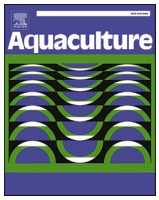
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Dietary β-conglycinin induces intestinal enteritis and affects glycerophospholipid and arginine metabolism in mirror carp (*Cyprinus carpio*)

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ABSTRACT

The use of soybean meal in aquafeed industry is partially limited by anti-nutritional factors such as β-conglycinin (β-CG). However, the effects of β-CG on mirror carp (*Cyprinus carpio*) growth performance and intestinal health has not been elucidated. This study investigated the effects of β-CG levels on growth performance, intestine immunity, and intestinal lipid and protein metabolism in mirror carp. Four hundred and fifty mirror carp (4.22 ± 0.03) g were fed a control (CON) diet containing fish meal as the protein source and four experimental diets containing β-CG at 40 (β-CG1), 80 (β-CG2), 120 (β-CG3), and 160 g/kg (β-CG4) replacing the fishmeal, respectively. Each treatment had 30 fish replicated three times and the feeding was done for six weeks. The results showed that weight gain, specific growth rate, survival rate, protein efficiency, feed conversion ratio, and condition factor were not affected by dietary β-CG ($P > 0.05$). Interestingly, the fish fed on the β-CG diets upregulated significantly the expression of tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β) and eIF4E-binding protein (4E-BP) in the proximal intestine (PI) than those fed on the CON diet ($P < 0.05$). The fish fed on the β-CG2 diet decreased significantly the expression of transforming growth factor beta 1 (TGFβ1) in the PI compared to those fed on the CON diet. The fish fed on the β-CG diets downregulated significantly the expression of target of rapamycin (TOR) in the PI than those fed on the CON diet. The fish fed on β-CG diets downregulated significantly the expression of AMP-activated protein kinase alpha (AMPK), acetyl-CoA carboxylase (ACC), TOR in the mid intestine (MI) while, claudin11 gene was significantly increased ($P < 0.05$). In the distal intestine (DI), feeding the fish on the β-CG diets downregulated significantly the expression of AMPK and ACC genes, while increased the expression TNF-α, IL-1β and claudin11 ($P < 0.05$). Feeding the fish with the β-CG diets mostly altered glycerophospholipid metabolism and arginine biosynthesis in intestinal mucosa. These results suggest that dietary β-CG induces enteritis in different intestinal segments and changes glycerophospholipid metabolism and arginine biosynthesis pathways. Fish farmers using soybean meal in the diets should process the ingredients to reduce β-CG concentration before use.

Abbreviations: β-conglycinin, β-CG; weight gain, WG; specific growth rate, SGR; survival rate, SR; protein efficiency, PE; feed conversion rate, FCR; condition factor, CF; proximal intestine, PI; mid intestine, MI; distal intestine, DI; forward primer sequence, F; reverse primer sequence, R; interleukin-1 beta, IL-1β; tumor necrosis factor alpha, TNF-α; transforming growth factor beta, TGF-β1; AMP-activated protein kinase alpha, AMPK-α; acetyl-CoA carboxylase, ACC; target of rapamycin, TOR; eIF4E-binding protein, 4E-BP; Kyoto Encyclopedia of Genes and Genomes, KEGG; initial body weight, IBW; final body weight, FBW; lysophosphatidylcholine, LysoPC; dihydroxyoctadecanoic acid, DiHOME.

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1. Introduction

Soybean meal is one of the primary protein sources in aquafeed due to its relatively high protein quantity and balanced amino acid composition (Taher et al., 2017). However, high-level replacement of fishmeal with soybean meal reduced growth in rice field eel (*Monopterus albus*) (Zhang et al., 2020a), causing lesions in the digestive tract in turbot (*Scophthalmus maximus L.*) (Peng et al., 2013), and decreased feeds utilization in Japanese seabass (*Lateolabrax japonicus*) (Zhang et al., 2018). This is partially due to the presence of anti-nutritional factors (Kokou et al., 2012), such as trypsin inhibitors, glycinin and β-conglycinin (β-CG) in soybean meal (Li et al., 2017a). β-CG is one major allergenic protein among the anti-nutrients existing in soybean (L'Hocine and Boye, 2007). β-CG have been reported to cause intestinal injuries in terrestrial animals by accelerating apoptosis (Zhao et al., 2010), decreasing villus height and causing pathological disruption (Hao et al., 2009) and inducing enteritis (L'Hocine and Boye, 2007), which decreased food intake, consequently slowing growth rate in piglets (Dréau and Lallès, 1999). These studies suggest that, soybean utilization in animal feeds is partly limited by the presence of β-CG.

The search for alternative cheap protein sources has led to extensive studies on β-CG in mammals (Dréau and Lallès, 1999; L'Hocine and Boye, 2007; Hao et al., 2009; Zhao et al., 2010). However, little attention has been paid on the effects of β-CG in fish. A few available studies in fish indicate that 4% to 8% β-CG supplementation reduced growth performance, destroyed intestine structural stability and caused various immune responses in turbot (*Scophthalmus maximus*), but did not induce proximal intestine inflammation (Li et al., 2017b). However, β-CG caused inflammation in grass carp (*Ctenopharyngodon Idella*) midintestine (MI) and distal intestine (DI) (Duan et al., 2020). Similarly, Zhang et al. (2014) reported that dietary 80 g/kg β-CG induced inflammation in the DI in Jian carp (*Cyprinus carpio* var. Jian). These studies indicate that, β-CG induces serious intestinal inflammation effects in the DI, MI and proximal intestine (PI). However, such a conclusion is based on a few studies, thus requires more studies to explore the effects of β-CG in fish by using other measured parameters such as pro-inflammatory cytokines and tight junction proteins.

The intestinal inflammation induced by β-CG impairs the epithelial barrier integrity (Shih and Targan, 2008), which stimulates the pro-inflammatory cytokines. Increased pro-inflammatory cytokines expression is usually accompanied by decreased expression of tight junction proteins in enteritis (Chen et al., 2018). However, the influence of β-CG on the inflammatory cytokines and tight junction dynamics during enteritis in fish are limited. This limits our knowledge on the effects of β-CG for formulating feeds, which do not impair fish health, an aspect requiring further studies to entangle. Metabolomics are great biomarkers for mammalian disease diagnosis (Casu et al., 2019). However, little information exists on the use of metabolomics analysis to inquire on differential metabolites and pathways enrichment when fishmeal is replaced by β-CG in fish.

Carp is the widespread and most cultured fish species worldwide (Rahman et al., 2020; FAO, 2022). It has been endorsed by the Organization for Economic Cooperation and Development for bioassay experiments (Mohamed et al., 2020). Nevertheless, the effects of β-CG in mirror carp growth performance and intestinal health have not been elucidated. Therefore, this study investigated the effects of 40 to 160 g/kg β-CG on the growth performance, intestinal health and glycerophospholipid and arginine metabolism in mirror carp. The results of this study enlighten our understanding on the biological function of β-CG for formulating appropriate feeds, which ensure fish intestine health.

2. Materials and methods

The study was conducted strictly according to China's Guidance of the Care and Use of Laboratory Animals. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Huzhou University.

2.1. Experimental diets and their proximate analysis

β-conglycinin was generously provided by the School of Food Science and Technology, Jiangnan University. The protein profile of β-CG fraction was revealed by the SDS-PAGE analysis (Fig.1.). The purity of β-CG fraction determined using quantitation software Quantity One is 67.9% β-CG. The control (CON) diet used fishmeal as the protein source, the experimental diets were supplemented with 40 (β-CG1), 80 (β-CG2), 120 (β-CG3), and 160 g/kg β-CG (β-CG4) replacing fishmeal. The formulation for all diets used in this study is shown in Table 1. Dietary ingredients were ground into fine powder and sieved through a 60-mesh sieve. The ingredients were mixed according to specific formulation for each diet and homogenized. The homogenized ingredients were thoroughly mixed with fish oil, soybean oil, phospholipid, and water to form a dough. Then, pellets with 2 mm particle size were produced by using a twin screw extruder (F-26, South China University of Technology, Guangzhou, China). The diets were dried in a ventilated oven at 40 °C and stored at −20 °C until needed for feeding fish. Three samples of each diet was collected for proximate analysis using standard methods (Table 1). Crude protein content was determined by Dumas nitrogen determination apparatus, while crude lipid was extracted by using Soxhlet extraction method (GB/T 6443–2006). All diets were isonitrogenous (34%) and isolipidic (7%) with relatively similar levels of essential amino acids.

