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Original Research Article

Yellow mealworm (*Tenebrio molitor*) meal in diets of grass carp (*Ctenopharyngodon idellus*): Effects on growth performance, antioxidant capacity, immunity, intestinal morphology, and intestinal microbiota

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ABSTRACT

The impacts of substituting dietary soybean meal (SBM) with yellow mealworm meal (YMM) were investigated during a 56-day feeding trial on growth, antioxidant capacity, immunity, intestinal morphology, and intestinal microbiota of grass carp. A total of 750 grass carp were divided into 5 groups (3 replications per group and 50 fish per replication) with different levels of YMM: SBM (control group), H25, H50, H75, and H100, for 8 weeks. The results showed that dietary YMM significantly increased final body weight (FW), weight gain (WG), and protein efficiency ratio (PER) in H25 group ($P < 0.05$); however, complete substitution showed the opposite trend ($P < 0.05$ for FW and WG). The liver antioxidant capacity was improved, manifested by enhanced superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activities, glutathione (GSH) content and up-regulated antioxidant-related genes mediated by the Keap1-Nrf2 signaling pathway in the H25 group ($P < 0.05$). However, in the H100 group oxidative stress occurred in parallel with impairment of hepatic function. Intestinal inflammation was aggravated in the H100 group as evidenced by the up-regulated pro-inflammatory gene expression mediated by the NF-κB pathway ($P < 0.05$). Additionally, the activity of intestinal digestive enzymes for the grass carp was significantly reduced and accompanied with intestinal mucosal barrier dysfunction in the H100 group ($P < 0.05$). In summary, replacement of SBM with 25% YMM showed positive influences on growth, antioxidant capacity, immunity, and intestinal health. Conversely, complete replacement of SBM with YMM triggered oxidative stress, caused liver function disorder, and impaired intestinal health in grass carp.

1. Introduction

It is well known that the most prevalent plant protein source for fish, especially herbivorous fish, is soybean meal (SBM). However, its high price, restricted supply, unbalanced amino acid profile, and the presence of anti-nutritional components limits its application in aquatic feed (Jia et al., 2022). Therefore, replacing SBM with an alternative protein is an effective way to promote sustainable development of aquaculture.

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In 2007, the European Union has authorized the use of 7 insect species as protein sources in aquafeed: yellow mealworm (*Tenebrio molitor*), common housefly (*Musca domestica*), black soldier fly (*Hermetia illucens*), banded cricket (*Gryllodes sigillatus*), field cricket (*Gryllus assimilis*), house cricket (*Acheta domesticus*) and lesser mealworm (*Alphitobius diaperinus*) (Giovanni et al., 2019). Compared to conventional protein sources, insects have the benefit of possessing a high feed conversion rate as well as a low water and land use, which may help to tackle the problem of protein deficit and environmental degradation (Wang et al., 2019). Therefore, humans are becoming more interested in applying insect meals in aquafeed.

Yellow mealworm meal (*T. molitor*, YMM) is one of the standout contenders for a viable alternative protein supply. YMM larvae and pupae (crude protein 69.01%, crude fat 5.88%) possesses abundant amino acids and polyunsaturated fatty acids (Shah et al., 2022). In addition, YMM contains functional active substances such as chitin, flavonoids, antioxidants peptides and antimicrobial peptides (Sergiy et al., 2016). Previous studies indicated effective replacement of fish meal with YMM was associated with better growth achievement in carnivorous and omnivorous fishes (Bai et al., 2023; Li et al., 2022a). Collectively, YMM could be considered as a potential alternative protein source. Some studies indicated the antioxidant capacity of carnivorous fishes increased with YMM supplementation (Zohreh et al., 2018; Guerreiro et al., 2023). However, excessive intake of YMM could cause oxidative stress (Song et al., 2018). Some studies indicated the immune system destroyed by YMM levels exceeding 4% in the diet (Gu et al., 2022). These findings verified the modulating effects of YMM on antioxidant capacity and immunity, but the mechanism has not clearly been found.

The grass carp (*Ctenopharyngodon idella*) is native to eastern Asia and widely distributed from the Heilongjiang River System. As an economically important freshwater fish, grass carp has been broadly cultivated worldwide, particularly in Asia and Europe. It has the advantages of rich protein, growing fast and a low breeding cost (Zhao et al., 2020). Studies have shown that herbivorous fish have obvious advantages in the preference and utilization of plant protein sources compared with carnivorous or omnivorous fish (Li et al., 2022b). Based on the function of herbal extract as an additive to stimulate immunity and enhance antioxidant status (Ahmadifar et al., 2020, 2021), medical plants also have the potential as a protein source. Comparatively speaking, insect protein is characterized by relatively comprehensive amino acids, no anti-nutritional factors, and a lower price than herbal extracts, and the substitution of insect protein source for SBM has not been investigated in juvenile grass carp. Therefore, the aim of this experiment was to assess the impacts of replacing SBM with YMM on growth, antioxidant capacity, immunity, and the intestinal microbiota of juvenile grass carp to explore whether YMM had the potential as a protein source.

2. Methods and materials

2.1. Animal ethics statement

The animal management procedures followed the guidelines of the Committee on Ethics of Animal Experiments at Hunan Agricultural University (No. 430723). The animal experiments comply with the ARRIVE guidelines.

2.2. Experimental fish, diets and conditions

The yellow mealworm meal was bought from Z&C biology Co., Ltd (Guangdong, China). Five isolipidic and isoprotein experimental diets were computed by substituting varying amounts of SBM with YMM (crude protein at 69.01%, crude fat at 5.88%): 0% (SBM), 25% (H25), 50% (H50), 75% (H75) and 100% (H100) (Table 1). After crushing and sieving through 60 meshes, the feed material was combined uniformly in a blender (BX-100 stuffing mixer) using the experimental recipe. Water was added to the mixture to attain the desired consistency before being pelletized with a 3.0-mm die head grinder. The feed material was air-dried at 25 °C until the moisture content was below 10%, before being reserved at −20 °C in a refrigerator for later use. The amino acid composition was shown in Table 2. The amino acid composition of the experimental diets was determined using an automatic amino acid analyzer (HITACHI L-8900, Tokyo, Japan) after acid hydrolysis with 6 mol/L HCl (reflux for 23 h at 110 °C). Tryptophan was not quantified due to its susceptibility to acid hydrolysis. Juvenile grass carp were purchased from Xidongting fishery (Changde, China) and the feeding experiment was carried out at Zhan Mao Lake (Changde, China). Firstly, the fish were given the basic diet for 2 weeks to adapt before the experiment. The 750 grass carp were divided into 5 groups, each with a similar initial weight (20.57 ± 0.34 g) which were kept in separate nylon net pond cages (2.0 m × 2.0 m × 2.0 m). There were 15 cages total with 50 fish per cage, with each group handling 3 cages. Each group received three daily meals (07:30, 12:30, 17:30) for 8 weeks. The amount of diet fed in the first week was 3% of the grass carp's starting weight. According to the feeding situation and the estimated weight of the grass carp, the feeding amount was adjusted once per week. The water temperature was 27.4 ± 3.5 °C, pH 7.3 ± 0.3, ammonia ≤0.2 mg/L, and dissolved oxygen 7.1 ± 0.2 mg/L (LH-M900 portable colorimeter).

2.3. Analyses of growth performance and proximate composition

After 24 h of starvation, fish were anesthetized with 40 mg/L clove oil (Nanjing Jiancheng CO., China). Fish in each net cage were

Table 1
Formulation and proximate composition of diets (g/kg, dry matter).

Item	Groups				
	SBM	H25	H50	H75	H100
Ingredients					
Fish meal	4.00	4.00	4.00	4.00	4.00
Distiller dried grains with solubles	9.00	9.00	9.00	9.00	9.00
Soybean meal (SBM)[1]	30.00	22.50	15.00	7.50	0.00
Yellow mealworm meal (YMM)[2]	0.00	5.24	10.48	15.73	20.97
Rapeseed meal	20.00	20.00	20.00	20.00	20.00
Rice bran	10.00	10.00	10.00	10.00	10.00
Wheat flour	22.00	22.00	22.00	22.00	22.00
Soybean oil	2.14	2.00	1.86	1.71	1.57
Bentonite	0.12	2.52	4.92	7.32	9.72
Choline chloride	0.20	0.20	0.20	0.20	0.20
$Ca(H_2PO_4)_2$	1.50	1.50	1.50	1.50	1.50
Premix[3]	1.00	1.00	1.00	1.00	1.00
Antioxidant	0.01	0.01	0.01	0.01	0.01
Anti-mildew agent	0.03	0.03	0.03	0.03	0.03
Total	100.00	100.00	100.00	100.00	100.00
Proximate analysis					
Crude protein	30.56	30.62	30.65	30.67	30.73
Crude fat	5.64	5.67	5.69	5.61	5.66
Crude ash	10.29	10.07	10.04	10.15	10.25

[1] SBM: crude protein at 46.35% and crude fat at 1.06%.
[2] YMM was provided by the Z&C Biotechnology Co. Ltd. (crude protein at 69.01%, crude fat at 5.88%).
[3] MGOTer Bio-Tech Co. Ltd (Shandong, China) provided the premix. Premix composition (per kilogram of diet): vitamin C phosphatase 6850 mg, vitamin D_3 40,000 IU, vitamin A 120,000 IU, iron 4800 mg, acid 3200 mg, magnesium 4000 mg, calcium pantothenate 720 mg, manganese 800 mg, nicotinic acid 1000 mg, zinc 2000 mg, vitamin B_2 280 mg, vitamin B_1 200 mg, vitamin E 480 mg, vitamin B_8 240 mg, vitamin K_3 200 mg, iodine 40 mg, copper 160 mg, selenium 4 mg, cobalt 12 mg, vitamin B_{12} 0.6 mg, folic acid 60 mg, biotin 1.2 mg.

Table 2
Amino acid composition of YMM, SBM and diets (%, dry matter)[1].

