoxide dismutase; CAT: catalase. [3] PSE: pooled standard error (*n*=3). [4] If statistical significance (P<0.05) was detected, the model that fits best the data was selected. [5] NOS: no structure

this study, optimal dietary CVM protein inclusion significantly increased the growth of grass carp, indicating a range of CVM could be effectively used by grass carp. Broken-line analysis on the relationship between SGR and CVM protein level showed that the optimal protein level was 36.54%. Kinds of literature indicate that the optimal protein content of juvenile grass carp is 35%~40% (Du et al., 2005; Liang et al., 2022). This indicates that protein requirements of juvenile grass carp could be met by CVM in this study. However, the growth performance of the CVM62 and CVM69 groups was nearly identical, which may be due to the fact that excessive protein intake may cause the body to convert it into energy instead of using it for growth and metabolic processes, resulting in wasted and inefficient energy use (Jin et al., 2015). The improvement of fish growth resulting from the increase in dietary protein level may be partly related to the activation of the TOR signaling pathway, an important nutrient-sensing process (Qin et al., 2019). In the present work, *tor* and *s6k1* expression significantly increased in grass carp fed with higher CVM protein diets, while the abundance of *4ebp1* mRNA decreased, which showed a positive effect on the muscle protein deposition (Fingar & Blenis, 2004).

The protein, energy, amino acid composition and digestibility of *C. vulgaris* are similar to that of soybean protein (Caporgno & Mathys, 2018). In previous studies, soybean meal and CPC were used as single protein sources to investigate their effects on the growth of grass carp, and the optimal protein content based on SGR was found to be 38.63% and 38.61%, respectively (Liu et al., 2022; Wang et al., 2022). However, in the current study, the optimal protein level (36.54%) was somewhat lower, and at the optimal protein level, the FE of CVM protein is higher than that of these two plant proteins, indicating that CVM has good protein quality and shows its potential use in aquafeed.

Table 6. Effect of dietary protein level from *C. vulgaris* meal on body proximate composition of grass carp (% in fresh sample)

Dietary protein level (%)	Moisture (%)	Crude protein (%)	Crude lipid (%)	Ash (%)
CVM35	73.77±0.41^{a}	13.13±0.12^{b}	8.15±0.31^{d}	3.27±0.03^{b}
CVM42	74.38±0.31^{b}	13.43±0.04^{c}	7.91±0.23^{d}	3.20±0.15^{b}
CVM49	74.70±0.22^{b}	13.37±0.17^{c}	7.44±0.10^{c}	3.18±0.12^{ab}
CVM55	75.28±0.04^{c}	13.12±0.13^{b}	7.25±0.35^{c}	3.09±0.07^{ab}
CVM62	75.73±0.18^{d}	13.07±0.03^{b}	6.69±0.07^{b}	3.00±0.11^{a}
CVM69	76.21±0.08^{e}	12.37±0.03^{a}	6.13±0.11^{a}	3.10±0.08^{ab}
PSE [2]	0.245	0.332	0.221	0.100
Orthogonal contrast (Pr >F) [3]				
Linear	0.000	0.000	0.000	0.006
Quadratic	0.861	0.000	0.688	0.376
Cubic	0.506	0.439	1.000	0.299
Regression				
Model	linear	quadratic	linear	linear
R^2	0.937	0.853	0.907	0.421
Pr>F [3]	0.000	0.000	0.000	0.004

[1] Values are means of three replicates (*n*=3). [2] PSE: pooled standard error (*n*=3). [4] If statistical significance (P<0.05) was detected, the model that fits best the data was selected.

Table 7. Effects of dietary protein level from *C. vulgaris* on muscle texture of grass carp