2.2. Experimental fish and rearing

Songpu mirror carp (*Cyprinus carpio*) were purchased from the

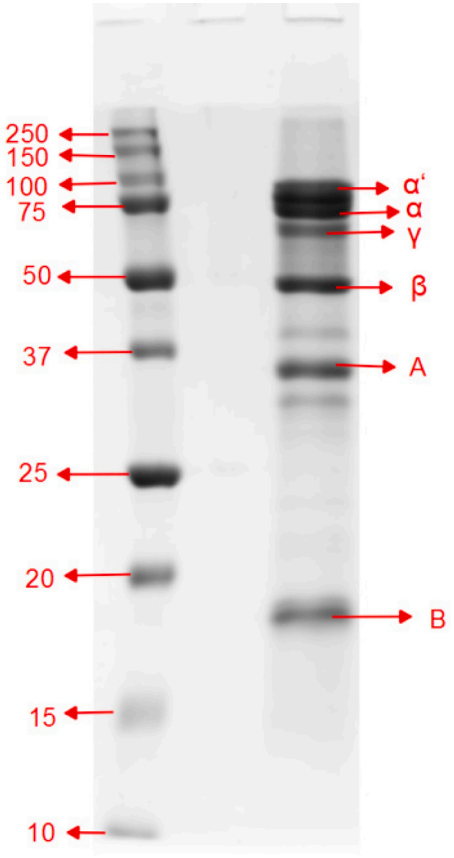


Fig. 1. SDS-PAGE profile of purified β-conglycinin fraction. Lane 1: protein marker; Lane 2: β-conglycinin fraction. α', α and β are subunits of β-conglycinin.

Table 1
Composition and nutrient levels of experimental diets.

Ingredients (g/kg)	Experimental diets				
	CON	β-CG1	β-CG2	β-CG3	β-CG4
Fish meal	412.0	354.0	297.0	239.0	182.0
β-conglycinin	0	40.0	80.0	120.0	160.0
Wheat flour	500.0	500.0	500.0	500.0	500.0
Fish oil	10.0	12.3	14.5	16.8	19.0
Soybean oil	10.0	12.2	14.4	16.7	18.9
Phospholipid	10.0	10.0	10.0	10.0	10.0
Vitamin premix[a]	3.00	3.00	3.00	3.00	3.00
Mineral Premix[b]	2.00	2.00	2.00	2.00	2.00
Choline chloride	5.00	5.00	5.00	5.00	5.00
Cellulose	2.50	12.9	22.9	33.6	43.6
Carboxymethyl cellulose	30.0	30.0	30.0	30.0	30.0
$Ca(H_2PO_4)_2$	13.6	15.5	17.0	18.5	20.0
Lysine	0	0.60	1.10	1.70	2.20
L-Threonine	1.90	2.50	3.10	3.70	4.30
Total	1000.0	1000.0	1000.0	1000.0	1000.0
Proximate analysis (%)					
Dry matter	90.29	91.11	91.17	91.17	91.51
Crude protein	34.03	34.01	34.03	34.01	34.03
Crude lipid	7.71	7.71	7.71	7.71	7.71
Ash	16.79	14.99	12.07	9.89	8.11

[a] The premix provided the following per kg of diets: VA 8000 IU, VC 500 mg, VD_3 3000 IU, VE 60 mg, VK_3 5 mg, VB_2 30 mg, VB_6 15 mg, VB_{12} 0.5 mg, Nicotinamide 175 mg, pantothenate acid 50 mg, Folic acid 5 mg, D-biotin 2.5 mg, Inositol 1000 mg.
[b] The premix provided the following per kg of diets:Zn 25 mg, Cu 3 mg, Fe 125 mg, Mn 15 mg, I 0.6 mg, Co 0.1 mg, Se 0.4 mg.

Heilongjiang River Fishery Research Institute of the Chinese Academy of Fishery Sciences, Harbin, China. Mirror carp were fed the CON diet thrice daily for one week to acclimatize them to experimental conditions. After the acclimation period, a total of 450 mirror carp with relatively similar weight (initial body weight 4.22 ± 0.03 g) were randomly distributed into 15 tanks in three replicates per treatment (each replicate tank contained 30 fish). The fish were fed with the CON and the experimental diets to apparent visual satiation thrice daily (09:00, 13:00 and 17:00 h) for six weeks. Fish in each tank were bulk weighed every two weeks to adjust the amount of feed. Uneaten diet from each tank was collected after 45 min feeding, dried and weighed to determine feed intake (Chen et al., 2020). During the experimental period, the mirror carp were cultured in a recirculating aquaculture system with continuous aeration. One third of the water by volume in each tank was changed daily with fresh dechlorinated water. The water temperature ranged from 23 to 27 °C, dissolved oxygen was not <6.0 mg L^{-1}, nitrite was lower than 0.1 mg L^{-1}, and ammonia nitrogen concentration was <0.03 mg L^{-1}. A natural photoperiod of 14 h light and 10 h dark was provided during the trial.

2.3. Determination of growth performance, survival rate, feed utilization efficiency and condition factor

At the end of the feeding trial, all fish in each tank were counted to estimate survival rate. Three fish per tank (nine per treatment) were randomly selected, individually weighed and measured for their body length to calculate growth performance indexes. Weight gain (WG), specific growth rate (SGR), survival rate (SR), protein efficiency (PE), feed conversion ratio (FCR), and condition factor (CF) were calculated according to the following formulae:

Weight gain (WG, %) = 100 × (final body weight − initial body weight)/initial body weight;

Specific growth rate (SGR, %/day) = 100 × (Ln final body weight − Ln initial body weight)/number of days;

Survival rate (SR, %) = 100 × (final fish number/initial fish number);

Protein efficiency (PE, %) = 100 × (weight gain/feed intake × feed crude protein content);

Feed conversion ratio (FCR) = feed intake/weight gain;

Condition factor (CF, g/cm^3) = 100 × (body weight, g)/(body length, cm)^3.

2.4. Total RNA extraction and quantitative real-time PCR analysis

After weight and length measurement, two fish from each tank (six per each treatment) were randomly sampled by immersing them in ice at 4 °C. The fish were then dissected aseptically to obtain the intestine. The intestine was immediately divided into PI, MI, and DI. The samples were immediately frozen in liquid nitrogen and stored at −80 °C for total RNA extraction and quantitative real-time PCR analysis.

Total RNA was extracted by Trizol Kit (Takara Bio Inc., Japan). Then the quantity and purity of RNA were measured by Nanodrop spectrophotometry (A260:A280 nm ratio) within the range specified by the kit (1.8 to 2.2). Genomic DNA was removed by using Prime Scrip™ RT reagent Kit (RR047A; Takara Bio Inc., Japan) with a gDNA eraser following manufacturer's instructions. The cDNAs were synthesized by using Prime Scrip™ RT reagent Kit according to the instructions. The SYBR Premix Ex TaqII reagents (RR420A; Takara Bio Inc., Japan) were used to determine mRNA level by quantitative real-time PCR (q-PCR) on a CFX-96 Real-Time PCR Detection System (Bio-rad Laboratories, Inc., China). The reactions were done in a volume of 25 μL containing 1.0 μL of 10 μM of reverse and forward primers, 2 μL of diluted cDNA, 12.5 μL of TB Green Premix Ex Taq II (Tli RNaseH Plus), and 8.5 μL of RNase Free dH_2O. PCR conditions were as follows: 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. Target gene mRNA relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). β-actin was used the housekeeping gene (Luo et al., 2014). The primers used for q-PCR analysis in this study are shown in Table 2.