Amino acid	Ingredients		Diets				
	YMM	SBM	SBM	H25	H50	H75	H100
EAA							
Arginine	3.52 ± 0.131	3.36 ± 0.104	2.19 ± 0.087	2.15 ± 0.121	2.12 ± 0.142	2.01 ± 0.046	1.95 ± 0.154
Histidine	0.68 ± 0.110	1.09 ± 0.097	0.96 ± 0.047	0.94 ± 0.08	0.86 ± 0.070	0.82 ± 0.052	0.82 ± 0.089
Isoleucine	2.29 ± 0.032	1.94 ± 0.015	1.57 ± 0.006	1.61 ± 0.005	1.66 ± 0.016	1.62 ± 0.006	1.62 ± 0.027
Leucine	3.68 ± 0.026	3.17 ± 0.021	2.72 ± 0.011	2.74 ± 0.023	2.74 ± 0.025	2.88 ± 0.042	2.88 ± 0.014
Lysine	4.85 ± 0.044	2.53 ± 0.061	2.03 ± 0.008	2.26 ± 0.022	2.29 ± 0.009	2.29 ± 0.030	2.29 ± 0.005
Methionine	1.19 ± 0.052	0.57 ± 0.030	0.53 ± 0.006	0.55 ± 0.020	0.55 ± 0.018	0.62 ± 0.009	0.61 ± 0.011
Phenylalanine	2.24 ± 0.007	2.41 ± 0.088	1.69 ± 0.077	1.64 ± 0.049	1.64 ± 0.072	1.64 ± 0.064	1.73 ± 0.112
Threonine	2.69 ± 0.036	1.82 ± 0.059	1.37 ± 0.124	1.38 ± 0.091	1.38 ± 0.107	1.38 ± 0.065	1.40 ± 0.051
Valine	3.05 ± 0.034	2.18 ± 0.037	1.77 ± 0.008	1.88 ± 0.025	1.96 ± 0.048	1.99 ± 0.022	1.99 ± 0.060
∑EAA	24.69 ± 0.431	19.82 ± 0.302	15.19 ± 0.045	15.32 ± 0.072	15.35 ± 0.021	15.31 ± 0.033	15.44 ± 0.022
NEAA							
Alanine	4.25 ± 0.071	2.03 ± 0.016	1.37 ± 0.005	1.49 ± 0.011	1.52 ± 0.008	1.51 ± 0.010	1.54 ± 0.017
Glycine	2.98 ± 0.045	1.86 ± 0.058	1.42 ± 0.093	1.40 ± 0.110	1.47 ± 0.061	1.51 ± 0.083	1.50 ± 0.096
Glutamic acid	7.69 ± 0.062	8.09 ± 0.029	7.41 ± 0.054	7.09 ± 0.067	6.91 ± 0.072	6.51 ± 0.094	6.51 ± 0.122
Proline	4.13 ± 0.112	2.41 ± 0.063	2.37 ± 0.015	2.37 ± 0.014	2.59 ± 0.008	2.51 ± 0.009	2.57 ± 0.017
Serine	2.26 ± 0.063	2.32 ± 0.088	1.76 ± 0.007	1.84 ± 0.007	1.84 ± 0.06	1.91 ± 0.021	2.10 ± 0.005
Aspartic acid	4.98 ± 0.076	5.11 ± 0.098	3.38 ± 0.012	3.28 ± 0.011	3.12 ± 0.006	2.84 ± 0.032	2.85 ± 0.019
∑NEAA	26.1 ± 0.281	22.03 ± 0.374	17.68 ± 0.047	17.48 ± 0.044	17.46 ± 0.006	16.92 ± 0.030	17.21 ± 0.018

YMM = yellow mealworm meal; SBM = soybean meal; EAA = essential amino acids; NEAA = non-essential amino acids.
[1] SBM was substituted by YMM at 0% (SBM), 25% (H25), 50% (H50), 75% (H75) and 100% (H100), respectively.

bulk weighed. The viscera mass was separated (9 fish per group), and the eviscerated body was weighed to calculate the viscerosomatic index, and the livers were then separated from the viscera mass and weighted to calculate the hepatosomatic index. Nutrient levels (crude ash, crude protein, and crude lipid) of the diets were determined according to the methods described by AOAC (2005). Crude lipid, crude protein, and crude ash were analyzed by petroleum ether Soxhlet extraction (method No. 2001.11), Kelley nitrogen determination (method No. 2001.11), and Muffle furnace (Tokyo, Japan) at 550 °C for 12 h (method No. 942.05), respectively. The amino acid content was measured using an L-8900 automatic analyzer (Hitachi, Japan) according to the GB/T 18246-2019 method. The biological data were counted using the following formulae:

$$\text{Weight gain (WG, \%)} = (W_1 - W_0) / W_0 \times 100;$$

$$\text{Feed conversion ratio (FCR)} = W_3 / (W_1N_1 - W_0N_2 + W_2);$$

$$\text{Survival rate (SR, \%)} = N_1/N_2 \times 100;$$

$$\text{Protein efficiency ratio (PER)} = (W_1N_1 - W_0N_2) / (W_3 \times P_1);$$

$$\text{Condition factor } \left(\text{CF, g}/\text{cm}^3\right) = W_1 / (L_1)^3 \times 100;$$

$$\text{Hepatosomatic index (HSI, \%)} = W_4/W_3 \times 100;$$

$$\text{Viscerosomatic index (VSI, \%)} = W_5/W_3 \times 100;$$

Where W_0, W_1, W_2, W_3, W_4, and W_5 were the initial and final fish average weights, dead fish weights (g), intake feed weights (g), liver weight (g), and visceral weight (g). L_1 was the fish body length (cm). N_1 and N_2 represented the final and initial numbers of fish. P_1 was the crude protein content of diet (%).

2.4. Serum biochemistry and antioxidant enzyme activity

The tail vein blood of 9 fish from each group was collected using a 1-mL syringe at the end of the experiment (approximately 1 mL/fish). The serum was produced by centrifugation (1699 × *g*, 4 °C) for 15 min. The liver and intestines from the same part of each fish were isolated on a sterile bench, which was frozen in −80 °C for antioxidant enzyme activity analysis. The liver and intestinal samples (9 fish from each group) were homogenized with 0.65% sodium chloride solution at a ratio of 1:9 using the homogenizer (XHF-D, Xinzhi, China) and centrifuge (1699 × *g*, 4 °C, 15 min) to determine antioxidant enzyme activity. The immunoglobulin M (IgM), complement 3 (C3), and complement 4 (C4) were determined by a turbidimetric inhibition immunoassay. Acid phosphatase (ACP) and alkaline phosphatase (AKP) were determined by the disodium phenyl phosphate colorimetric method. Glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) were determined by Reitman-Frankel assay. Superoxide dismutase (SOD) was determined by the xanthine oxidase method. Catalase (CAT) was determined by the ammonium molybdenum assay. Malondialdehyde (MDA) was determined by the thiobarbituric acid method. Glutathione peroxidase (GPx) and glutathione (GSH) were determined by a colorimetric method. Reactive oxygen species (ROS) was determined by 2,7-dichlorofluorescein diacetate. All operations were carried out in accordance with the instructions (Nanjing Jiancheng CO., China). The results were calculated according to the formulae within the instructions. The absorbance of the samples was measured using a microplate reader (Thermo-1510).

2.5. Digestive enzyme activity, intestine histology and real-time quantitative PCR analysis

For each group of 9 fish, the midgut was removed and cleaned of intestinal contents and surrounding fat, washed with normal saline, dried with filter paper, weighed, and homogenized. The samples were homogenized with 0.65% sodium chloride solution at a ratio of 1:9 using the homogenizer (XHF-D, Xinzhi, China) to determine antioxidant enzyme activity. Samples were centrifuged at 4 °C (1699 × *g*, 10 min), and supernatant was taken. The amylase, lipase, and trypsin activities were determined by commercial assay kits (Nanjing Jiancheng Technology Co., Ltd., Nanjing, China) within 24 h. Segments 2 to 3 cm above the midgut at the same part were removed for histological investigation. The midgut was fixed for

24 h in 4% buffered paraformaldehyde before being dried in a graded ethanol series. Following that, the samples were xylene-infiltrated, paraffin-embedded, and sectioned with a rotary microtome. Hematoxylin-eosin solution (Nanjing Jiancheng Co., China) was used to stain the samples, and photographs were taken using a digital camera fitted to a microscope (3DHistech Ltd., Budapest, Hungary). The soft of Case Viewer 2.0 analysis was used. Each treatment had 3 sections. Each slice was selected with three views, each of which measured the three intestinal folds of height, muscle thickness, and goblet cell numbers.

Following the results for growth performance and intestinal morphology, the SBM, H25, and H100 groups were chosen to assess the liver and intestine associated gene expression (each group of 9 fish). The TRNzol reagent was used to lyse the liver and intestines to extract the total RNA (Invitrogen, USA). Spectrophotometry (NanoDrop ND-2000) and electrophoresis on 2% agarose gels were used to determine the quantity and quality of RNA, respectively. A reagent kit was used to perform reverse transcription of the whole RNA into cDNA (Monad Biotech Co., Ltd., Suzhou, China). Real-time qPCR was carried out with the aid of the CFX 96 real-time PCR detection instrument (Bio-Rad, Hercules, CA, USA) using a Prime-Script RT reagent kit (Monad, Beijing China). The delta−delta CT technique ($2^{-\Delta\Delta CT}$) was used to determine the levels of expression. Table 3 displays quantitative PCR primer sequences and GenBank accession codes (Tsingke Biotech Co., Ltd. Beijing, China). Since β-actin has been utilized before and has undergone no notable alterations, it was employed as a nonregulated reference gene (Su et al., 2011).