Diet treatments	Hardness (g)	Springiness	Chewiness (g)	Resilience (g/s)	Gumminess	Cohesiveness	Cooking loss (%)
CVM35	650.80±17.37^{a}	0.55±0.02^{ab}	168.46±2.35^{b}	0.15±0.01^{b}	342.72±2.38^{d}	0.43±0.01	18.31±0.53
CVM42	732.32±36.75^{b}	0.59±0.07^{bc}	214.57±10.26^{c}	0.15±0.00^{bc}	276.03±3.33^{b}	0.41±0.00	16.45±0.11
CVM49	828.94±15.15^{c}	0.65±0.03^{bc}	191.48±21.94^{bc}	0.17±0.01^{c}	229.63±9.52^{a}	0.40±0.01	17.26±0.34
CVM55	881.28±33.09^{c}	0.71±0.01^{c}	211.65±1.83^{c}	0.14±0.00^{ab}	272.73±13.64^{b}	0.42±0.00	17.13±1.00
CVM62	817.60±8.97^{c}	0.56±0.06^{ab}	197.06±2.27^{bc}	0.13±0.01^{ab}	298.50±16.28^{bc}	0.42±0.02	17.83±0.02
CVM69	671.99±15.19^{ab}	0.44±0.03^{a}	130.054±7.17^{a}	0.13±0.01^{a}	331.34±18.25^{cd}	0.40±0.01	17.38±0.70
PSE [2]	40.543	0.077	18.057	0.000	21.114	0.000	0.978
Orthogonal Contrast (Pr>F) [3]							
Linear	0.037	0.182	0.031	0.003	0.688	0.171	0.928
Quadratic	0.000	0.001	0.000	0.012	0.000	0.495	0.213
Cubic	0.026	0.250	0.239	0.180	0.064	0.103	0.100
Regression							
Model [4]	Quadratic	Quadratic	Quadratic	Quadratic	Quadratic	NOS	NOS
R^2	0.795	0.567	0.595	0.534	0.733		
Pr>F [3]	0.000	0.002	0.001	0.003	0.000		

[1] Values are means of three replicates (*n*=3). [2] PSE: pooled standard error (*n*=3). [3] If statistical significance (P<0.05) was detected, the model that fits best the data was selected. [4] NOS: no structure

Table 8. Effects of dietary protein level from CVM on histomorphological indices of muscle fiber of grass carp

Diet treatments	Area (μm²)	Diameter (μm)	Density (N/mm²)
CVM35	2024.69±106.55^{c}	48.07±2.40^{bc}	161.66±1.18^{a}
CVM42	1624.34±93.26^{b}	44.41±1.81^{b}	174.55±2.43^{bc}
CVM49	1288.203±55.89^{a}	35.70±3.88^{a}	181.88±0.63^{c}
CVM55	1263.87±129.85^{a}	35.98±1.68^{a}	179.86±4.48^{c}
CVM62	1892.54±83.94^{bc}	48.53±1.11^{bc}	165.66±3.11^{ab}
CVM69	2179.07±75.17^{c}	51.76±0.94^{c}	161.41±2.03^{a}
PSE [2]	162.38	3.81	4.56
Orthogonal Contrast (Pr>F) [3]			
Linear	0.050	0.139	0.552
Quadratic	0.000	0.000	0.000
Cubic	0.829	0.918	0.000
Regression			
Model	Quadratic	Quadratic	Quadratic
R [2]	0.829	0.682	0.771
Pr>F [3]	0.000	0.000	0.000

[1] Values are means of three replicates (*n*=3). [2] PSE=pooled standard error (*n*=3). [3] If statistical significance (P<0.05) was detected, the model that fits best the data was selected.