2.5. Liquid chromatography–mass spectrometry (LC-MS) untargeted metabolomics

The intestinal mucosa of 18 fish of each treatment (every 3 samples were pooled as a sample) was scraped and stored at −80 °C for untargeted metabolomics analysis. Six intestinal mucosa samples from each treatment were used for untargeted metabolomics analysis by using liquid chromatography–mass spectrometry (LC-MS) (Shanghai Majorbio

Table 2
Primer sequence of target genes in quantitative real-time PCR.

Target genes		Primer sequence (5′-3′)	Gene accession number
IL-1β	F	AACTTCACACTTGAGGAT	XM_019080073.2
	R	GACAGAACAATAACAACAAC	
TNF-α	F	AAGTCTCAGAACAATCAGGAA	XM_019088899.2
	R	TGCCTTGGAAGTGACATT	
TGF-β1	F	GCCTGTTGGGACTTGTGCTC	XM_019072371.2
	R	CCTCGAATGGCTTCAATGCG	
Occludin	F	ATCGGTTCAGTACAATCAGG	KF975606.1
	R	GACAATGAAGCCCATAACAA	
Claudin3c	F	GCACCAACTGTATCGAGGATG	XM_042710912.1
	R	GGTTGTAGAAGTCCCGAATGG	
Claudin7	F	CTTCTATAACCCCTTCACACCAG	XM_042732468.1
	R	ACATGCCTCCACCCATTATG	
Claudin11	F	TCGGAAGTGAACCAGAAAGC	XM_042773688.1
	R	GAAGCCAAAGGACATCAAGC	
AMPK-α	F	CAGAAACACGAAGGCAGGGT	XM_042777568.1
	R	CCTGTTAGCTGGTGCTCTCC	
ACC	F	TGGCTGCACTGCACTCTCACT	XM_042724983.1
	R	GGTCCAGCTTCCCTGCGGTC	
TOR	F	ATCATACGCATCCAGTCCATTG	XM_042761449.1
	R	GGTCATTAGCCAGTAGAGTGTTC	
4E-BP	F	GCTACCTCACGACTATTGC	XM_042737175.1
	R	TTCTTGCTTGTCACTCCTG	

Bio-pharm Technology Co., Ltd., Shanghai, China).

2.6. Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis

A targeted data analysis approach was employed after identifying the significant differential metabolites between the fish fed on CON and β-CG3 diets. The statistically significant metabolite in the profile of intestinal metabolites for fish fed on CON and β-CG3 diets were sequentially used and extracted. The annotation information of the metabolites in the database was obtained, and their annotations were counted. Fold change (FC) > 1 indicated that the metabolite was upregulated, whereas FC < 1 indicated the metabolite was downregulated.

2.7. Statistical analyses

Data for growth performance and related parameters and intestine immunity were tested for normality by using Shapiro Wilk test and homogeneity of variances by Levene's test. After confirming parametric statistics assumptions, the data were subjected to one-way analysis of variance (ANOVA) using SPSS, version 24.0 for mac followed by Duncan's test. A significant difference was considered when $P < 0.05$. The results of growth performance and related parameters and intestine immunity are presented as means ± standard deviation (SD).

The data analysis for metabolomics were conducted by using partial least squares discriminant analysis (PLS-DA) and orthogonal PLS-DA (OPLS-DA). All metabolites identified by mass spectrometry were compared with the KEGG (https://www.kegg.jp/kegg/compound/) and HMDB (http://www.hmdb.ca) databases. Intestinal mucosa metabolites passing the VIP-values threshold (VIP > 1) and the *P*-values <0.05 were considered as significantly different. *P*-values on the normalized peak areas were determined by using Student's *t*-test at $P \leq 0.05$ for significant difference.

3. Results

3.1. Growth performance, survival rate, feed utilization efficiency, and condition factor

The results on the effect dietary β-CG on growth performance and related parameters are shown on Table 3. Feeding the fish with all the experimental diets did not affect significantly FBW, WG, and SGR ($P > 0.05$). No fish mortalities occurred throughout the study. The SR was 100% in all diets, with no significant difference among them ($P > 0.05$). Feeding the fish with the experimental diets did not affect significantly PER and FCR ($P > 0.05$). The CF was not significantly affected by dietary β-CG among all the treatments ($P > 0.05$).

Table 3
Effects of β-conglycinin on growth performance of mirror carp.

Indexes	Experimental diets				
	CON	β-CG1	β-CG2	β-CG3	β-CG4
IBW (g/fish)	4.20 ± 0.03	4.25 ± 0.04	4.18 ± 0.01	4.22 ± 0.03	4.23 ± 0.04
FBW (g/fish)	25.40 ± 1.34	26.24 ± 0.13	27.19 ± 1.21	24.88 ± 1.90	26.02 ± 1.36
WG (%)	504.71 ± 28.13	516.64 ± 9.44	550.91 ± 28.31	490.05 ± 43.61	515.39 ± 38.11
SGR (%)	4.28 ± 0.11	4.33 ± 0.04	4.46 ± 0.10	4.22 ± 0.17	4.32 ± 0.15
SR (%)	100.00 ± 00	100.00 ± 00	100.00 ± 00	100.00 ± 00	100.00 ± 00
PE (%)	2.39 ± 0.12	2.44 ± 0.02	2.51 ± 0.08	2.31 ± 0.14	2.42 ± 0.11
FCR	1.23 ± 0.61	1.21 ± 0.01	1.17 ± 0.04	1.27 ± 0.07	1.22 ± 0.05
CF (g/cm^3)	3.29 ± 0.10	3.43 ± 0.15	3.72 ± 0.41	3.28 ± 0.21	3.37 ± 0.24

Initial body weight (IBW), final body weight (FBW), weight gain (WG), specific growth rate (SGR), survival rate (SR), Protein efficiency (PE), feed conversion ratio (FCR), and condition factor (CF). Values are mean ± SD. (n = 3).

3.2. Inflammatory cytokines in the intestine

Feeding the mirror carp with β-CG diets affected significantly the mRNA expression of genes related to inflammatory cytokines in the intestine ($P < 0.05$; Fig. 2). The mirror carp fed on the β-CG2 and β-CG3 diets upregulated significantly the mRNA expression of tumor necrosis factor alpha (TNF-α) gene in the PI (Fig. 2A), MI (Fig. 2B), and DI (Fig. 2C) than those fed on the CON diet ($P < 0.05$). Similarly, the fish fed on the β-CG4 diet increased significantly the mRNA expression of TNF-α gene in the PI and DI than those fed on the CON diet ($P < 0.05$). The mirror carp fed on β-CG1 diet also enhanced significantly the mRNA expression of TNF-α gene only in the DI than those fed on the CON diet ($P < 0.05$). The fish fed on the β-CG3 and β-CG4 diets upregulated the mRNA expression of interleukin-1 beta (IL-1β) gene in the PI (Fig. 2A) and DI (Fig. 2C) than those fed on the CON diet ($P < 0.05$). Likewise, the mirror carp fed on the β-CG1 and β-CG2 upregulated significantly the mRNA expression of IL-1β gene in the PI and MI, respectively than those fed on CON diet ($P < 0.05$). The mirror carp fed on the β-CG2 diet downregulated significantly the mRNA expression of transforming growth factor beta 1 (TGFβ1) gene in the PI (Fig. 2A) and MI (Fig. 2B) than those fed on the CON diet ($P < 0.05$). However, the fish fed on β-CG4 diet upregulated significantly the mRNA expression of TGFβ1 gene only in the PI than those fed on the CON diet ($P < 0.05$).

