Effects of Clostridium autoethanogenum Protein as a Replacement for Soybean Meal on Growth Performance, Plasma Biochemical Indices, and Hepatopancreatic and Intestinal Histopathology in Grass Carp (Postprint)

Authors: Wei Hongcheng, Yu Huanhuan, Chen Xiaoming, Chao Wei, Zou Fangqi, Chen Pei, Zheng Yinhuai, Wu Xiufeng, Liang Xufang, Xue Min

Date: 2018-12-25T00:00:00+00:00

Abstract

This study aimed to investigate the effects of a novel protein source, ethanol clostridial protein, as a replacement for soybean meal on growth performance, plasma biochemical indices, and hepatopancreatic and intestinal histopathology in grass carp. A total of 540 grass carp with an initial body weight of (25.70±0.03) g were selected and randomly divided into 3 groups with 6 replicates per group and 30 fish per replicate. The control group (group J0) was fed a basal diet, while the experimental groups were fed diets in which 5% (group J5) and 10% (group J10) ethanol clostridial protein replaced 27.5% and 55.0% of the soybean meal in the basal diet, respectively. The experimental period lasted 10 weeks. The results showed: 1) The survival rate of grass carp in group J5 was significantly higher than that in group J10 ($P<0.05$), with no significant difference from group J0 ($P>0.05$). The weight gain rate of grass carp in group J5 was significantly higher than that in group J0 ($P<0.05$), and the feed conversion ratio was significantly lower than that in group J0 ($P<0.05$). 2) The plasma total cholesterol (TC) content in groups J5 and J10 was significantly lower than that in group J0 ($P<0.05$); the plasma glucose content in group J10 was significantly higher than that in group J0 ($P<0.05$); the plasma aspartate aminotransferase (AST) activity in group J10 was significantly higher than that in group J5 ($P<0.05$), and that in group J5 was significantly higher than that in group J0 ($P<0.05$); the plasma alanine aminotransferase (ALT) activity in group J10 was significantly lower than that in groups J0 and J5 ($P<0.05$); the plasma AST/ALT ratio in group J10 was significantly higher than that in groups J0 and J5 ($P<0.05$); the plasma malondialdehyde (MDA) content in group J5 was significantly lower than that in groups J0 and J10 ($P<0.05$). There

were no significant differences in plasma immunoglobulin M (IgM), interleukin-8 (IL-8), and interleukin-1 (IL-1) contents among all groups (P>0.05). 3) The whole-body moisture content in group J10 was significantly higher than that in group J5 (P<0.05). There were no significant differences in whole-body crude ash, crude protein, and crude fat contents among all groups (P>0.05). 4) Histopathological sections of the hepatopancreas and intestine revealed that with increasing ethanol clostridial protein supplementation, nuclear aggregation in the hepatopancreas of grass carp in the experimental groups was exacerbated, while intestinal lesions were improved compared with the control group. It can be concluded that dietary supplementation with 5% ethanol clostridial protein is beneficial for the growth performance of grass carp, but increasing the supplementation level to 10% adversely affects growth performance, reduces survival rate, and causes liver damage. Therefore, the recommended inclusion level of ethanol clostridial protein in grass carp diets is 5%.

Full Text

Effects of Soybean Meal Replacement by *Clostridium autoethanogenum* Protein on Growth Performance, Plasma Biochemical Indexes, and Hepatopancreas and Intestinal Histopathology of Grass Carp (*Ctenopharyngodon idellus*)

WEI Hongcheng[1], YU Huanhuan[1], CHEN Xiaoming[1], CHAO Wei[2], ZOU Fangqi[2], CHEN Pei[1], ZHENG Yinhua[1], WU Xiufeng[1], LIANG Xufang[3], XUE Min[1], *

[1]National Aquatic Feed Safety Assessment Center, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China
[2]Beijing Shoulang Bio-Technology Co., Ltd., Beijing 100041, China
[3]College of Fisheries, Huazhong Agricultural University, Wuhan 430070, China
Key Laboratory of Feed Biotechnology, Ministry of Agriculture, Beijing 100081, China

Abstract: This experiment was conducted to investigate the effects of replacing soybean meal with a novel protein source—*Clostridium autoethanogenum* protein—on growth performance, plasma biochemical indexes, and hepatopancreas and intestinal histopathology in grass carp. A total of 540 grass carp with an initial body weight of (25.70±0.03) g were randomly divided into three groups, each with six replicates of 30 fish. The control group (J0) was fed a basal diet, while the experimental groups were fed diets in which 5% (J5) and 10% (J10) *Clostridium autoethanogenum* protein replaced 27.5% and 55.0% of the soybean meal in the basal diet, respectively. The trial lasted for 10 weeks. The results showed: (1) The survival rate of grass carp in the J5 group was significantly higher than that in the J10 group (P<0.05) but did not differ significantly from the J0 group (P>0.05). The weight gain rate of grass carp in the J5 group was significantly higher than that in the J0 group (P<0.05),