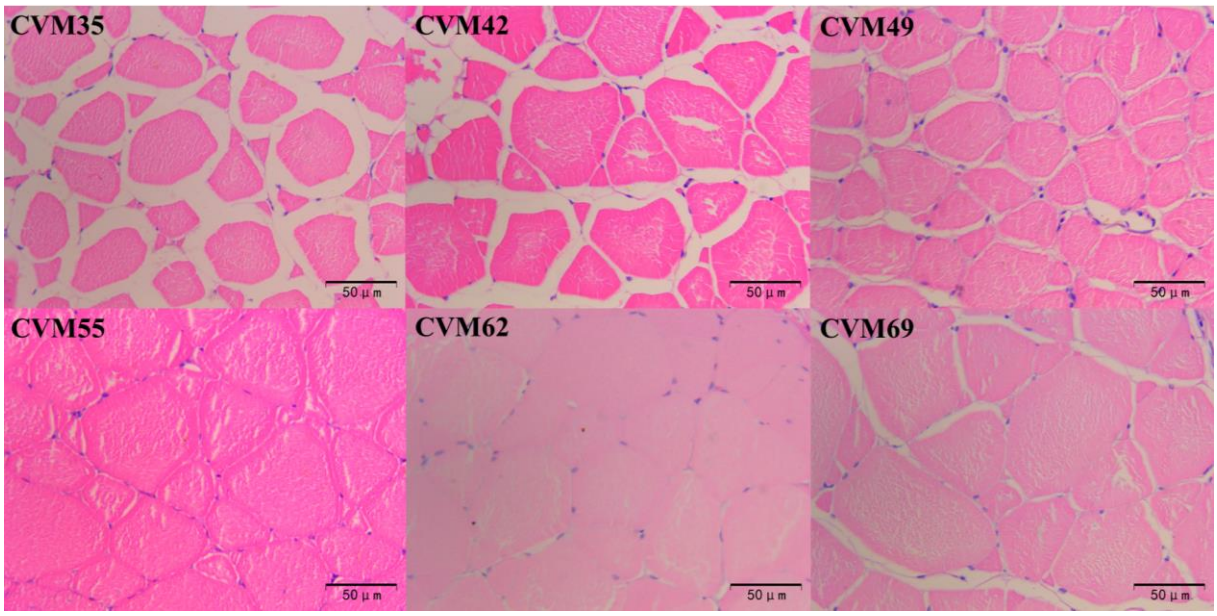

Figure 2 Muscle histology of grass carp fed different CVM protein level for 8 weeks (hematoxylin and eosin, ×400).

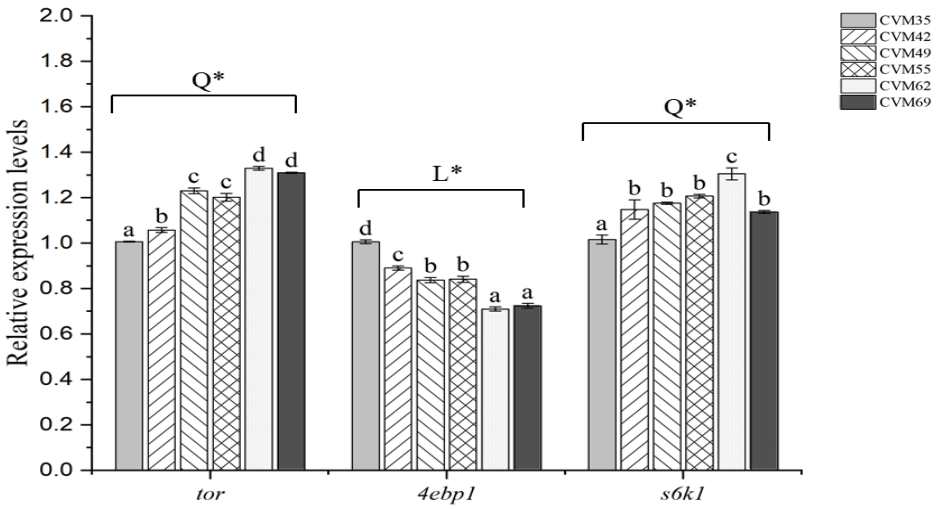

Figure 3 Effect of dietary *C. vulgaris* protein level on muscle protein synthesis gene expression. Q (quadratic) and L (linear) means the model of the dependent variable across the graded level of protein by the orthogonal polynomial contrast, * means P<0.05. Different letters (a ~ d) indicate significant differences among means (P<0.05).