3.3. Tight junction protein in the intestine

Feeding the mirror carp on the β-CG diets affected significantly the mRNA expression of genes related to tight junction protein in the intestine ($P < 0.05$; Fig. 3). The fish fed on the β-CG3 diet upregulated significantly the mRNA expression of claudin3c gene in the PI (Fig. 3A) than those fed on CON diet ($P < 0.05$). Similarly, the mirror carp fed on the β-CG2 and β-CG3 diets enhanced significantly the mRNA expression of claudin3c gene only in the MI(Fig. 3B) than those fed on CON diet ($P < 0.05$). However, the mirror carp fed on β-CG2 and β-CG3 downregulated significantly the mRNA expression of claudin3c gene only in the DI than those fed on CON diet ($P < 0.05$).

The mirror carp fed on the β-CG4 diet upregulated significantly the mRNA expression of claudin7 gene in the DI (Fig. 3C) than those fed on CON diet($P < 0.05$). However, the fish fed on the β-CG3 and β-CG4 diets downregulated the mRNA expression of claudin7 gene in the MI (Fig. 3B) than those fed on CON diet ($P < 0.05$). The mirror carp fed on the β-CG3 and β-CG4 diets upregulated significantly the mRNA expression of claudin11 gene in the MI (Fig. 3B) and DI (Fig. 3C) than those fed on CON diet ($P < 0.05$). Moreover, the fish fed on β-CG1 and β-CG2 diets upregulated significantly the mRNA expression of claudin11 gene in the DI and MI, respectively than those fed on the CON diet ($P < 0.05$). The mirror carp fed on β-CG2 and β-CG3 diets upregulated significantly the mRNA expression of occludin gene in the MI (Fig. 3B) and PI (Fig. 3C), respectively compared to those fed on CON diet ($P < 0.05$). However, the fish fed on on β-CG2 and β-CG3 diets downregulated significantly the mRNA expression of occludin gene in the DI than those fed on CON diet ($P < 0.05$). Interestingly, the fish fed on the β-CG4 diet upregulated significantly the mRNA expression of claudin3c, claudin7, claudin11 and occludin genes in the DI than those fed on β-CG3 diet ($P < 0.05$).

3.4. Protein and lipid signal pathway in the intestine

Feeding the mirror carp on the β-CG diets affected significantly the mRNA expression of genes related to protein and lipid signal pathway in the intestine ($P < 0.05$; Fig. 4). The mirror carp fed on the β-CG2, β-CG3 and β-CG4 downregulated significantly the mRNA expression of AMP-

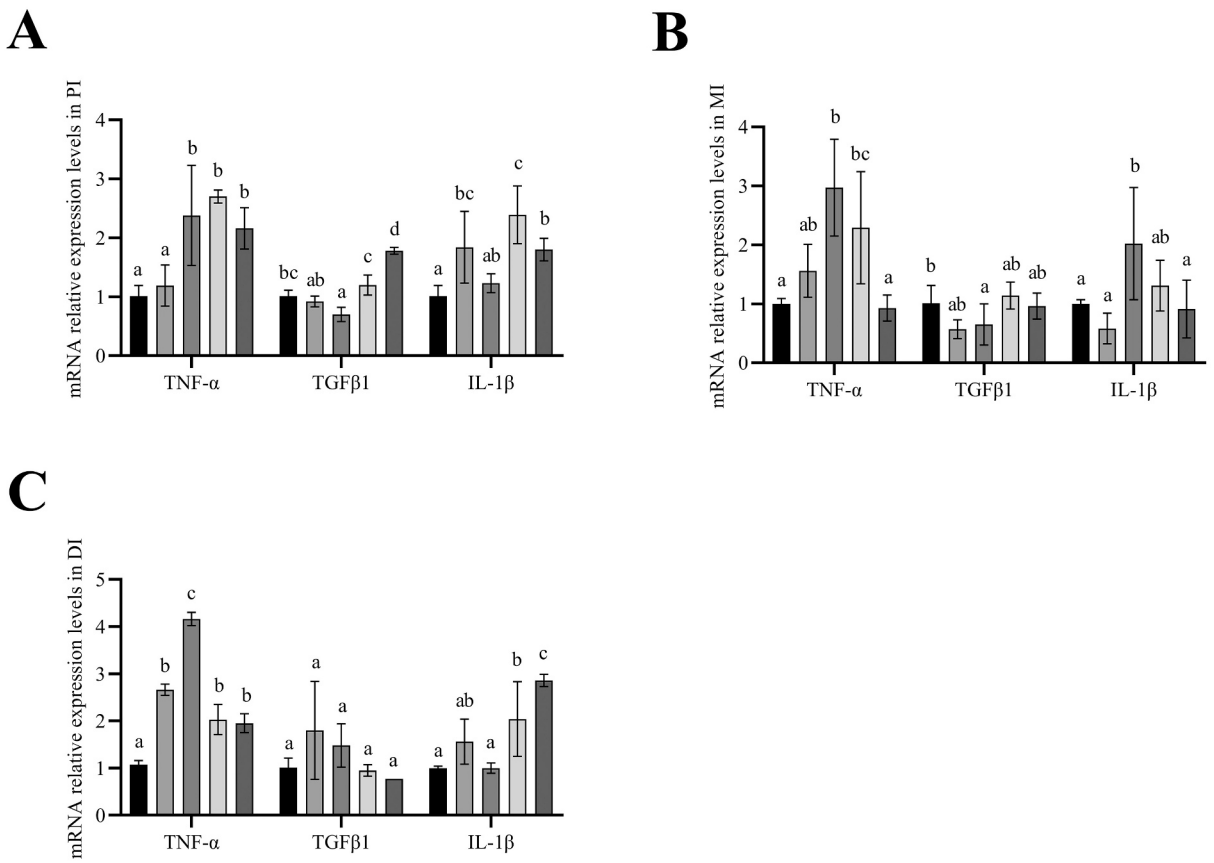


Fig. 2. Effects of different treatments on the mRNA levels of TNF-α, TGFβ1, and IL-1β in the proximal intestine (A), mid intestine (B), and distal intestine (C) of mirror carp. Data are presented as means ± SD, $n = 3$. ^{a,b,c} Mean values with different letters were significantly different ($P < 0.05$).
■ CON, □ β-CG1, ▨ β-CG2, □ β-CG3, ▨ β-CG4.

activated protein kinase alpha (AMPK) gene in the MI (Fig. 4B) and DI (Fig. 4C) than those fed on the CON diet ($P < 0.05$). Moreover, the fish fed on the β-CG1 diet reduced significantly the mRNA expression of AMPK gene in the DI (Fig. 4C) compared to those fed on CON diet ($P < 0.05$). Interestingly, the mirror carp fed on all the β-CG diets downregulated significantly the mRNA expression of acetyl-CoA carboxylase (ACC) gene in the MI (Fig. 4B) and DI (Fig. 4C) than those fed on CON diet ($P < 0.05$).

The mirror carp fed on β-CG1, β-CG2 and β-CG3 diets downregulated significantly the expression mRNA of target of rapamycin (TOR) gene in the PI (Fig. 4A) and MI (Fig. 4B) than those fed on CON diet ($P < 0.05$). Furthermore, the fish fed on the β-CG4 diet reduced significantly the expression mRNA of TOR gene in the PI, MI and DI (Fig. 4C) than those fed on the CON diet ($P < 0.05$). The mirror carp fed on the β-CG2 and β-CG3 diets upregulated significantly the mRNA expression of eIF4E-binding protein (4E-BP) gene in the PI (Fig. 4A) and MI (Fig. 4B) than those fed on the CON diet ($P < 0.05$). Moreover, the fish fed on the β-CG4 diet enhanced significantly the mRNA expression of 4E-BP gene in the PI and DI compared to those fed on CON diet ($P < 0.05$). The mirror carp fed on the β-CG1 diet increased significantly the mRNA expression of 4E-BP gene in the PI than those fed on the CON diet ($P < 0.05$). However, the feeding the fish on the experimental diets did not affect significantly the mRNA expression of AMPK and ACC genes in the PI ($P > 0.05$).