2.6. Analysis of gut microbiome

Following the results for growth performance and intestinal morphology, the SBM, H25, and H100 groups were chosen to assess the intestinal microbiome (9 fish per group). In a sterile environment using a scalpel, the intestinal contents were scraped into sterile Eppendorf tubes. Samples were stored in liquid nitrogen and then kept at −80 °C. Following the findings for growth performance and intestinal morphology, The SBM, H25, and H100 groups were chosen to participate in 16S rRNA sequencing. Using the PowerFecal DNA Isolation Kit (MoBio Laboratories, Inc.), DNA was isolated from the contents of the intestines. Amplification of the V3 to V4 region was carried out using barcode fusion primers 341F and 805R, after which high-throughput sequencing was performed using the Illumina MiSeq platform. Effective tags were produced by applying the DADA2 method to filter out chimeric sequences and denoise the pair-end reads. The BIPES pipeline was used to divide the raw sequences into distinct samples according to barcodes, and UCHIME was then used to filter chimeric sequences (Edgar et al., 2011). Utilizing FASTX-Toolkit (Hannon Lab, USA) to eliminate low-quality scores (Q score, 20), all sequences were categorized into operational taxonomic units (OTUs) with 97% similarity using QIIME 2. Classification hierarchies were assigned to each OTU using uclust. Relevant data were submitted to the NovoMagic system (Beijing Novogene Technology Co., Ltd).

2.7. Statistical analysis

Data conforming to normality and Homogeneity of Variances were statistically analyzed by one-way ANOVA with Tukey HSD multiple comparisons test. The data was employed to mixed linear model using R 4.1.2 software as follows:

$$Y_{ij\kappa} = \mu + T_i + P_j + S_\kappa + e_{ij\kappa},$$

where $Y_{ij\kappa}$, μ, T_i, P_j, S_κ, *and* $e_{ij\kappa}$ were the dependent variable, overall mean, fixed treatment effect, period effect, steer effect, and random error.

The significance level was $P < 0.05$. The findings were given as means ± standard error (SE). The raw sequence was submitted to the NCBI Sequence Read Archive database with the accession number PRJNA854610.

3. Results

3.1. Amino acid composition of YMM, soybean meal and diets

The amino acid composition of YMM, soybean meal and diets are shown in Table 2. Compared to soybean meal, the contents of the essential amino acids (EAA) isoleucine, leucine, lysine, methionine, threonine, valine, and non-essential amino acids (NEAA) alanine, glycine, and proline were higher in YMM. With the increase of YMM substitution level, the contents of EAA (isoleucine, leucine, lysine, methionine, and valine) and NEAA (alanine, proline, and serine) were increased. While NEAA (glutamic acid and

Table 3
Primer sequence of real-time quantitative PCR.

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)	Amplicon, bp	Primer efficiency	Accession No.
β-actin	GATGATGAAATTGCCGCACTG	ACCGACCATGACGCCCTGATGT	135	0.95	M25013
gpx1	AGGGGCTGGTTATTCTGGG	GGGCGTTCTCACCATTCAC	162	0.99	EU828796.2
gpx4	TACGCTGAGAGAGGTTTACAC	CTTTTCCATTGGGTTGTTCC	196	0.94	KU255598
Gsto	GGTGCTCAATGCCAAGGGAA	CTCAAACGGGTCGGATGGAA	208	1.02	KT757314
Gr	GTGTCCAACTTCTCCTGTG	ACTCTGGGGTCCAAAACG	221	0.98	JX854448
Cat	GAAGTTCTACACCGATGAGG	CCAGAAATCCCAAACCAT	157	1.03	FJ560431
Cuznsod	CGCACTTCAACCCTTACA	ACTTTCCTCATTGCCTCC	218	0.99	GU901214
Mnsod	ACGACCCAAGTCTCCCTA	ACCCTGTGGTTCTCCTCC	111	0.93	GU218534
nrf2	CTGGACGAGGAGACTGGA	ATCTGTGGTAGGTGGAAC	234	1.05	KF733814
keap1	TTCCACGCCCTCCTCAA	TGTACCCTCCCGCTATG	205	1.03	KF811013
il-1β	AGAGTTTGGTGAAGAAGAGG	TTATTGTGGTTACGCTGGA	234	0.96	JQ692172
il-6	CAGCAGAATGGGGGAGTTATC	CTCGCAGAGTCTTGACATCCTT	134	0.99	KC535507.1
tnf-α	ACGCTCAACAAGTCTCAG	CTGGCTGTAGACGAAGTAA	252	1.01	HQ696609
nf-κb	GAAGAAGGATGTGGGAGATG	TGTTGTCGTAGATGGGCTGAG	124	1.04	AC131025.1
Occludin	TATCTGTATCACTACTGCGTCG	CATTCACCCAATCCTCCA	208	0.93	KF193855.1
Claudin12	CCCTGAAGTGCCCACAA	GCGTATGTCACGGGAGAA	81	1.01	KF998571
zo-1	CGGTGTCTTCGTAGTCGG	CAGTTGGTTTGGGTTTCAG	154	0.97	KF193852.1
zo-2	TACAGCGGGACTCTAAAATGG	TCACACGGTCGTTCTCAAAG	150	0.99	KM112095

β-actin = beta-actin; *gpx1* = glutathione peroxidase 1; *gpx4* = glutathione peroxidase 4; *gsto* = glutathione S-transferase omega; *gr* = glutathione reductase; *cat* = catalase; *cuznsod* = copper/zinc superoxide dismutase; *mnsod* = manganese superoxide dismutase; *nrf2* = nuclear factor erythroid 2-related factor 2; *keap1* = kelch-like ECH-associated protein 1; *il-1β* = interleukin-1β; *il-6* = interleukin-6; *tnf-α* = tumor necrosis factor alpha; *nf-κb* = nuclear factor kappa-B; *zo-1* = zonula occludens-1; *zo-2* = zonula occludens-2.

aspartic acid) were decreased. Overall, soybean meal replacement with YMM appeared to elevate the total EAA content and lower the total NEAA content, even for the H25 group.

3.2. Growth performance and body compositions

The growth and morphometric parameters of grass carp are displayed in Table 4. When the content of YMM increased gradually, the FW, WG and PER showed an increasing trend before decreasing. The FW and WG were significantly higher in H25 than other groups ($P < 0.05$), and the PER was significantly higher in the H25 group than the SBM group ($P = 0.009$). Dietary YMM inclusion had a linear ($P = 0.008$) and quadratic ($P = 0.018$) impact on the FW. The WG revealed a significantly linear relationship ($P = 0.012$), and the PER revealed a significantly quadratic relationship ($P = 0.037$). The FCR, SR, CF, HSI, and VSI did not differ significantly over the five groups ($P > 0.05$).

The results of body composition were presented in Table 5. Crude protein and moisture were significantly higher in YMM addition groups than that of the SBM group ($P < 0.05$). The trend of crude fat was opposite to that of crude protein, which was significantly lower in H25, H50, H75 groups than in the control group ($P = 0.001$). The results suggested a significant increase of moisture with YMM addition. The crude protein revealed a significantly linear relationship ($P < 0.001$), and the crude fat and moisture revealed a significantly quadratic relationship ($P < 0.05$). The ash had not significantly changed ($P = 0.440$).

3.3. Serum biochemical parameters

In Table 6, as compared to the SBM group, the levels of IgM, C3 contents, and AKP activity significantly increased with further inclusion of YMM ($P < 0.05$), excepting the H100 group. Compared with the SBM group, C4 content and ACP activity were significantly increased in the H25 group ($P < 0.05$). Dietary YMM inclusion had a linear and quadratic impact on the IgM and C4 contents ($P < 0.05$). The content of C3 and activities of ACP and AKP revealed a significantly quadratic relationship ($P < 0.05$).

3.4. Antioxidant indexes in the liver

As shown in Table 7, in the liver, the GOT and GPT activities and ROS content for groups H75 and H100 were significantly increased compared to those of the SBM group ($P < 0.05$). Compared with the SBM group, the MDA content in the H25 group was significantly reduced, while that in the H100 group was significantly increased ($P = 0.001$). In addition, the GPx, CAT activities and GSH content of the H25 and H50 groups were significantly increased compared with that of the SBM group ($P < 0.05$). Compared with the SBM group, the SOD activity of H25 was significantly increased ($P = 0.001$). The activities of SOD, CAT, and content of GSH of fish in the H100 group were significantly lower than that of SBM group ($P < 0.05$). Dietary YMM inclusion had a linear and quadratic impact on the activities of GOT, SOD, GPx, and CAT and contents of GSH, ROS, and MDA ($P < 0.05$). The activity of GPT revealed a significantly linear relationship ($P = 0.004$).

The results of mRNA expression levels in the liver were shown in Fig. 1. The results for the H25 group showed remarkably up-regulated manganese superoxide dismutase (*mnsod*), catalase (*cat*), nuclear factor erythroid 2-related factor 2 (*nrf2*), glutathione peroxidase 1 (*gpx1*), glutathione peroxidase 4 (*gpx4*), glutathione S-transferase omega (*gsto*), and glutathione reductase (*gr*) mRNA expression ($P < 0.05$), and down-regulated kelch-like ECH-associated protein 1 (*keap1*) mRNA expression compared with the SBM group ($P < 0.05$). Whereas the results for the H100 group showed significantly downregulated copper/zinc superoxide dismutase (*cuznsod*), *gpx1*, *gpx4*, and *gr* compared with the SBM group ($P < 0.05$). There was no significant difference in *mnsod*, *cat*, *keap1*,

Table 4
Effect of substituting SBM with YMM on growth performance and biological indices of the grass carp after 8 weeks[1].