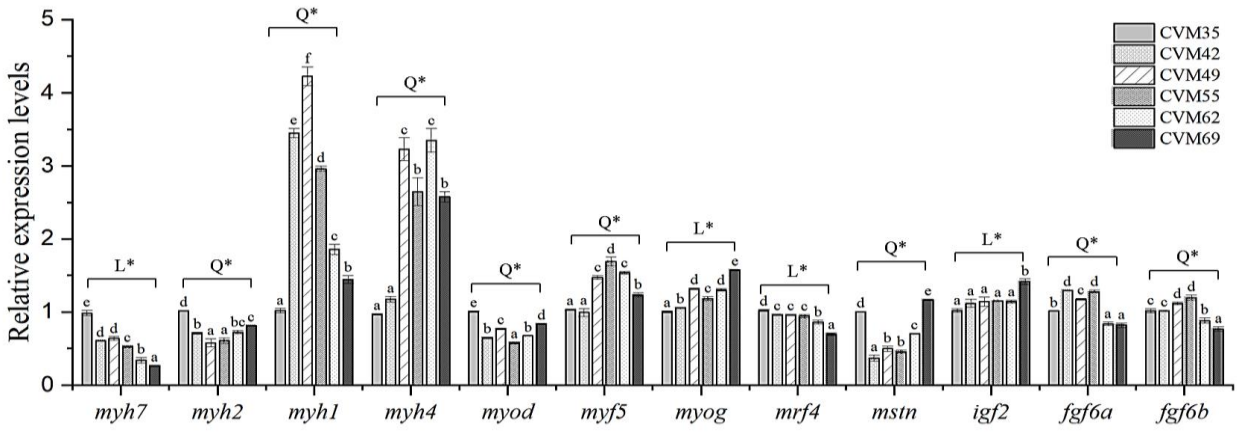

Figure 4. Effects of dietary *C. vulgaris* protein level on muscle growth related gene expressions. Q (quadratic) and L (linear) means the model of the dependent variable across the graded level of protein by the orthogonal polynomial contrast, * means P<0.05. Different letters (a ~ f) indicate significant differences among means (P<0.05).

According to the increase in HSI and the decrease in AST, the increase in CVM protein in grass carp diet improved the energy supply and health status of the hepatopancreas(Raji et al., 2018). Moreover, increase in dietary CVM protein level decreased the serum TG and TC of grass carp. Dietary CVM may reduce blood lipids by increasing protein phosphorylation and improving insulin signaling pathway in hepatopancreas, skeletal muscle and adipose tissue(Vecina et al., 2014). And this lipid metabolism improvement effect was not observed in previous studies (Liu et al., 2022; Wang et al., 2022).

Serum Immunity Capacity and Antioxidant Capacity

In teleost fish, IgM is the main antibody in the serum that binds to specific pathogens to obtain specific immunity (Abdelhamid et al., 2020), LYZ and C3, the essential components in non-specific immune system of teleost fish, are widely used to assess the immune response (Zhu et al., 2019). In this study, a moderate dietary protein inclusion (CVM49 and CVM55) showed the highest IgM content, while lower or excess protein level decreased the IgM, and the LYZ was significantly lower in CVM69 group than in other groups, which indicated that fish immunity was not consistent with growth in response to dietary protein level variation and the CVM has an immunomodulatory effect. Similar results were also reported in grass carp using other protein sources (Huang et al., 2016; Liu et al., 2022). Possible explanation for the immune-enhancing effect of appropriate dietary protein could the increasing protein intake and feed utilization (Liu et al., 2022) . Moreover, it was demonstrated that unsaturated fatty acids and polysaccharides in *C. vulgaris* activate the immune system of Nile tilapia after subacute exposure to diazinon increasing IgM content (Abdelhamid et al., 2020).

MDA indicates the extent of oxidative damage to lipids and proteins (Maliwat et al., 2017). T-AOC reflects the ability of antioxidants to scavenge harmful free radicals in the blood and cells. GSH is essential for eliminating free radicals as a non-enzymatic antioxidant (Livingstone, 2001). In this study, with the increase in dietary CVM protein level, MDA decreased first and then increased while GSH-Px showed the opposite trend and T-AOC showed a linear increase trend, which indicted the moderate CVM protein inclusion also enhanced the antioxidant capacity, similar with the immune response. In another study, supplementing CVM in the diet of young rainbow trout resulted in a significant reduction of plasma MDA content and increased activity of GPX (Chen et al., 2022). Similarly, dietary supplementation of 10% CVM in tilapia significantly increased serum GSH-PX content and reduced MDA content (Galal et al., 2018).

Fish Chemical Composition

It was suggested that water and lipids tended to change in opposite directions (Schulz & Oslage, 1976), which was confirmed in this study. Fish lipid content tended to decreased while moisture content increased with the CVM protein level, which partly explains the fast growth in the high protein groups. Similarly, largemouth bass fed with a *C. vulgaris* diet had more protein (Yu et al., 2022).

Flesh Quality and Related Gene Expression

Muscle firmness is an important determinant of fish meat quality influencing the flavor of meat (Silva et al., 2009). The current study showed that optimal dietary CVM protein level increased muscle hardness, springiness of grass carp. Similar results were reported when using soybean meal or cottonseed protein concentrate as dietary protein source in grass carp diet (Liu et al., 2022; Wang et al., 2022). Changes in dietary protein levels mainly lead to changes in muscle fiber diameter and density, which affect fish hardness (Silva

et al., 2009). The current study confirmed this, as optimal dietary protein inclusion showed a relatively higher value of hardness may be due to the higher fiber density. In fish, myogenesis involves the specific regulation of MRFs, with *myod* and *myf5* controlling myogenic cell proliferation and *myog* and *mrf4* acting on cell differentiation (Hernández-Hernández et al., 2017). Dietary CVM protein level altered the expression of MRFs genes with enhanced expression of *myf5* and *myog* genes and reduced expression of *myod* and *mrf4* at optimal protein inclusion compared with low protein diet, which finally regulate the muscle fiber diameter and density and the muscle quality. Moreover, *myog* plays a crucial role in muscle growth and homeostasis (Ganassi et al., 2020). Though the reduction of *myod* expression limited the transcription of myog gene, high expression of *myf5* has a complementary effect on *myog* (Rudnicki et al., 1993).