3.5. Differential metabolites

Intestinal mucosa from the fish fed on the CON and β-CG3 diets were profiled in negative and positive electrospray ionization modes. The OPLS-DA score plots performed to verify the differential metabolites between the β-CG3 and CON and multivariate analysis supervision (Fig. 5A, B). R^2X and R^2Y represent cumulative interpretation rates. In all comparisons, R^2Y was close to 1, indicating that the model is reliable (Zhao and Xu, 2022). In all the OPLS-DA permutation testing, Q^2 had values below zero, suggesting that the model derived by OPLS-DA had a good fit and high predictability and thus was used in subsequent analysis (Fig. 5C, D).

The fish fed on the β-CG3 diet upregulated 42 metabolites compared to those fed on the CON diet ($P < 0.05$) (data not shown). Moreover, the fish fed on the β-CG3 diet downregulated 12 metabolites compared to those fed on the CON diet ($P < 0.05$). Many of the downregulated 12 metabolites were lipids and lipid-like molecules (50.0%), organic acids and derivatives (17.39%), phenylpropanoids and polyketides (15.22%), organic nitrogen compounds:2 (4.35%), organic oxygen compounds:2 (4.35%), organoheterocyclic compounds:2 (4.35%), benzenoids:1 (2.17%) and organonitrogen compounds:1 (2.17%) (Fig. 6).

3.6. KEGG enriched pathways

Feeding the fish on the on the β-CG3 diet changed eight critical metabolic pathways (Fig. 7). These were alpha-linolenic acid metabolism, sphingolipid signaling pathway, sphingolipid metabolism, linoleic acid metabolism, glycerophospholipid metabolism, choline metabolism in cancer, arginine biosynthesis and apoptosis (Fig. 7). The primary differential metabolites of the KEGG pathway are shown in Table 4.

4. Discussion

The current study indicated that feeding the fish on the β-CG diets did not affect growth performance and related parameters contrary to

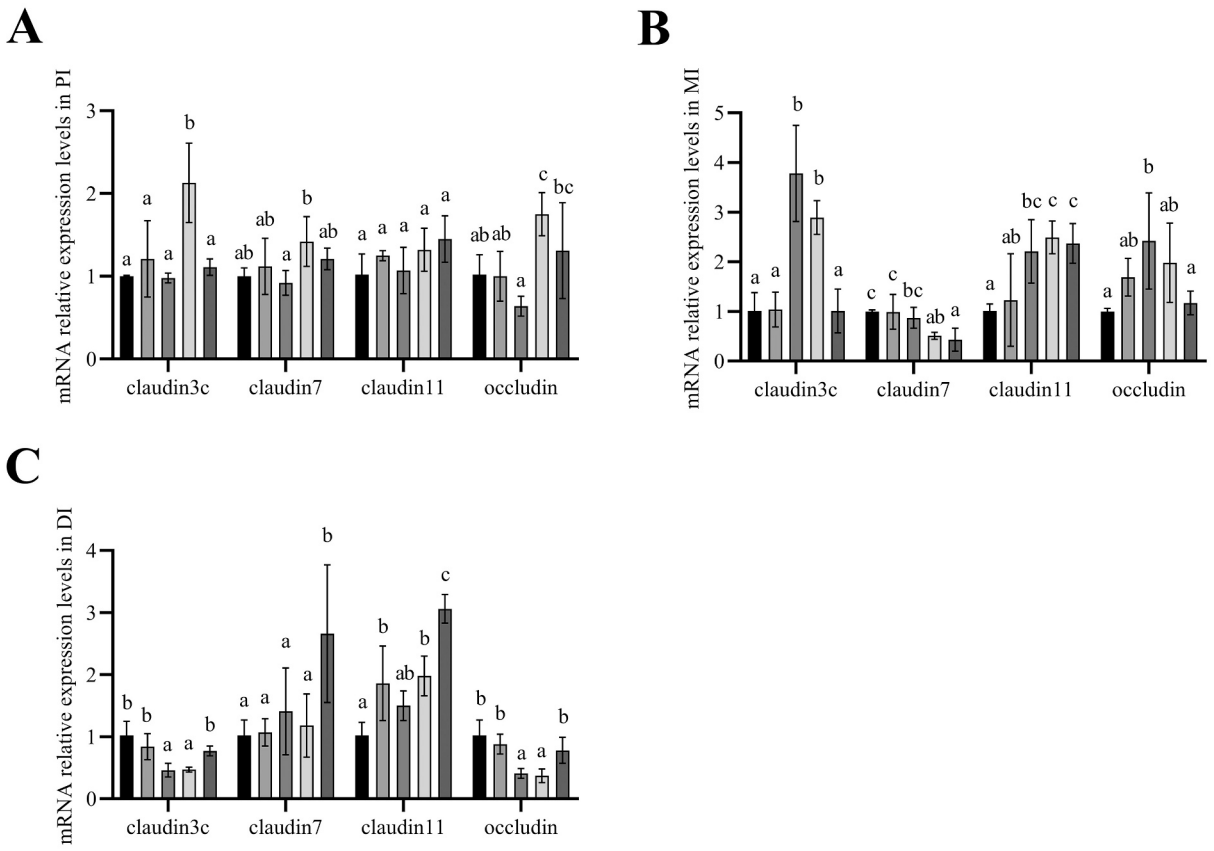


Fig. 3. Effects of different treatments on the mRNA levels of tight junction protein in the proximal intestine (A), mid intestine (B), and distal intestine (C) of carp. Data are presented as means ± SD, n = 3.

previous studies in various fish species. For instance, growth was impaired after feeding 20 g/kg β-CG for eight weeks in golden crucian carp (*Carassius auratus*) (Li et al., 2022), 4% β-CG for twelve weeks in turbot (Li et al., 2017b), and 70 g/kg β-CG for seven weeks in Chinese mitten crabs (*Eriocheir sinensis*) (Han et al., 2019). Moreover, growth performance was suppressed after feeding 80.0 g/kg β-CG for six weeks in Jian carp (Zhang et al., 2014) and grass carp for seven weeks (Duan et al., 2020). The inability of 40 to 160 g/kg β-CG to influence growth during the six weeks in this study is beyond our expectation. Probably, mirror carp has more robust adaptations because cyprinids have been considered more tolerant to soybean anti-nutritional factors (Viola et al., 1982). Indeed, β-CG diet had lower effect on growth performance in carp compared with grass carp and Egyptian catfish (*Clarias lazera*) (Wu et al., 2011). Further studies are required on the effect of β-CG on growth in various fish species to understand the reasons. These results indicate that, β-CG at the levels used in this study did not affect growth and survival rate of the mirror carps.

Soybean antigenic protein enters the body after feeding destroying the structural stability of the intestine, activating the immune system and causing intestinal inflammation (Francis et al., 2001). TNF-α plays an essential role in mediating the inflammatory, regulating immune function and other pathophysiological reactions (Cho et al., 2001). IL-1β plays a vital role in the inflammatory process and regulation of the immune regulation (Huising et al., 2004). Both TNF-α and IL-1β are important pro-inflammatory factors (Dinarello, 2000). TGF-β1 is an essential anti-inflammatory factor inhibiting inflammation (Zhu et al., 2021). In the present study, the expression of pro-inflammatory factors (TNF-α or IL-1β) were upregulated by dietary β-CG in the PI, MI, and DI and β-CG1 and β-CG4 in the MI, similar to results in grass carp (Zhang et al., 2020b), Chinese mitten crabs (Han et al., 2019), juvenile golden crucian carp (Li et al., 2020; Li et al., 2022) and turbot (Li et al., 2017b). However, TNF-α and IL-1β were downregulated in most β-CG4 diet except IL-1β in the DI, compared with those fed on β-CG3 diet. Meanwhile, the fish fed on β-CG2 downregulated the TGF-β1 gene in PI and MI. Up-regulation of pro-inflammatory factors suggest increased inflammatory response while downregulation of anti-inflammatory factor indicates completion of inflammation (Limbu et al., 2018).