Item	Groups					*P*-value		
	SBM	H25	H50	H75	H100	ANOVA	Linear	Quadratic
IW, g	20.73 ± 0.534	20.27 ± 0.500	20.24 ± 0.334	20.60 ± 0.193	21.02 ± 0.070	0.564	0.304	0.150
FW, g	60.11 ± 0.669^{b}	64.16 ± 0.557^{c}	58.56 ± 1.011^{b}	58.20 ± 0.367^{b}	55.77 ± 0.670^{a}	0.001	0.008	0.018
WG, %	190.25 ± 4.364^{b}	217.09 ± 10.328^{c}	189.58 ± 9.399^{b}	182.60 ± 2.405^{ab}	165.29 ± 4.026^{a}	0.005	0.012	0.067
Feed intake, g/d per fish	0.94 ± 0.006	0.96 ± 0.043	0.87 ± 0.063	0.92 ± 0.036	0.89 ± 0.066	0.718	0.159	0.418
FCR	1.71 ± 0.044	1.62 ± 0.033	1.90 ± 0.172	1.90 ± 0.015	1.80 ± 0.054	0.157	0.046	0.179
SR, %	87.33 ± 0.667	90.00 ± 1.155	88.67 ± 0.667	90.00 ± 3.055	86.00 ± 3.055	0.596	0.407	0.107
PER	1.98 ± 0.035^{ab}	2.29 ± 0.059^{c}	2.15 ± 0.124^{bc}	2.04 ± 0.044^{ab}	1.83 ± 0.049^{a}	0.009	0.078	0.037
CF, g/cm^{3}	2.18 ± 0.052	2.08 ± 0.046	2.03 ± 0.108	2.06 ± 0.041	2.03 ± 0.051	0.420	0.023	0.280
HSI, %	3.45 ± 0.124	3.64 ± 0.119	3.75 ± 0.116	3.72 ± 0.161	3.47 ± 0.192	0.226	0.188	0.018
VSI, %	20.63 ± 0.622	21.99 ± 1.841	20.35 ± 0.776	17.91 ± 2.313	19.06 ± 1.544	0.058	0.103	0.374

YMM = yellow mealworm meal; SBM = soybean meal; IW = initial body weight; FW = final body weight; WG = weight gain; FCR = feed conversion rate; SR = survival rate; PER = protein efficiency ratio; CF = condition factor; HSI = hepatopancreas index; VSI = viscerosomatic index.
Different superscripts were used to show statistical differences at $P < 0.05$.
[1] SBM was substituted by YMM at 0% (SBM), 25% (H25), 50% (H50), 75% (H75) and 100% (H100), respectively.

Table 5
Effect of substituting SBM with YMM on body composition of grass carp after 8 weeks[1].

Item	Groups					*P*-value		
	SBM	H25	H50	H75	H100	ANOVA	Linear	Quadratic
Crude protein, %	17.63 ± 0.414^{a}	19.44 ± 0.311^{b}	19.43 ± 0.220^{b}	20.1 ± 0.578^{b}	19.45 ± 0807^{b}	0.029	<0.001	0.086
Crude fat, %	8.27 ± 0.496^{b}	5.54 ± 0.370^{a}	5.78 ± 0.393^{a}	5.97 ± 0.374^{a}	7.86 ± 0.411^{b}	0.001	0.445	0.011
Ash, %	3.95 ± 0.260	3.36 ± 0.122	4.51 ± 0.069	4.39 ± 0.114	3.44 ± 1.786	0.440	0.177	0.144
Moisture, %	68.61 ± 0.165^{a}	70.99 ± 0.152^{b}	70.29 ± 0.261^{b}	70.52 ± 0.212^{b}	69.98 ± 0.795^{b}	0.017	0.178	0.019

YMM = yellow mealworm meal; SBM = soybean meal.
Different superscripts were used to show statistical differences at $P < 0.05$.
[1] SBM was substituted by YMM at 0% (SBM), 25% (H25), 50% (H50), 75% (H75) and 100% (H100), respectively.

Table 6
Effect of substituting SBM with YMM on serum biochemistry of grass carp after 8 weeks[1].

Item	Groups					*P*-value		
	SBM	H25	H50	H75	H100	ANOVA	Linear	Quadratic
IgM, g/L	1.02 ± 0.035^{a}	1.74 ± 0.046^{c}	1.63 ± 0.068^{c}	1.34 ± 0.061^{b}	1.06 ± 0.036^{a}	0.001	0.005	0.004
C3, g/L	1.17 ± 0.097^{a}	2.02 ± 0.102^{b}	2.93 ± 0.067^{c}	1.98 ± 0.032^{b}	1.21 ± 0.094^{a}	0.001	0.446	<0.001
C4, g/L	0.18 ± 0.006^{a}	0.33 ± 0.008^{b}	0.31 ± 0.008^{ab}	0.28 ± 0.008^{ab}	0.19 ± 0.008^{a}	0.001	0.047	0.002
ACP, U/L	2.49 ± 0.017^{ab}	2.66 ± 0.013^{c}	2.57 ± 0.018^{bc}	2.46 ± 0.033^{ab}	2.44 ± 0.042^{a}	0.001	0.054	0.038
AKP, U/L	0.20 ± 0.004^{a}	0.25 ± 0.008^{b}	0.24 ± 0.003^{b}	0.25 ± 0.010^{b}	0.21 ± 0.004^{a}	0.001	0.270	0.007

YMM = yellow mealworm meal; SBM = soybean meal; IgM = immunoglobulin M; C3 = complement 3; C4 = complement 4; ACP = acid phosphatase; AKP = alkaline phosphatase.
Different superscripts were used to show statistical differences at $P < 0.05$.
[1] SBM was substituted by YMM at 0% (SBM), 25% (H25), 50% (H50), 75% (H75) and 100% (H100), respectively.

Table 7
Effect of substituting SBM with YMM on liver antioxidant enzyme activities of grass carp after 8 weeks[1].

Item	Groups					*P*-value		
	SBM	H25	H50	H75	H100	ANOVA	Linear	Quadratic
GOT, U/mL	17.80 ± 0.289^{a}	18.76 ± 0.630^{a}	19.36 ± 0.512^{a}	26.25 ± 0.462^{b}	26.65 ± 0.775^{b}	0.001	0.002	0.029
GPT, U/mL	5.30 ± 0.371^{a}	5.87 ± 0.588^{a}	5.56 ± 0.463^{a}	8.32 ± 0.550^{b}	9.50 ± 0.601^{b}	0.001	0.004	0.064
ROS, U/mg	2.03 ± 0.148^{a}	1.94 ± 0.049^{a}	1.89 ± 0.111^{a}	2.90 ± 0.107^{b}	3.15 ± 0.117^{b}	0.001	0.007	0.007
MDA, nmol/mg	1.75 ± 0.020^{bc}	1.49 ± 0.050^{a}	1.57 ± 0.032^{ab}	1.87 ± 0.058^{cd}	2.06 ± 0.059^{d}	0.001	0.022	0.011
SOD, U/mg	29.84 ± 0.540^{b}	33.15 ± 0.644^{c}	31.07 ± 0.874^{bc}	28.79 ± 0.474^{ab}	26.12 ± 0.220^{a}	0.001	0.004	0.002
GPx, U/mg	73.95 ± 1.876^{a}	87.83 ± 1.573^{c}	82.95 ± 1.388^{bc}	76.22 ± 1.578^{ab}	70.89 ± 1.787^{a}	0.001	0.035	0.007
CAT, U/mg	3.42 ± 0.246^{b}	6.12 ± 0.103^{c}	5.46 ± 0.272^{c}	3.20 ± 0.483^{ab}	2.13 ± 0.082^{a}	0.001	0.002	0.003
GSH, μmol/mg	37.88 ± 1.016^{b}	43.43 ± 1.662^{c}	41.99 ± 0.374^{c}	38.65 ± 0.621^{bc}	32.29 ± 0.289^{a}	0.001	0.031	0.002

YMM = yellow mealworm meal; SBM = soybean meal; GOT = glutamic oxaloacetic transaminase; GPT = glutamic pyruvic transaminase; ROS = reactive oxygen species; MDA = malondialdehyde; SOD = superoxide dismutase; GPx = glutathione peroxidase; CAT = catalase; GSH = glutathione.
Different superscripts were used to show statistical differences at $P < 0.05$.
[1] SBM was substituted by YMM at 0% (SBM), 25% (H25), 50% (H50), 75% (H75) and 100% (H100), respectively.

nrf2, and *gsto* gene expression for the H100 group compared with the SBM group ($P > 0.05$). Dietary YMM inclusion had a linear and quadratic impact on *mnsod*, *cat*, *nrf2*, and *gsto* ($P < 0.05$). The *cuznsod*, *keap1*, *gpx1*, *gpx4*, and *gr* revealed a significantly quadratic relationship ($P < 0.05$).

3.5. Digestive enzymes and intestine histology

As shown in Table 8, compared with the SBM group, the amylase activity of the H50, H75 and H100 groups was significantly decreased ($P = 0.001$). Trypsin was not impacted by the YMM supplementation ($P = 0.333$). By including YMM, lipase activity was significantly reduced as compared to SBM group ($P = 0.001$). Dietary YMM inclusion had a linear ($P = 0.012$) and quadratic ($P = 0.020$) impact on amylase activity. The lipase activity revealed a significantly linear relationship ($P = 0.002$).

In the midgut, the H25, H50 and H75 groups showed significant increases in intestinal fold height ($P = 0.001$). However, when compared to SBM group, the H100 group demonstrated significant reduction ($P = 0.001$). The muscle thickness in the H75 and H100 groups was significantly reduced ($P = 0.022$). The intestinal goblet cell numbers significantly decreased when the replacement amount of YMM was greater than 50% ($P = 0.001$) (Table 9). Dietary YMM inclusion had a linear ($P = 0.003$) and quadratic ($P = 0.001$) impact on goblet cell numbers. The muscle thickness revealed a significantly linear relationship ($P = 0.003$), and the intestinal fold height revealed a significantly quadratic relationship ($P < 0.001$). When compared to the SBM group, intestinal folds and breakages were more common in the H75 and H100 groups (Fig. 2).

3.6. Antioxidant and immunity indexes in the intestine

As shown in Table 10, the ROS and MDA contents in the intestine of the H25 and H50 groups were significantly reduced compared to the SBM group ($P < 0.05$). Compared to the SBM group, the SOD activity in groups H25, H50 and H75 was significantly increased ($P = 0.001$). The CAT activity in groups H25 and H50 was significantly increased compared to the SBM group ($P = 0.001$). The activity of GPx and content of GSH in the H25 group were significantly higher than the SBM group ($P < 0.05$), similar to the results for the liver. However, for group H100, significantly increased ROS activity and decreased SOD activity compared with the SBM group was observed ($P < 0.05$). Dietary YMM inclusion had a linear and quadratic impact on ROS, MDA, and GSH contents and SOD, GPx, and CAT activities ($P < 0.05$).