Myosin is the most abundant muscle protein, and subtypes of *myh* are considered as major and reliable molecular markers of muscle fiber types (Valente et al., 2013). Expression of *myh* may be controlled by the expression of *MRFs* and then affect the composition type of muscle fibers (Wang et al., 2022). The increased expression of *myh7* is related to the enhancement of *myog* (Long et al., 2022). The greater number of type IIb muscle fibers encoded by *myh4* also further corroborate the higher hardness (Essén-Gustavsson et al., 1994). Some studies have shown that *mrf4* knockdown can up-regulate the expression of genes encoding myosin heavy chains, especially *myh1, myhc4, myh2* (Moretti et al., 2016). Moreover, the optimal CVM protein levels significantly up-regulated *fgf6a*, *fgf6b* and down-regulated *mstn*. Based on our previous work that *fgf6a* plays a role in muscle regeneration by stimulating the proliferation and migration of myoblasts, and *fgf6b* plays a regulatory role in muscle hypertrophy (Xu et al., 2019). Muscle growth is also regulated by *mstn*, which normally inhibits skeletal muscle growth (McPherron et al., 1997).

Conclusions

In conclusion, dietary CVM protein level significantly affected various indices of grass carp. Appropriate inclusion of dietary CVM protein can promote the growth performance, antioxidant capacity and immunity of grass carp. Furthermore, appropriate CVM protein inclusion regulates *tor*, *s6k1* and *4ebp1* expression as well as gene expression associated with myosin types and muscle development, partially increasing grass carp body protein content and meat quality. Based on the SGR of grass carp, the optimum protein level provided by CVM in the diet is 36.54%. Moreover, the results indicated that CVM has the similar nutritional value and improves the feed efficiency of grass carp compared to the traditional feed protein source, such as soybean meal.

Ethical Statement

The animal study was reviewed and approved by the Animal Care and Use Ethics Committee of Huazhong Agricultural University.

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Author Contribution

Y.W. carried out the experiment and drafted the manuscript; Q.T. designed the experiment, drafted and revised the manuscript; C.W. and X.W. helped to culture the fish; X.L. and Y. N. analysed the data; S.X. helped to get the funding and carry out the sample analysis; All authors have read and agreed to the published version ofthe manuscript.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Abdelhamid, F. M., Elshopakey, G. E., & Aziza, A. E. (2020). Ameliorative effects of dietary *Chlorella vulgaris* and β-glucan against diazinon-induced toxicity in Nile tilapia (*Oreochromis niloticus*). *Fish & Shellfish Immunology*, *96*, 213-222. https://doi.org/10.1016/j.fsi.2019.12.009

Alagawany, M., Taha, A. E., Noreldin, A., El-Tarabily, K. A., & Abd El-Hack, M. E. (2021). Nutritional applications of species of Spirulina and Chlorella in farmed fish: A review. *Aquaculture*, *542*, 736841. https://doi.org/10.1016/j.aquaculture.2021.736841

AOAC. (1995). *Official Methods of Analysis of Association of Official Analytical Chemist*. AOAC International. Virginia, USA.

Bostock, J., McAndrew, B., Richards, R., Jauncey, K., Telfer, T., Lorenzen, K., Little, D., Ross, L., Handisyde, N., Gatward, I., & Corner, R. (2010). Aquaculture: global status and trends. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1554), 2897-2912. https://doi.org/10.1098/rstb.2010.0170

Bourne, M. C. (1978). Texture profile analysis. *Food Technology*, *32*, 62-66, 67.