The fish fed on the β-CG had enteritis, which was aggravated by up-regulating pro-inflammatory cytokines as reported previously (Wang et al., 2019). The enteritis was caused by the allergic effects of β-CG. However, the enteritis was reduced at higher levels of β-CG. We presume that the fish body established protective mechanisms to reduce allergic reactions due to dietary antigens. Low doses antigen results in priming while high doses antigen results in tolerance; tolerance appeared to take some time to develop (Bailey and Haverson, 2006). The mechanism for generating tolerance has yet to be explained, but is considered to be an effective means to protect the host from immune responses against innocuous food antigens (Klipper et al., 2000). Urán et al. (2008) reported a slower start of the enteritis process and recovery of enteritis after four weeks in common carp. Similarly, the body of mirror carp in the present study adapted due to the higher adaptability in omnivorous fish to plant ingredients (Zheng et al., 2020), which helped to retain intestinal health (Yao et al., 2019), indicating that there was a balance of immune regulation in the body adjusted by many cytokines (Yao et al., 2019). However, the ability of mirror carp to adapt to the allergic effects at high levels of β-CG requires further studies to explore. In general, feeding the mirror carp with β-CG induced enteritis in the intestine.

The intestinal epithelial barrier is the first line of defense against pathogenic microorganisms and toxins in fish the intestine (Yu and Li, 2014). The connection between intestinal epithelial cells primarily relies on tight junction proteins, mainly composed of occludin, claudins and others. Occludin is essential for some mechanisms of tight junction

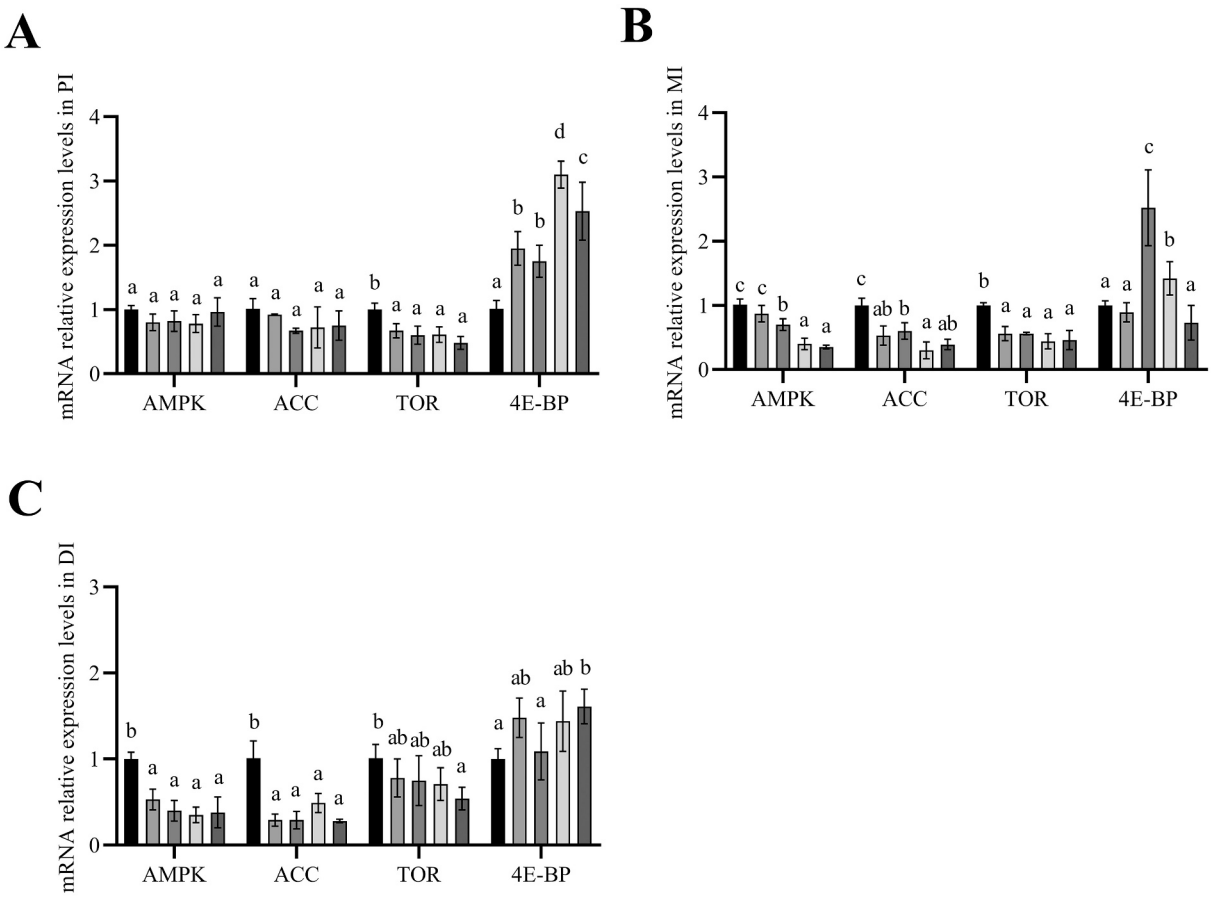


Fig. 4. Effects of different treatments on the mRNA levels of AMPK, ACC, TOR, and 4E-BP in the proximal intestine (A), mid intestine (B), and distal intestine (C). Data are presented as means ± SD, n = 3.

regulation (Buckley and Turner, 2018), while claudins are the major barrier-forming proteins of tight junction structure (Wang et al., 2017), which forms a highly selective barrier and protect animal health. In our study, we found that feeding the mirror carp with β-CG2 and β-CG3 diets reduced the mRNA expression of claudin3c and occludin genes in the DI, similar to results obtained by Zheng et al., (2020). In addition, the mirror carp fed on β-CG4 diet increased the relative expression of occludin, claudin11, claudin7, and claudin3c in the DI compared with those fed on β-CG2 and β-CG3 diets.

The decreased expression of genes related to tight junction proteins usually represents the weakened intestinal barrier function (Takiishi et al., 2017). This results indicate impaired intestinal barrier function in fish fed on β-CG diets. Similarly, Zhao et al. (2014) reported changes in tight junction expression induced by β-CG leading to intestinal epithelial barrier dysfunction in porcine intestinal epithelial cells from jejunum (IPEC-J2). Soybean antigen proteins are known to cause adverse effects on tight junction proteins in grass carp DI, MI, and PI (Zhang et al., 2021). The decreased tight junction proteins in this study is due to increased expression of pro-inflammatory cytokines as reported previously in grass carp (Chen et al., 2018) and turbot (Liu et al., 2018). Tight junction proteins are affected by pro-inflammatory cytokines (Bruewer et al., 2013), which cause an osmotic pressure imbalance (He et al., 2022a, 2022b), leading to increased permeability reducing tight junction expression (Groschwitz and Hogan, 2009). However, the β-CG4 diet enhanced the intestinal barrier function in DI. The precise reason for increased tight junction proteins in fish at high β-CG level is unavailable, requiring further studies to scrutinize. In summary, feeding the mirror carp with β-CG diets at lower level impaired the tight junction proteins.