The results of mRNA expression levels in the intestine were shown in Fig. 3. The levels of mRNA expression for the *cuznsod*, *gpx1*, and claudin12 genes were not significantly affected by the addition of YMM ($P > 0.05$). The mRNA expression levels of *mnsod*, *cat*, *nrf2*, *gpx4*, *gr*, and occludin genes in the H25 group were significantly up-regulated compared with SBM group ($P < 0.05$), while kelch-like ECH-associated protein 1 (*keap1*) in H25 group was significantly down-regulated ($P = 0.002$). Conversely, the mRNA expression levels of *gpx4*, *gsto*, and *gr* were significantly downregulated in the H100 group ($P < 0.05$). The mRNA expression levels of *mnsod*, interleukin-1β (*il-1β*), interleukin-6 (*il-6*), tumor necrosis factor alpha (*tnf-α*), and nuclear factor kappa-B (*nf-κb*) genes were considerably up-regulated in the H100 group compared to the SBM group ($P < 0.05$), whereas tight junction protein zonula occludens-1 (*zo-1*) and zonula occludens-2 (*zo-2*) genes were dramatically down-regulated ($P < 0.05$). Dietary YMM inclusion had a linear ($P = 0.015$) and quadratic ($P = 0.035$) impact on mRNA expression level of *il-1β*. The mRNA expression level of *mnsod*, *cat*, *nrf2*, *keap1*, *gpx4*, *gsto*, *gr*, *il-6*, *tnf-α*, *nf-κb*, occludin, *zo-1*, and *zo-2* revealed a significantly quadratic relationship ($P < 0.05$), and the intestinal fold height revealed a significantly quadratic relationship ($P < 0.001$).

3.7. Gut microbiota

The Chao1 index was one of the alpha diversity indexes widely used in ecology to estimate the total number of species contained in

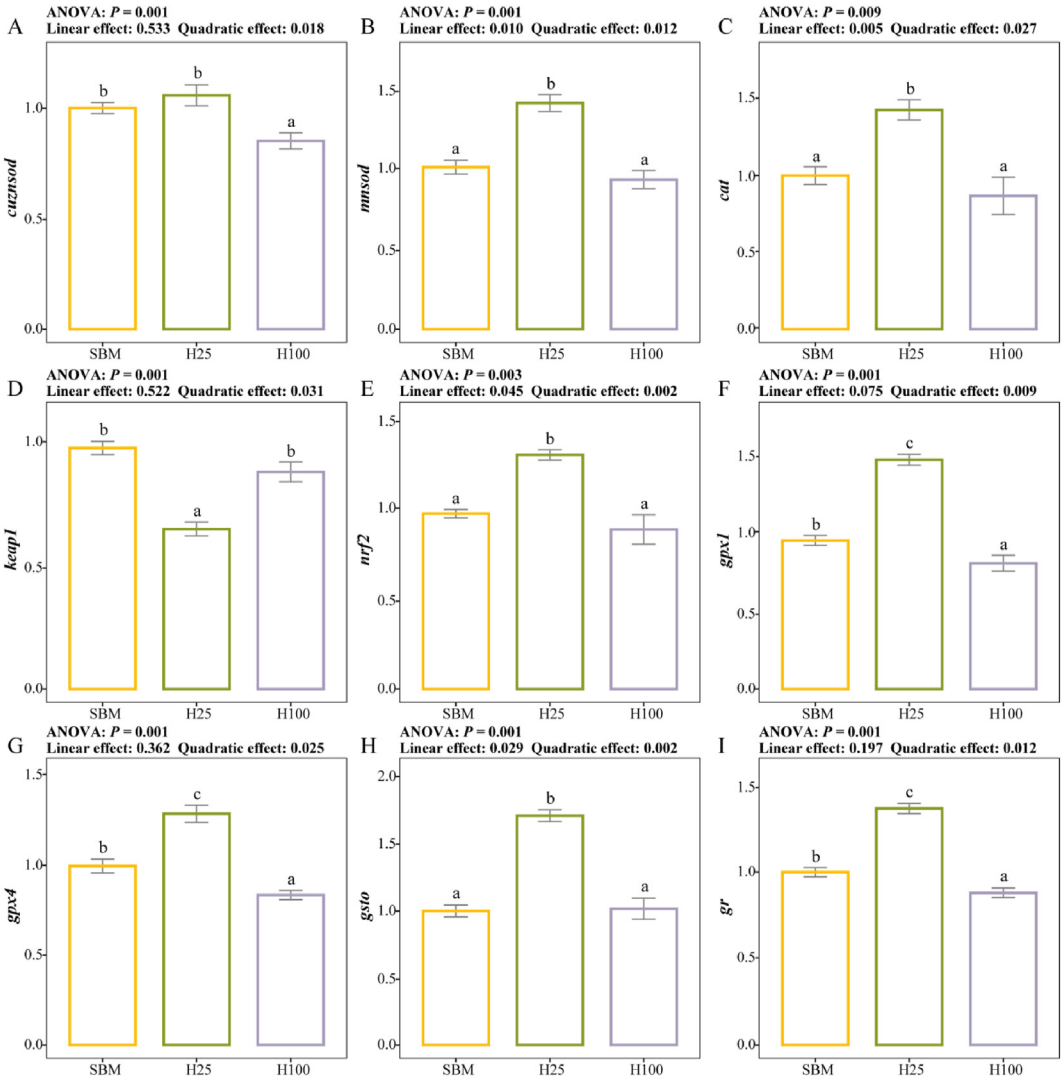

Fig. 1. Expression levels of genes in the liver of grass carp fed diets substituting SBM with YMM. YMM = yellow mealworm meal; SBM = soybean meal. SBM was substituted by YMM at 0% (SBM), 25% (H25) and 100% (H100), respectively. Different letters were used to illustrate the statistical differences ($P < 0.05$). (A) Copper/zinc superoxide dismutase (*cuznsod*); (B) manganese superoxide dismutase (*mnsod*); (C) catalase (*cat*); (D) kelch-like ECH-associated protein 1 (*keap1*); (E) nuclear factor erythroid 2-related factor 2 (*nrf2*); (F) glutathione peroxidase 1 (*gpx1*); (G) glutathione peroxidase 4 (*gpx4*); (H) glutathione S-transferase omega (*gsto*); (I) glutathione reductase (*gr*).

Table 8
Effect of substituting SBM with YMM on digestive enzymes activity in the intestine of grass carp after 8 weeks[1].

Item	Groups					*P*-value		
	SBM	H25	H50	H75	H100	ANOVA	Linear	Quadratic
Amylase, U/mg prot	36.13 ± 1.237^{c}	31.96 ± 1.300^{bc}	30.95 ± 0.393^{b}	25.52 ± 0.747^{a}	21.90 ± 0.595^{a}	0.001	0.012	0.020
Trypsin, U/mg prot	545.13 ± 29.176	536.41 ± 33.004	507.47 ± 47.160	550.61 ± 13.265	602.57 ± 15.291	0.333	0.154	0.082
Lipases, U/g prot	22.10 ± 0.337^{c}	18.05 ± 1.042^{b}	17.91 ± 1.041^{b}	14.13 ± 0.474^{a}	12.89 ± 0.350^{a}	0.001	0.002	0.297

YMM = yellow mealworm meal; SBM = soybean meal.
Different superscripts were used to show statistical differences at $P < 0.05$.
[1] SBM was substituted by YMM at 0% (SBM), 25% (H25), 50% (H50), 75% (H75) and 100% (H100), respectively.

a community sample. The Ace index was an index used to estimate the number of OTUs in a community and was also one of the common indexes used to estimate the total number of species. The Shannon index was calculated considering the total number of classifications in the sample and the proportion of each classification. The Simpson index is a measure of the diversity and evenness of species distribution in a community calculating the probability that two randomly sampled individuals belong to different species. The Ace, Chao1, Shannon, and Simpson indexes were not affected by the dietary treatment ($P > 0.05$). It meant that there was no significant change with YMM inclusion in operational taxonomic unit (OTU) diversity (Shannon and Simpson indexes) and richness (Ace and Chao1 indexes) ($P > 0.05$) (Fig. 4). The addition of YMM to the alpha diversity indexes had no significant effect ($P > 0.05$) (Fig. 4). There were 342 unique OTUs in the control group, 147 unique OTUs in the H25 group, 522 unique OTUs in the H100 group

Table 9
Effects of substituting SBM with YMM on the intestinal morphology of grass carp after 8 weeks[1].

Item	Groups					*P*-value		
	SBM	H25	H50	H75	H100	ANOVA	Linear	Quadratic
FH, μm	510.03 ± 24.414[b]	596.43 ± 6.941[c]	634.23 ± 41.329[c]	783.80 ± 4.401[d]	423.70 ± 9.102[a]	0.001	0.423	<0.001
MT, μm	206.60 ± 13.611[b]	213.93 ± 4.573[b]	205.80 ± 7.367[b]	176.37 ± 3.149[a]	171.57 ± 10.186[a]	0.022	0.003	0.109
GC, number of cells/villus	43.00 ± 3.211[b]	41.67 ± 0.877[b]	43.33 ± 2.851[b]	18.67 ± 1.204[a]	17.33 ± 0.333[a]	0.001	0.003	0.001

YMM = yellow mealworm meal; SBM = soybean meal; FH = fold height; MT = muscle thickness; GC = goblet cell.
Different superscripts were used to show statistical differences at $P < 0.05$.
[1] SBM was substituted by YMM at 0% (SBM), 25% (H25), 50% (H50), 75% (H75) and 100% (H100), respectively.