Caporgno, M. P., & Mathys, A. (2018). Trends in microalgae incorporation into innovative food products with potential health benefits. *Frontiers in Nutrition*, *5*, 58. https://doi.org/10.3389/fnut.2018.00058

Chen, W., Gao, S., Huang, Y., Chang, K., & Zhao, X. (2022). Addition of *Chlorella sorokiniana* meal in the diet of juvenile rainbow trout (*Oncorhynchus mykiss*): Influence on fish growth, gut histology, oxidative stress, immune response, and disease resistance against *Aeromonas salmonicida*. *Fish & Shellfish Immunology*, *129*, 243-250. https://doi.org/10.1016/j.fsi.2022.08.062

Dong, M., Zhang, L., Wu, P., Feng, L., Jiang, W., Liu, Y., Kuang, S., Li, S., Mi, H., Tang, L., & Zhou, X. (2022). Dietary protein levels changed the hardness of muscle by acting on muscle fiber growth and the metabolism of collagen in sub-adult grass carp (*Ctenopharyngodon idella*). *Journal of Animal Science and Biotechnology*, *13*(1), 109. https://doi.org/10.1186/s40104-022-00747-7

Du, Z.-Y., Liu, Y.-J., Tian, L.-X., Wang, J.-T., Wang, Y., & Liang, G.-Y. (2005). Effect of dietary lipid level on growth, feed utilization and body composition by juvenile grass carp (Ctenopharyngodon idella). *Aquaculture Nutrition*, *11*(2), 139-146. https://doi.org/10.1111/j.1365-2095.2004.00333.x

Essén-Gustavsson, B., Karlsson, A., Lundström, K., & Enfält, A. C. (1994). Intramuscular fat and muscle fibre lipid contents in halothane-gene-free pigs fed high or low protein diets and its relation to meat quality. *Meat Science*, *38*(2), 269-277. https://doi.org/10.1016/0309-1740(94)90116-3

FAO. (2020). *The State of World Fisheries and Aquaculture 2020. Sustainability in action.* https://doi.org/10.4060/ca9229en

Fingar, D. C., & Blenis, J. (2004). Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene*, *23*(18), 3151-3171. https://doi.org/10.1038/sj.onc.1207542

Galal, A. A. A., Reda, R. M., & Abdel-Rahman Mohamed, A. (2018). Influences of *Chlorella vulgaris* dietary supplementation on growth performance, hematology, immune response and disease resistance in *Oreochromis niloticus* exposed to sub-lethal concentrations of penoxsulam herbicide. *Fish & Shellfish Immunology*, *77*, 445-456. https://doi.org/10.1016/j.fsi.2018.04.011

Ganassi, M., Badodi, S., Wanders, K., Zammit, P. S., & Hughes, S. M. (2020). Myogenin is an essential regulator of adult myofibre growth and muscle stem cell homeostasis. *eLife*, *9*, e60445. https://doi.org/10.7554/eLife.60445

Gunathilaka, B. E., Jeong, S.-M., Cho, M.-U., Kim, K.-W., Hur, S.-W., Lee, S., You, S.-G., & Lee, S.-M. (2023). Effects of dietary fish meal replacement with alternative protein ingredients and their combinations on growth performance, feed Utilization, fillet composition, and biochemical parameters of red seabream (*Pagrus major*). *Aquaculture Nutrition*, *2023*(1), 8883739. https://doi.org/10.1155/2023/8883739

Hernández-Hernández, J. M., García-González, E. G., Brun, C. E., & Rudnicki, M. A. (2017). The myogenic regulatory factors, determinants of muscle development, cell identity and regeneration. *Seminars in Cell & Developmental Biology*, *72*, 10-18. https://doi.org/10.1016/j.semcdb.2017.11.010

Huang, J. F., Xu, Q. Y., & Chang, Y. M. (2016). Effects of temperature and dietary protein on the growth performance and IGF-I mRNA expression of juvenile mirror carp (*Cyprinus carpio*). *Aquaculture Nutrition*, *22*(2), 283-292. https://doi.org/10.1111/anu.12254

Jin, Y., Tian, L.-x., Xie, S.-w., Guo, D.-q., Yang, H.-j., Liang, G.-y., & Liu, Y.-j. (2015). Interactions between dietary protein levels, growth performance, feed utilization, gene expression and metabolic products in juvenile grass carp (*Ctenopharyngodon idella*). *Aquaculture*, *437*, 75-83. https://doi.org/10.1016/j.aquaculture.2014.11.031

Jobling, M. (2012). National Research Council (NRC): Nutrient requirements of fish and shrimp. *Aquaculture International*, *20*(3), 601-602. https://doi.org/10.1007/s10499-011-9480-6