The AMPK is a highly conserved sensor of cellular energy status found in all eukaryotes. It plays a crucial role in systemic energy balance (Kahn et al., 2005). AMPK pathway suppresses ATP-consuming biosynthetic processes while stimulates ATP-generating processes (Daval et al., 2006). In the current study, the mRNA expression of AMPK was decreased by dietary β-CG. This might be attributed to the fact that soybean antigen protein destroyed the structural stability of the intestine through pro-inflammatory and tight junction proteins (Jiang et al., 2015), which required more energy for maintenance (Ho et al., 2018), thus decreased AMPK gene expression. Similarly, previous studies demonstrated increased mRNA expression of pro-inflammatory genes, which inhibited phosphorylated AMPK levels in mouse (Park et al., 2018) and piglets (He et al., 2022a, 2022b). Our results indicate that β-CG-induced intestinal inflammation was partially related to the inhibition of AMPK signaling.

ACC is one of the first proteins identified as a target of AMPK. ACC is a critical enzyme of the lipogenic pathway (Sim and Hardie, 1988) and is simultaneously regulated by AMPK (Park et al., 2002). The mRNA expression of ACC gene declined with increasing β-CG content in the MI and DI in this study. In addition, we also observed decrease in AMPK gene accompanied by downregulation of ACC gene, which was inconsistent with the results reported by Kudo et al. (1996). This might be attributed to the fact that another pathway, such as the glycolytic pathway, may inhibit AMPK activity (Kemp and Oakhill, 2017).

The mTOR is also one of the target proteins of the downstream signaling pathway after AMPK (Saxton and Sabatini, 2017). It is a limiting step in animal protein synthesis (Preiss and Hentze, 2003). The multifactor regulated multifunctional protein, 4E-BP1 is an inhibitory factor of eukaryotic translation initiation factor, which affects protein synthesis by binding to eIF4E (Saxton and Sabatini, 2017). The 4E-BP is one of the mTOR-regulated target sites. In the present study, feeding the mirror carp on the β-CG2 and β-CG3 diets; β-CG4 diet; and β-CG1 diet

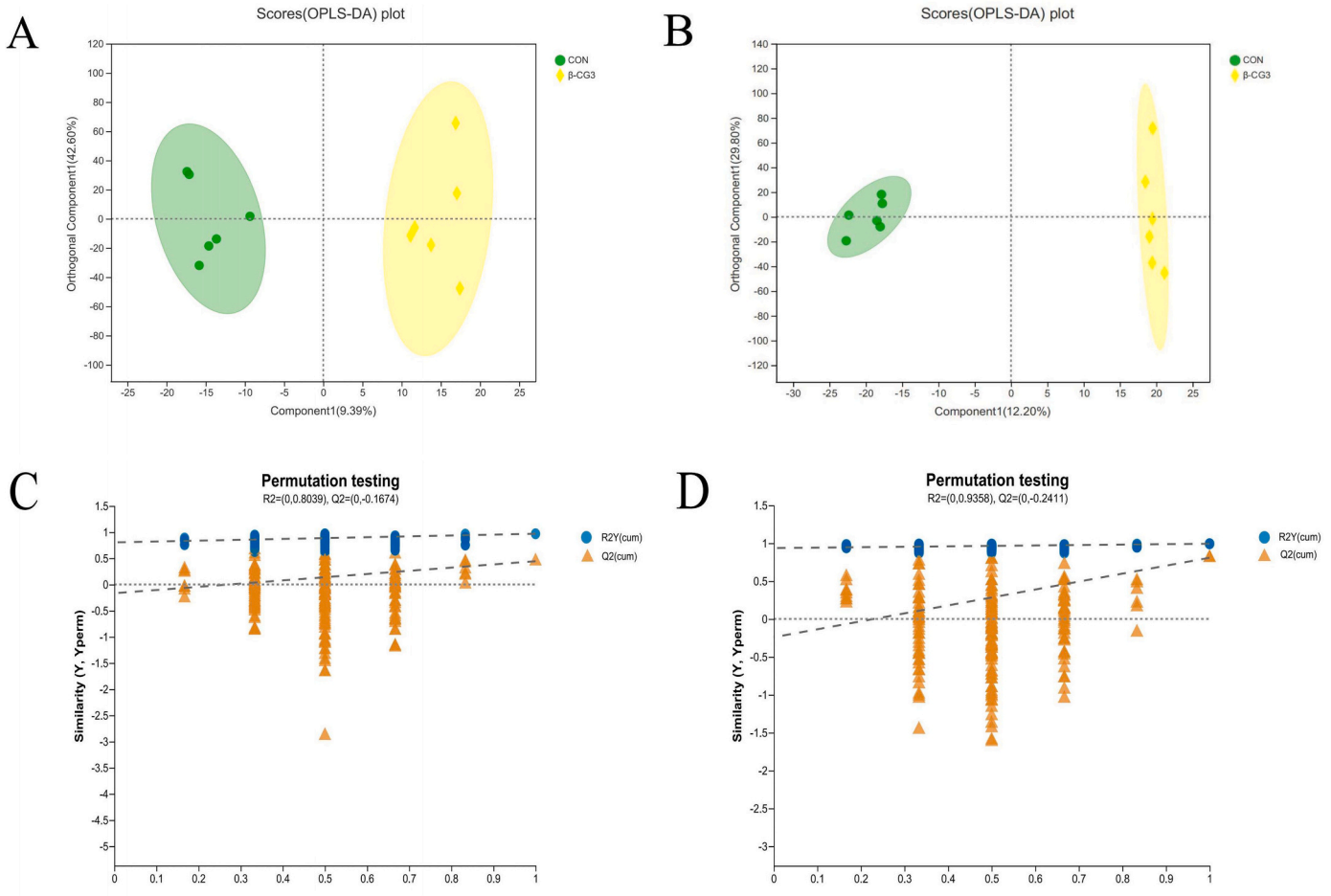


Fig. 5. OPLS-DA score plot in positive (A) and negative (B) ion mode based on the normalized data. Panels (C,D) are OPLA-DA permutationtests. n = 3. The R^2Y value represents the goodness of fit of the model. The Q^2 value represents the predictability of the models.

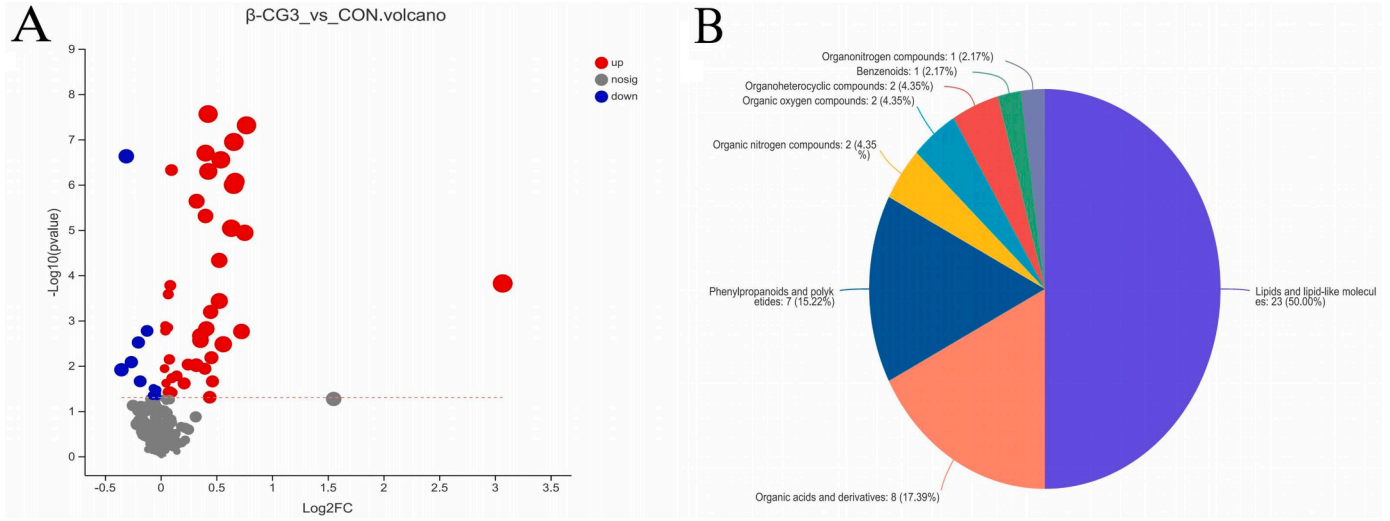


Fig. 6. The volcano plots (A) and HMDB superclass (B) of the intestinal mucosa. The blue, red, and gray dots represent the significantly down-regulated and up-regulated metabolites and no significantly differential metabolites. The VIP numeric value was represented by dot size. n = 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