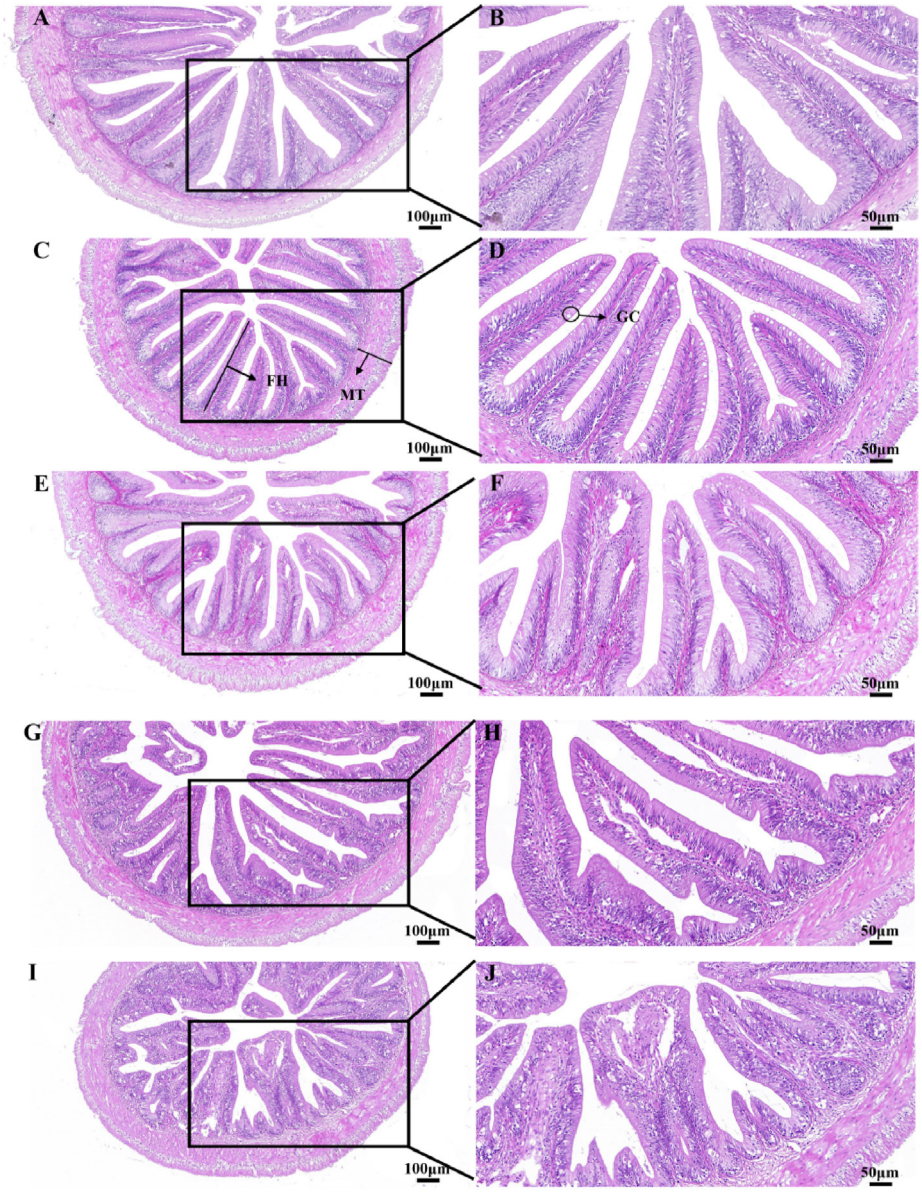

Fig. 2. Mucosa morphology of middle intestine. (A, C, E, G, and I) SBM, H25, H50, H75, and H100 groups (Magnification 100 ×), respectively. (B, D, F, H, and J) Partially enlarged images (magnification 200 ×). YMM = yellow mealworm meal; SBM = soybean meal; FH = fold height; MT = muscle thickness; GC = goblet cell. SBM was substituted by YMM at 0% (SBM), 25% (H25), 50% (H50), 75% (H75) and 100% (H100), respectively.

and 427 shared OTUs in the 3 groups (Fig. 5A). After merging, 1794 OTUs overall were found with a 97% similarity.

The findings demonstrated that the phyla Firmicutes, Bacteroidota, Spirochaetota, and Proteobacteria were mostly represented by the relative abundance of bacterial OTUs discovered in the gut microbiota. In the H25 group, *Spirochaetes* dramatically increased while *Proteobacteria* significantly lessened as compared to the SBM group ($P < 0.05$) (Fig. 5B). On the genus level, *Clostridium_sensu_stricto_1*, *Brevinema*, and *Bacteroides* accounted for the majority of OTUs. Compared to SBM diet, *Brevinema*,

Table 10
Effect of substituting SBM with YMM on intestinal antioxidant enzyme activities of grass carp after 8 weeks[1].

Item	Groups					*P*-value		
	SBM	H25	H50	H75	H100	ANOVA	Linear	Quadratic
ROS, U/mg	8.81 ± 0.115[b]	4.60 ± 0.194[a]	4.91 ± 0.188[a]	8.81 ± 0.607[b]	10.00 ± 0.141[c]	0.001	0.008	0.001
MDA, nmol/mg	15.57 ± 0.563[b]	8.88 ± 0.378[a]	9.89 ± 0.240[a]	15.01 ± 0.726[ab]	16.52 ± 0.743[b]	0.001	0.041	<0.001
SOD, U/mg	55.78 ± 1.679[b]	75.54 ± 2.042[d]	69.26 ± 0.987[c]	66.18 ± 1.712[c]	43.18 ± 0.966[a]	0.001	0.004	0.002
GPx, U/mg	107.66 ± 0.680[a]	117.81 ± 0.417[b]	111.11 ± 0.910[ab]	108.90 ± 0.767[a]	108.99 ± 0.893[a]	0.001	0.005	0.026
CAT, U/mg	5.96 ± 0.404[a]	12.02 ± 0.302[b]	10.77 ± 0.295[b]	6.73 ± 0.561[a]	5.03 ± 0.520[a]	0.001	0.006	0.003
GSH, μmol/mg	175.84 ± 3.361[a]	221.31 ± 2.450[b]	193.77 ± 3.459[a]	179.52 ± 1.968[a]	171.67 ± 0.999[a]	0.001	0.018	0.012

YMM = yellow mealworm meal; SBM = soybean meal; ROS = reactive oxygen species; MDA = malondialdehyde; SOD = superoxide dismutase; GPx = Glutathione peroxidase; CAT = catalase; GSH = Glutathione.
Different superscripts were used to show statistical differences at $P < 0.05$.
[1] SBM was substituted by YMM at 0% (SBM), 25% (H25), 50% (H50), 75% (H75) and 100% (H100), respectively.

Lactobacillus and *Fusobacterium* significantly improved in the H25 group ($P < 0.05$), and *Lactobacillus* significantly increased in the H100 group ($P < 0.05$) (Fig. 5C).

4. Discussion

As known, the EAA (methionine and lysine) in SBM are limited and cannot meet the needs of fish, which is a restraint on fish growth. Conversely the two amino acids in YMM are higher. The content of EAA (isoleucine, leucine, lysine, methionine, threonine, valine) in the diets of YMM treatment groups were higher than that in the control group. This maybe one of the main reasons why grass carp's growth performance was improved when dietary SBM was replaced by 25% YMM in the current experiment. Similar results were also found in mirror carp (Li et al., 2022a). There were adverse consequences observed on growth performance when the SBM was completely replaced. Chitin content in YMM varies from 3.8% to 6.8% (Janssen et al., 2017). High concentration of chitin in diet was poorly digested by several fish species, leading reduced lipid absorption and fat digestion, thus resulting in an inferior growth rate (Adeniyi and Folorunsho, 2015). The growth performance of common carp (*Cyprinus carpio*) and Nile tilapia (*Oreochromis niloticus*) was negatively affected by adding 1 to 2% chitin (Gopalakannan and Arul, 2006; Shiau and Yu, 1999). Similar to previous studies, complete substitution of soybean meal by black soldier fly had a negative effect on grass carp growth performance (Hu et al., 2023). The decreased fat utilization might partially account for the impaired growth performance in the H100 group. Previous studies indicated substitution of SBM by YMM without effect on growth in grass carp (Li et al., 2023a). This discrepancy may be related to different growth periods of the fish and different insect meal processing methods.

The changes of serum biochemical indexes reflect the nutritional metabolism and physiological balance of the fish, which is regarded as a useful measure for assessing the effects of nutritional factors on the health status of various fish species (Abdel et al., 2020). IgM is a kind of globulin with antibody activity, which is an important part of the specific immune system as a class of immunoactive molecules (Gong et al., 2021). C3 and C4 are important protein factors in the immune response, which produces biological effects such as cytolysis, adhesion, immune regulation, neutralization of toxins and removal of pathogens when activated (Copenhaver et al., 2018). The findings of this research revealed replacing 25 to 75% of SBM with YMM increased IgM and C3 contents, consistent with results for large yellow croakers (*Larimichthys crocea*) (Zhang et al., 2022a). Furthermore, AKP and ACP are important as component of phagocytic lysosomes, which have anti-inflammatory and immunomodulatory actions through effecting a decrease in neutrophil production (Lallès, 2019). In present study, ACP and AKP activities were enhanced to varied degrees in H25, which was consistent with results in Pacific white shrimp (*Litopenaeus vannamei*) (He et al., 2022). Nevertheless, the complete substitution of SBM by YMM had no significant effect on serum immunity of grass carp, which was different from the study of fresh black soldier fly in Pacific white shrimp. This may be caused by the difference in fat levels between the fresh black soldier fly and YMM (Zhang et al., 2013). It was reported that the antimicrobial peptides and fibrinolytic protein in YMM was involved in the modulation of the immune system (Huang et al., 2012; Shafique et al., 2021). These findings indicated moderate addition of YMM leading to an improvement in the immune function of the grass carp.