Liang, H., Xu, H., Ge, X., Zhu, J., Ren, M., & Mi, H. (2022). Water temperature affects the protein requirements, growth performance, and nutritional metabolism of grass carp (*Ctenopharyngodon idella*) juveniles. *Aquaculture Reports*, *25*. https://doi.org/10.1016/j.aqrep.2022.101267

Liu, G., Zhou, M., Wang, X., Mao, X., Long, X., Xie, S., Han, D., & Tan, Q. (2022). Effects of dietary cottonseed protein concentrate levels on growth performance, health status, flesh quality and intestinal microbiota of grass carp (*Ctenopharyngodon idellus*). *Metabolites*, *12*(11). https://doi.org/10.3390/metabo12111046

Livingstone, D. R. (2001). Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Marine Pollution Bulletin*, *42*(8), 656-666. https://doi.org/10.1016/s0025-326x(01)00060-1

Long, K., Su, D., Li, X., Li, H., Zeng, S., Zhang, Y., Zhong, Z., Lin, Y., Li, X., Lu, L., Jin, L., Ma, J., Tang, Q., & Li, M. (2022). Identification of enhancers responsible for the coordinated expression of myosin heavy chain isoforms in skeletal muscle. *BMC Genomics*, *23*(1), 519. https://doi.org/10.1186/s12864-022-08737-9

Maliwat, G. C., Velasquez, S., Robil, J. L., Chan, M., Traifalgar, R. F., Tayamen, M., & Ragaza, J. A. (2017). Growth and immune response of giant freshwater prawn *Macrobrachium rosenbergii* (De Man) postlarvae fed diets containing *Chlorella vulgaris* (Beijerinck). *Aquaculture Research*, *48*(4), 1666-1676. https://doi.org/10.1111/are.13004

McPherron, A. C., Lawler, A. M., & Lee, S. J. (1997). Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature*, *387*(6628), 83-90. https://doi.org/10.1038/387083a0

Moretti, I., Ciciliot, S., Dyar, K. A., Abraham, R., Murgia, M., Agatea, L., Akimoto, T., Bicciato, S., Forcato, M., Pierre, P., Uhlenhaut, N. H., Rigby, P. W., Carvajal, J. J., Blaauw, B., Calabria, E., & Schiaffino, S. (2016). MRF4 negatively regulates adult skeletal muscle growth by repressing MEF2 activity. *Nature Communication*, *7*, 12397. https://doi.org/10.1038/ncomms12397

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research*, *29*(9), e45-e45. https://doi.org/10.1093/nar/29.9.e45

Qin, Q., Cao, X. F., Dai, Y. J., Wang, L. N., Zhang, D. D., Jiang, G. Z., & Liu, W. B. (2019). Effects of dietary protein level on growth performance, digestive enzyme activity, and gene expressions of the TOR signaling pathway in fingerling *Pelteobagrus fulvidraco*. *Fish Physiology and Biochemistry*, *45*(5), 1747-1757. https://doi.org/10.1007/s10695-019-00664-z

Raji, A. A., Alaba, P. A., Yusuf, H., Abu Bakar, N. H., Mohd Taufek, N., Muin, H., Alias, Z., Milow, P., & Abdul Razak, S. (2018). Fishmeal replacement with *Spirulina Platensis* and *Chlorella vulgaris* in African catfish (*Clarias gariepinus*) diet: Effect on antioxidant enzyme activities and haematological parameters. *Research in Veterinary Science*, *119*, 67-75. https://doi.org/10.1016/j.rvsc.2018.05.013

Rudnicki, M. A., Schnegelsberg, P. N., Stead, R. H., Braun, T., Arnold, H. H., & Jaenisch, R. (1993). MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell*, *75*(7), 1351-1359. https://doi.org/10.1016/0092-8674(93)90621-v

Safi, C., Zebib, B., Merah, O., Pontalier, P.-Y., & Vaca-Garcia, C. (2014). Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. *Renewable and Sustainable Energy Reviews*, *35*, 265-278. https://doi.org/10.1016/j.rser.2014.04.007

Sahin, T., & Ergün, S. (2021). Incorporation of Rapa Whelk (*Rapana venosa*) meal in diets for rainbow trout (*Oncorhynchus mykiss*) fry. *Aquaculture Research*, *52*(2), 678-692. https://doi.org/10.1111/are.14925

Schulz, E., & Oslage, H. J. (1976). Composition and nutritive value of single-cell protein (SCP). *Animal Feed Science and Technology*, *1*(1), 9-24. https://doi.org/10.1016/0377-8401(76)90003-1