decreased the relative expression of TOR accompanied by an increase in the relative expression of 4E-BP in the PI and MI; PI and DI; and PI respectively, similar to previous results reported by Duan et al. (2020) in grass carp and reduced protein content in Jian carp (Zhang et al., 2014). The reduced mRNA expression of TOR was due to increased pro-inflammatory cytokines, which inhibited TOR signaling because the latter are regulated by the former (Weichhart et al., 2008). Accordingly, Hu et al. (2015) reported that TOR signaling inhibition induced pro-

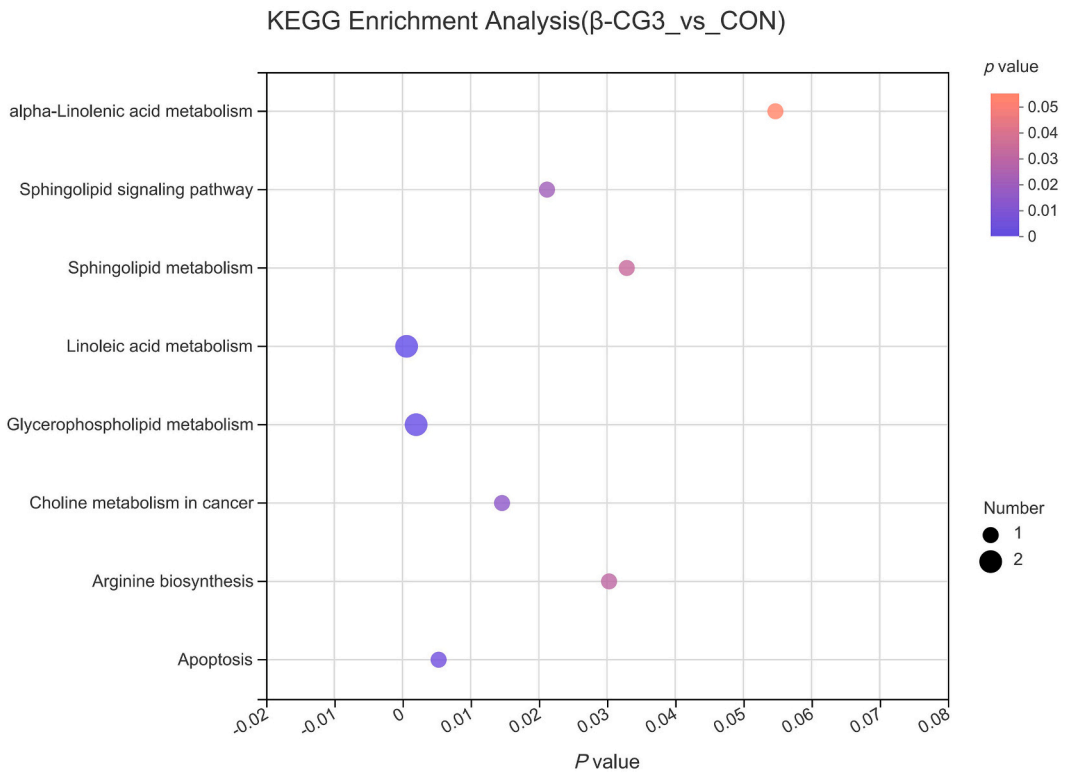


Fig. 7. The KEGG Topology analysis of β-CG3 vs. CON. The X-axis represents the pathway impact, and the Y-axis represents the pathway enrichment. The size of the bubble indicates the importance, n = 3.

Table 4
Main differential metabolites of KEGG pathway: β-CG3 vs. CON, data representative of three replicates.

KEGG Pathway ID	Pathway Description	Metabolite	FC	Trend
map00564	Glycerophospholipid metabolism	LysoPC(20:3 (5Z,8Z,11Z))	1.06	↑
		Dimethylethanolamine	1.03	↑
map05231	Choline metabolism in cancer	LysoPC(20:3 (5Z,8Z,11Z))	1.06	↑
map00591	Linoleic acid metabolism	9,10-DiHOME	1.06	↑
		9,10,13-TriHOME	1.05	↑
map00592	alpha-Linolenic acid metabolism	Traumatic Acid	0.96	↓
map00600	Sphingolipid metabolism	Sphingosine	1.05	↑
map04071	Sphingolipid signaling pathway			
map04210	Apoptosis			
map00220	Arginine biosynthesis	Citrulline	0.88	↓

Fold change (FC), ↑ means upregulated while ↓ means downregulated.

inflammatory cytokine production. In general, feed the mirror carp with β-CG diets suppress TOR expression.

In this study, the mirror carp fed on the β-CG3 diet upregulated metabolites related to lipids and lipid-like molecules, organic acids, and derivatives, consistency with the results on decreased protein synthesis (TOR) and lipogenesis (ACC), destroyed intestinal structure, and induced inflammation. The lipid related metabolites were upregulated to supply more energy required to repair the intestinal barrier (Ho et al., 2018) because they yield more energy. Glycerophospholipid metabolism is the major pathway involved in both general systemic-immune and low-grade inflammatory states, suggesting that phospholipids are potential inflammatory mediators (Zhu et al., 2022). An individual phospholipid species substituted by choline at the end group yield a phosphatidylcholine (PC) (Guo et al., 2005). An earlier study showed that n-3 polyunsaturated fatty acids (PUFAs), which are components of PC, had a modulating effect on inflammatory reactions (Das, 2011). PC inhibits inflammation during various conditions (Tokés et al., 2011). Accordingly, glycerophospholipid was enhanced in intestinal mucosa of fish fed on β-CG3 diet to alleviate intestinal inflammation. This might be reason for mirror carp to adapt to the allergic effects at high levels and the increased tight junction proteins reported previously in this study. In mammals, citrulline is precursor of arginine (Wakabayashi et al., 1993). Accordingly, supplementing citrulline increased circulating arginine (Dhanakoti et al., 1990). Moreover, citrulline is considered a more reliable marker of gastrointestinal (GI) damage (Papadia et al., 2007). The results indicated downregulation of citrulline in intestinal mucosa for fish fed on β-CG3 diet due to intestinal inflammation. Taken together, the fish fed on β-CG3 diet enhanced glycerophospholipid in the intestinal mucosa to alleviate intestinal inflammation while citrulline was downregulated due to intestinal inflammation.

5. Conclusion

In conclusion, dietary β-CG does not affect growth performance of mirror carp. However, the β-CG diets induce enteritis in different intestinal segments due to allergic effects. High levels of β-CG diets alleviates the enteritis due to enhanced glycerophospholipid, which reduces intestinal inflammation.

Author statement

Qiaohua Luo contributed in writing the original draft preparation and formal analysis; Zuliang Zhou contributed in experimental operation and field sampling; Jianhua Zhao and Hong Xu contributed in experiment design and manuscript revision; Samwel Mchele Limbu contributed in manuscript revision; Qiyou Xu contributed in funding acquisition, experiment design, management and manuscript revision.

All authors contributed to the article and approved the submitted version

Declaration of Competing 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.

Data availability

Data will be made available on request.

Acknowledgments

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