Fish have an innate antioxidant defense system, consisting of antioxidant enzymes and non-enzymatic antioxidants. However, when the antioxidant defense is overwhelmed, or aquatic animals are under prolonged stress, this equilibrium is easily upset and oxidative stress occurs, compromising tissue function (Lushchak, 2016). SOD and CAT act on superoxide free radical and hydrogen peroxide respectively, and the variation of their activity imply the dynamic shift in the free radical response and tissue injury *in vivo*. MDA is the end product of fatty acid peroxidation, and its concentration can be measured to figure out the level of lipid oxidation. MDA and ROS are oxidative stress indicators (Shi et al., 2020). In addition, GSH and GPx are crucial in the process of mitigating oxidative damage (Dinu et al., 2020). In the current study, ROS and MDA contents were significantly increased, and antioxidant-related enzymatic activities and gene mRNA expression levels were significantly reduced in the livers of grass carp in the H100 group, suggesting the occurrence of oxidative stress. Simultaneously, it was accompanied by the increased serum GOT and GPT, which have always been used as effective indicators to reflect the degree of liver damage in fish (Liang et al., 2022). Previous studies have found a high proportion of YMM replacing SBM causes the feed to be more prone to rankness, which may eventually lead to increased oxidative stress (Zhang et al., 2023). Due to the presence of multi-unsaturated bonds, polyunsaturated fatty acids are readily susceptible to lipid peroxidation when exposed to higher temperatures and humid environments. A series of toxic products including lipid hydroperoxides, volatile organic acids, ketones, aldehydes, hydrocarbons, and epoxides were generated. These metabolites contribute to oxidative stress, negatively influencing the nutritive value of the feed and the health status of the fish (Zuzanna et al., 2020). The increased deposition of unsaturated fatty acid in the liver after high YMM diets maybe also increase oxidative stress. This study found that liver SOD, GPx, CAT activities and GSH content antioxidant-related gene (*cat*, *mnsod*, *gpx1*, *gpx4*, *gsto* and *gr*) expression increased in the H25 group. Similar findings in previous research argued that the addition of YMM to a meal boosts antioxidant capacity (Henry et al., 2018; Zhang et al., 2022b). This effect

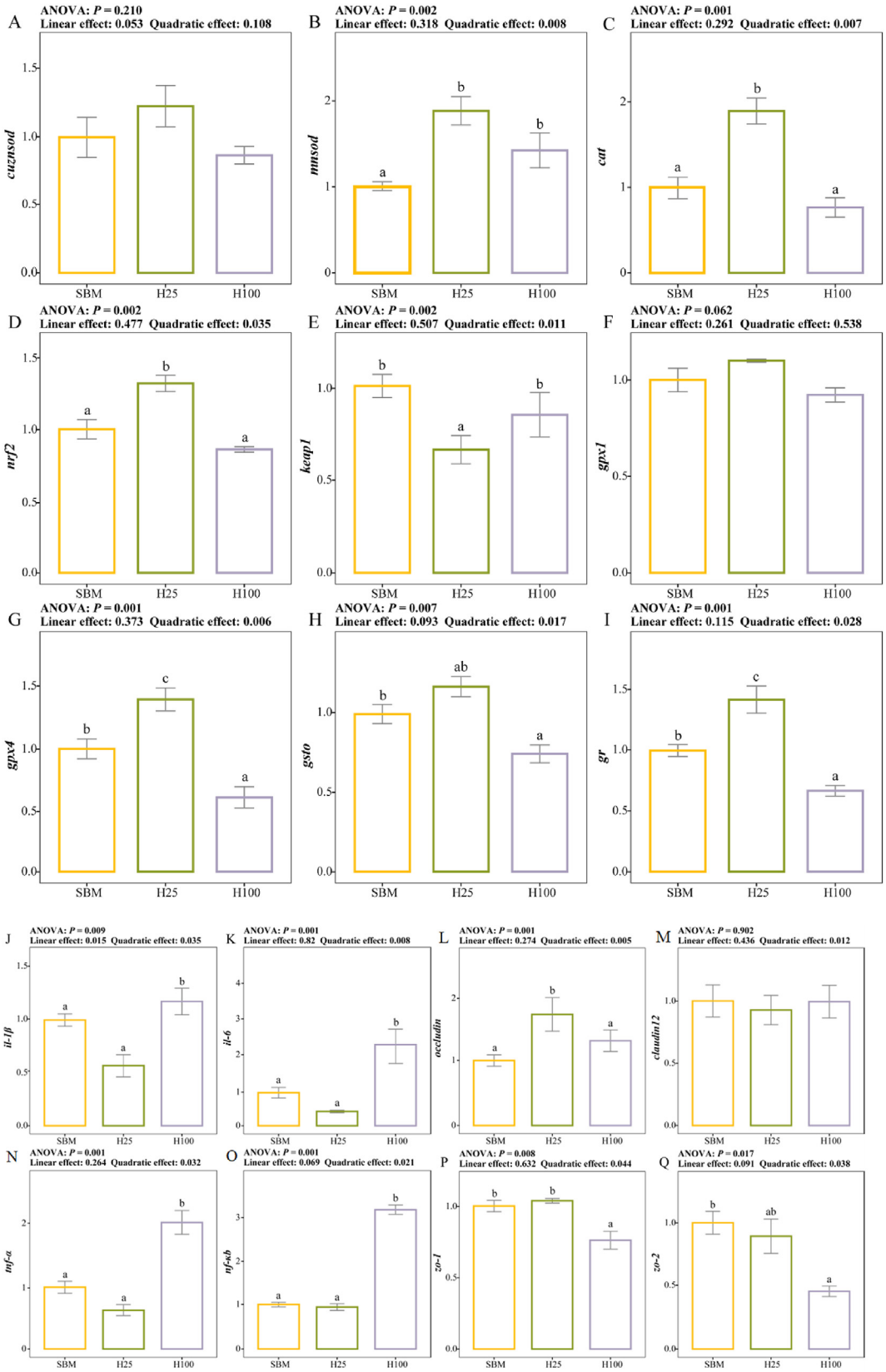

Fig. 3. Expression levels of genes in the intestines of grass carp fed diets substituting SBM with YMM. YMM = yellow mealworm meal; SBM = soybean meal. (A) Copper/zinc superoxide dismutase (*cuznsod*); (B) manganese superoxide dismutase (*mnsod*); (C) catalase (*cat*); (D) nuclear factor erythroid 2-related factor 2 (*nrf2*); (E) kelch-like ECH-associated protein 1 (*keap1*); (F) glutathione peroxidase 1 (*gpx1*); (G) glutathione peroxidase 4 (*gpx4*); (H) glutathione S-transferase omega (*gsto*) (I) glutathione reductase (*gr*); (J) interleukin-1β (*il-1β*); (K) interleukin-6 (*il-6*); (L) occludin; (M) claudin12; (N) tumor necrosis factor alpha (*tnf-α*); (O) nuclear factor kappa-B (*nf-κb*); (P) zonula occludens-1 (*zo-1*); (Q) zonula occludens-2 (*zo-2*). Different letters were used to illustrate the statistical differences ($P < 0.05$). SBM was substituted by YMM at 0% (SBM), 25% (H25) and 100% (H100), respectively.

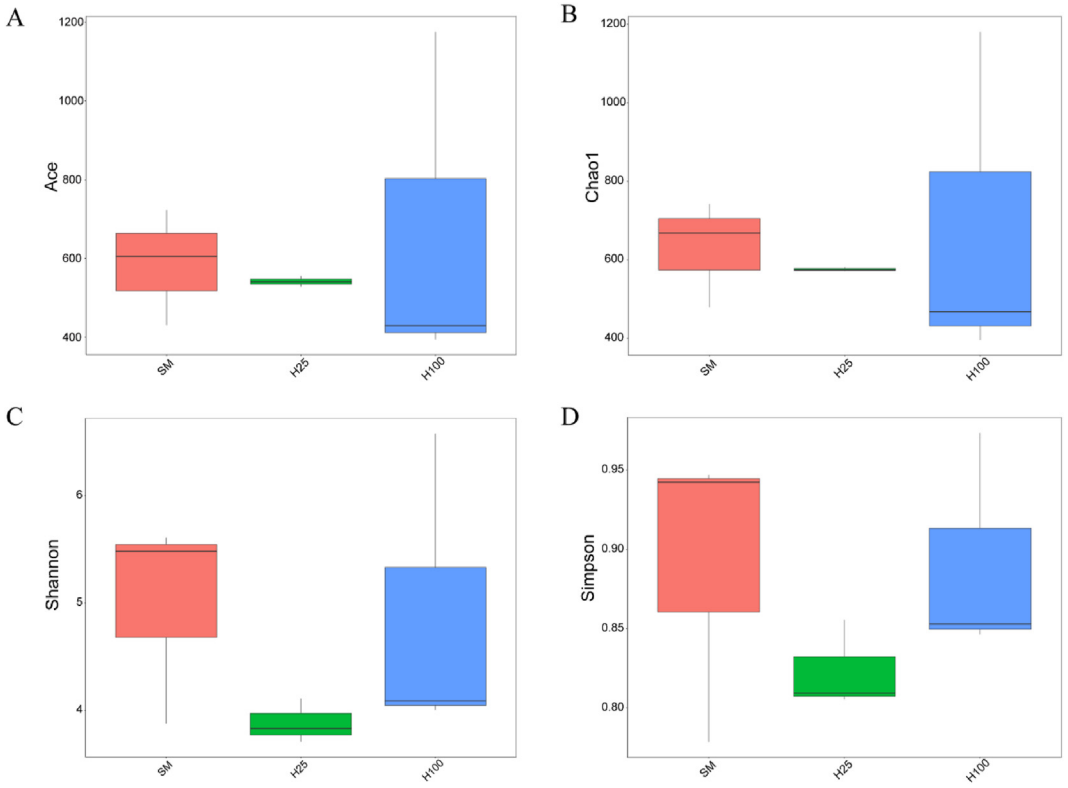

Fig. 4. Intestinal bacterial diversity richness and diversity of grass carp with different levels of YMM substitution for SBM and box plots showing Ace (A), Chao1 (B), Shannon (C), and Simpson richness and diversity indices (D). YMM = yellow mealworm meal; SBM = soybean meal. The wilcox rank sum test was used to analyze the difference between groups. No letters were used to illustrate no statistical differences ($P > 0.05$). SBM was substituted by YMM at 0% (SBM), 25% (H25) and 100% (H100), respectively.