Shi, X., Luo, Z., Chen, F., Wei, C. C., Wu, K., Zhu, X. M., & Liu, X. (2017). Effect of fish meal replacement by Chlorella meal with dietary cellulase addition on growth performance, digestive enzymatic activities, histology and myogenic genes' expression for crucian carp *Carassius auratus*. *Aquaculture Research*, *48*(6), 3244-3256. https://doi.org/10.1111/are.13154

Silva, P., Valente, L. M., Olmedo, M., Galante, M. H., Monteiro, R. A., & Rocha, E. (2009). Hyperplastic and hypertrophic growth of lateral muscle in blackspot seabream *Pagellus bogaraveo* from hatching to juvenile. *Journal of Fish Biology*, *74*(1), 37-53. https://doi.org/10.1111/j.1095-8649.2008.02122.x

Valente, L. M. P., Moutou, K. A., Conceição, L. E. C., Engrola, S., Fernandes, J. M. O., & Johnston, I. A. (2013). What determines growth potential and juvenile quality of farmed fish species? *Reviews in Aquaculture*, *5*(s1), S168-S193. https://doi.org/10.1111/raq.12020

Vecina, J. F., Oliveira, A. G., Araujo, T. G., Baggio, S. R., Torello, C. O., Saad, M. J. A., & Queiroz, M. L. d. S. (2014). Chlorella modulates insulin signaling pathway and prevents high-fat diet-induced insulin resistance in mice. *Life Sciences*, *95*(1), 45-52. https://doi.org/10.1016/j.lfs.2013.11.020

Wang, X., Liu, G., Xie, S., Pan, L., & Tan, Q. (2022). Growth and meat quality of grass carp (*Ctenopharyngodon idellus*) responded to dietary protein (soybean meal) level through the muscle metabolism and gene expression of myosin heavy chains. *Frontiers in nutrition*, *9*, 833924. https://doi.org/10.3389/fnut.2022.833924

Xie, C., Li, J., Li, D., Shen, Y., Gao, Y., & Zhang, Z. (2018). Grass Carp: The Fish that Feeds Half of China. In J. Gui, Q. Tang, Z. Li, J. Liu, & S. S. De Silva (Eds.), *Aquaculture in China* (pp. 93-115). https://doi.org/10.1002/9781119120759.ch2_1

Xu, Y., Tan, Q., Hu, P., & Yao, J. (2019). Characterization and expression analysis of FGF6 (fibroblast growth factor 6) genes of grass carp (*Ctenopharyngodon idellus*) reveal their regulation on muscle growth. *Fish Physiology and Biochemistry*, *45*(5), 1649-1662. https://doi.org/10.1007/s10695-019-00655-0

Yu, H., Liang, H., Ge, X., Zhu, J., Wang, Y., Ren, M., & Chen, X. (2022). Dietary chlorella (*Chlorella vulgaris*) supplementation effectively improves body color, alleviates muscle inflammation and inhibits apoptosis in largemouth bass (*Micropterus salmoides*). *Fish & Shellfish Immunology*, *127*, 140-147. https://doi.org/10.1016/j.fsi.2022.06.017

Zarantoniello, M., de Oliveira, A. A., Sahin, T., Freddi, L., Torregiani, M., Tucciarone, I., Chemello, G., Cardinaletti, G., Gatto, E., Parisi, G., Bertolucci, C., Riolo, P., Nartea, A., Gioacchini, G., & Olivotto, I. (2023). Enhancing rearing of European seabass (*Dicentrarchus labrax*) in aquaponic systems: Investigating the effects of enriched black soldier fly (*Hermetia illucens*) prepupae meal on fish welfare and quality traits. *Animals*, *13*(12), 1921. https://doi.org/10.3390/ani13121921

Zhao, H., Chong, J., Tang, R., Li, L., Xia, J., & Li, D. (2018). Metabolomics investigation of dietary effects on flesh quality in grass carp (*Ctenopharyngodon idellus*). *GigaScience*, *7*(10), giy111. https://doi.org/10.1093/gigascience/giy111

Zhu, Y., Hu, P., Yao, J., Xu, D., Xu, Y., & Tan, Q. (2019). Optimal dietary alcoholic extract of lotus leaf improved growth performance and health status of grass carp (*Ctenopharyngodon idellus*). *Fish & Shellfish Immunology*, *93*, 1-7. https://doi.org/10. 1016/j. fsi. 2019. 07. 039