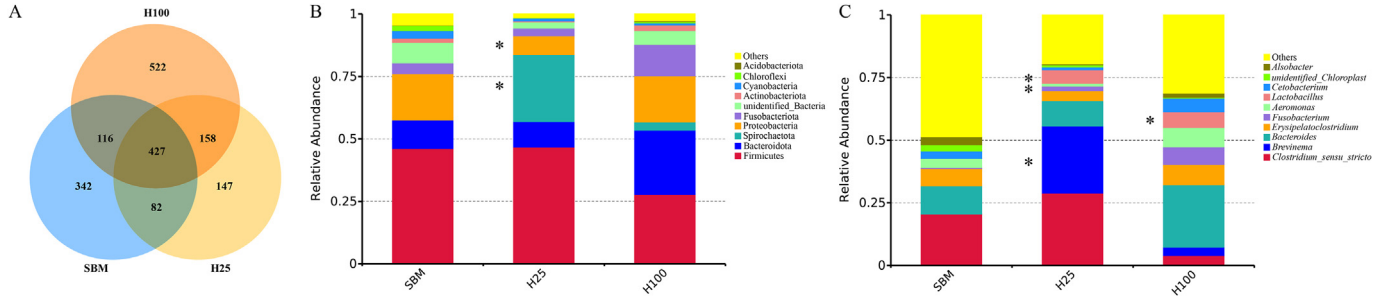

Fig. 5. The unique and shared OTUs (A) and the average relative abundance of bacterial OTUs at phylum (B) and genus level (C) in gut of grass carp. OTUs = operational taxonomic units; YMM = yellow mealworm meal; SBM = soybean meal. The asterisk (*) denotes a considerable deviation from the SBM group ($P < 0.05$). SBM was substituted by YMM at 0% (SBM), 25% (H25) and 100% (H100), respectively.

may be related to the flavonoid and chitin content of YMM which have been known to be potential antioxidants (Reihaneh and Pooran, 2023). The antioxidant enzyme activity and gene expression of grass carp were decreased in the H100 group, in contrast to the study of black soldier fly completely replacing fish meal in snakehead (*Channa striata*) (Siddaiah et al., 2023). This may be due to differences in antioxidant capacity between herbivorous and carnivorous fish. Under homeostatic condition, the master antioxidant transcriptional factor, nuclear factor-erythroid 2-related factor 2 (Nrf2) is maintained in the cytoplasm by *keap1*. Upon excess oxidative stress, Keap1 is degraded and allows *nrf2* to translocate to the nucleus to upregulate the transcription of downstream antioxidant targets, reinforcing the intracellular antioxidant capacity (Wu et al., 2022). To further characterize the mechanism underlying the oxidative modulation effect of YMM, we examined the responses of the Nrf2 signaling pathway to the YMM addition. The *keap1* mRNA level was down-regulated and the *nrf2* mRNA level was up-regulated in the H25 group, whereas opposite results were observed in the H100 group. The variations within the Keap1/Nrf2 signaling pathway were in accordance with the mRNA results for antioxidant related genes. Therefore, we speculated that the oxidative modulation effect of YMM was conferred through regulating Keap1/Nrf2 signaling pathway.

Fish growth is intimately tied to the digestive system's ability to break down and absorb nutrients, which is influenced by digestive enzyme activity, gut absorption area and structural integrity (Niklasson et al., 2011). In the present study, YMM had no effect on intestinal trypsin activity of grass carp, but 100% substitution was shown to reduce the activities of amylase and lipase. However, Hoffmann et al. found no considerable differences in the intestinal

enzyme activities in sea trout (*Salmo trutta*) fed diets supplemented with YMM (Hoffmann et al., 2020). Mucus released by intestinal goblet cells serves as the initial line of defense against pathogens and feed antigens, and its integrity is crucial for maintaining tissue homeostasis (Sahlmann et al., 2013). In the H100 group, the intestinal fold height, muscle thickness and amount of goblet cells was significantly reduced, and the intestinal folds showed breakage. The observation was similar in the results on turbot fed diets with a YMM replacement level of 60% to 75% (Bai et al., 2023). The lessened nutrient availability related to reduced intestinal digestive enzyme activity, reduced absorption area and breakages in the structure of the intestinal folds were additional important reasons for the decreased growth performance of the H100 group.

The epithelium of the intestinal mucosa acts as a physical barrier by forming tight connections that prevent pathogens from entering. Therefore, tight junctions (TJs) and enterocyte structural integrity are fundamental components of intestinal barrier function. TJs are structural connection proteins between intestinal epithelial cells, comprised of integral transmembrane proteins (occludin, tricelluin, claudins, and junctional adhesion molecule) and cytosolic scaffold proteins such as zonula occludens proteins (Deluco et al., 2021). Occludin is essential in the upkeep and assembly of TJs. Zonula occludens-1 oversees attaching these transmembrane proteins to the actin cytoskeleton to give structural support (Chen et al., 2022). In the current study, high substitution levels of YMM in the diet caused intestinal epithelial barrier dysfunction as demonstrated by the decreased mRNA expression of the tight junction proteins in the H100 group. Similar findings were obtained with black soldier fly treatment in golden pompano (*Trachinotus ovatus*) (Li et al., 2023b). Under the condition of oxidative stress, the special structure of intestinal mucosa and the convective exchange mechanism of oxygen determines that it is more prone to oxidative damage, accompanied by mucosal permeability increasing, electrolyte secretion, and intestinal epithelial morphology and function abnormalities (Kamal et al., 2017). Indeed, markers of oxidative stress were observed in the intestines of the H100 group. SOD activity and antioxidant gene expression (*gpx4*, *gsto*, *gr*) was decreased and ROS content was boosted in the H100 group. Therefore, the observed intestinal mucosal barrier dysfunction might have been induced by the oxidative stress caused by high levels of YMM.

The gut also participates in the process of immune regulation. Cytokines act as crucial roles in evoking the inflammatory responses. Previous research demonstrated an increase in pro-inflammatory cytokines and a decrease in anti-inflammatory cytokines exacerbating inflammation and disrupting intestinal health (Tian et al., 2017). NF-κB mediates the inflammatory response by recruiting and activating immune cells and regulating transcription of other cytokines. This study showed that *nf-κb*, *il-β*, *il-6* and *tnf-α* were upregulated in the H100 group, demonstrating the activation of the inflammation response. A similar trend was observed in channel catfish (*Lctalurus punctatus*) fed diets including cricket meal (*Gryllus bimaculatus*) (Fan et al., 2023). In normal physiological states, Nrf2 and NF-κB signaling pathways coordinate to maintain cell homeostasis. Under conditions of oxidative stress, complex molecular mechanisms (transcriptional and post-transcriptional) link Nrf2 and NF-κB in a cell-type dependent manner. Nrf2 inhibits NF-κB by competing for the transcriptional co-activator CBP (CREB-binding protein)—p300 complex. Abrogation of Nrf2 increases NF-κB activity leading to increased cytokine production (Li et al., 2019). Thus, the activation of inflammation response mediated through NF-κB signaling pathway in the H100 group may be related to suppressed Nrf2 expression.

Intestinal microorganisms can impact the physiological, nutritional, immunological, and metabolic functions of the host organism (Hong et al., 2020; Huang et al., 2022). Our study revealed that YMM had no significant impact on alpha diversity indexes of the gut microbiome in grass carp, consistent with European perch (*Perca fluviatilis*) (Tran et al., 2022). Nevertheless, the relative abundances of bacterial phyla and genera fluctuated during dietary interventions. In our study, the major phyla in the gut of grass carp were Firmicutes, Bacteroidota, Spirochaetota, and Proteobacteria. *Clostridium_sensu_stricto_1*, *Brevinema*, and *Bacteroides* were the major genera of bacteria. It was discovered that the growth performance was favorably connected with the ratio of Firmicutes and Bacteroidota (Fan and Li, 2019). The proportion of Firmicutes and Bacteroidota as well as the growth performance of grass carp declined in the H100 group where YMM totally replaced SBM. In addition, the abundances of Firmicutes and Bacteroidota also differed with the change of antioxidant capacity in channel catfish (Yang et al., 2023). The H100 groups showed an increase in the abundance of the harmful gram-positive bacteria genus *Brevinema*. A previous study discovered that the content of *Brevinema* increases with intestinal oxidative damage of largemouth bass (*Micropterus salmoides*) (Wu et al., 2023). Under oxidative stress, reactive oxygen species can directly act on intestinal epithelial cells, leading to lipid peroxidation in the phospholipid layer of cell membrane. Many harmful substances destroy the bacterial environment, and eventually lead to the imbalance of gut microbiota structure (Muccioli et al., 2010). The relative abundances of Proteobacteria significantly decreased and Spirochaetota increased and in the H25 group compared to SBM, which proved to decrease of pathogenic bacteria (Reuvers et al., 2023). Previous studies have shown that grass carp observed similar changes in gut microbiota during oxidative stress (Shi et al., 2023; Li et al., 2023c). This suggested that oxidation capacity may affect the intestinal flora composition of grass carp, which may warrant further study to elucidate a mechanism. Finally, excessive replacement of SBM with YMM may cause intestinal flora disturbance of grass carp, which was unfavorable to growth.

5. Conclusions

In summary, substitution of dietary SBM with up to 25% YMM was beneficial for growth performance, whereas complete substitution negatively affected growth performance. High amounts of YMM caused oxidative stress accompanied by liver function disorder, and aggravated intestinal inflammation, damaging the intestinal mucosal barrier, ultimately depressing growth performance.

Credit Author Statement

Linlin Yang: Conceptualization, Methodology, Writing – original draft. **Minglang Cai:** Software. **Lei Zhong:** Writing – review & editing. **Yulong Yin:** Methodology. **Yonghong Xie:** Funding acquisition. **Shouqi Xie:** Writing – review & editing. **Yi Hu:** Funding acquisition. **Junzhi Zhang:** Writing – review & editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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