and the feed conversion ratio was significantly lower ($P<0.05$). (2) Plasma total cholesterol (TC) content in the J5 and J10 groups was significantly lower than in the J0 group ($P<0.05$). Plasma glucose content in the J10 group was significantly higher than in the J0 group ($P<0.05$). Plasma aspartate aminotransferase (AST) activity in the J10 group was significantly higher than in the J5 group ($P<0.05$), which in turn was significantly higher than in the J0 group ($P<0.05$). Plasma alanine aminotransferase (ALT) activity in the J10 group was significantly lower than in the J0 and J5 groups ($P<0.05$). The plasma AST/ALT ratio in the J10 group was significantly higher than in the J0 and J5 groups ($P<0.05$). Plasma malondialdehyde (MDA) content in the J5 group was significantly lower than in the J0 and J10 groups ($P<0.05$). No significant differences were observed in plasma immunoglobulin M (IgM), interleukin-8 (IL-8), or interleukin-1 (IL-1) contents among all groups ($P>0.05$). (3) Whole-body moisture content in the J10 group was significantly higher than in the J5 group ($P<0.05$), though no significant differences were found in whole-body crude ash, crude protein, or crude lipid contents among groups ($P>0.05$). (4) Histopathological examination of the hepatopancreas and intestine revealed that as the inclusion level of *Clostridium autoethanogenum* protein increased, nuclear aggregation in the hepatopancreas intensified, while intestinal lesions improved compared to the control. These findings indicate that dietary supplementation with 5% *Clostridium autoethanogenum* protein benefits growth performance in grass carp, whereas increasing the level to 10% adversely affects growth, reduces survival, and causes liver damage. Therefore, the recommended inclusion level of *Clostridium autoethanogenum* protein in grass carp diets is 5%.

Keywords: *Clostridium autoethanogenum* protein; grass carp; soybean meal; growth; histopathology

1. Introduction

China is a major aquaculture producer, with grass carp (*Ctenopharyngodon idellus*) ranking first in production volume, exceeding 5 million tons annually [1]. As a herbivorous species, grass carp utilizes minimal amounts of animal-based proteins such as fishmeal in formulated feeds, relying primarily on plant protein sources including soybean meal, cottonseed meal, and rapeseed meal. Compared to cottonseed and rapeseed meals, soybean meal is most widely used due to its high availability, good palatability, and high digestibility, as the enzymatic digestion capacity of grass carp' s intestinal hepatopancreas for soybean meal surpasses that for other plant proteins [2-3]. However, numerous studies have demonstrated that plant proteins present challenges for fish, including imbalanced essential amino acids [4], presence of antinutritional factors (such as urease and trypsin inhibitors) [5-6], and inconsistent product quality [7-9]. Soybean meal contains protease inhibitors and soy lectins that inhibit digestive enzyme activity in fish [10-11], and excessive use can be detrimental to aquatic animal growth and health. Although fermentation, physical processing,

multi-protein source blending, and exogenous amino acid supplementation can partially compensate for these deficiencies, reports indicate that for herbivorous fish, intestinal and hepatopancreatic lesions caused by excessive soybean meal remain difficult to resolve effectively [12-13]. Therefore, identifying non-animal-derived feed protein sources with low antinutritional factor content, such as single-cell proteins, is crucial for maintaining digestive system health in herbivorous fish.

With the gradual application of microbial fermentation technology in feed industry production, several countries have established microbial biomass protein (MBP) industries. MBP is characterized by high protein content, relatively complete amino acid profiles, and abundant vitamins and trace elements [14], while also imparting umami flavor to improve feed palatability [15]. Additional advantages include high production efficiency, wide availability of raw materials, and suitability for industrial-scale production, with considerable portions already being applied in livestock and aquaculture feeds [16]. However, MBP quality is largely affected by non-protein nitrogen content, and some products exhibit poor quality. For instance, monosodium glutamate bacterial protein suffers from low true protein content, amino acid imbalance, and low amino acid digestibility [17].

Clostridium autoethanogenum protein is a novel bacterial protein produced through fermentation using *Clostridium autoethanogenum* as the strain. First isolated from rabbit feces by Abrini et al. [18], this bacterium utilizes carbon monoxide (CO) as a raw material in liquid fermentation. The fermentation broth is centrifuged and dried to obtain a high-protein biological feed ingredient. Currently, no clear evaluation data exist for *Clostridium autoethanogenum* protein in aquaculture feeds. Therefore, this study designed a gradient replacement trial to investigate its effects on growth performance, plasma biochemical indexes, and hepatopancreas and intestinal histopathology in grass carp, providing a reference for its application in aquafeeds.

1.1 Experimental Materials

The *Clostridium autoethanogenum* protein used in this experiment was a light yellow powder provided by Beijing Shoulang Bio-Technology Co., Ltd., containing 84.69% crude protein with a pepsin digestibility of 90.2%.

1.2 Experimental Design and Diets

A total of 540 grass carp with an initial body weight of (25.70±0.03) g were randomly divided into three groups, each consisting of six replicates of 30 fish. The control group (J0) received a basal diet, while experimental groups received diets in which 5% (J5) and 10% (J10) *Clostridium autoethanogenum* protein replaced 27.5% and 55.0% of the soybean meal in the basal diet, respectively.

Soybean meal is deficient in methionine but rich in arginine, whereas *Clostridium autoethanogenum* protein shows the opposite pattern. Consequently, as

the inclusion level of *Clostridium autoethanogenum* protein increased, dietary DL-methionine (DL-Met) supplementation gradually decreased while L-arginine (L-Arg) supplementation increased. Feed ingredients were mixed sequentially according to inclusion levels from low to high, and processed into 2 mm extruded floating pellets using a twin-screw extruder (TSE65 type, Beijing Xiandai Yanggong Machinery Technology Development Co., Ltd.). All three diets were isonitrogenous, isoenergetic, and contained essential amino acids meeting grass carp requirements [19]. Dietary composition and nutrient levels are presented in Table 1 , and amino acid composition is shown in Table 2 .

1.3 Feeding Management

Prior to the formal experiment, fish were acclimated in the culture system for two weeks and fed the control diet during this period. The trial was conducted at the Beijing Nankou National Aquatic Feed Safety Assessment Center using an indoor recirculating aquaculture system with aerated well water at a flow rate of 9.64 L/min. After disinfecting the fish and system with 2% salt water, healthy and uniformly sized grass carp were randomly stocked into conical culture tanks (0.26 m^3 volume). The experiment ran from September 26 to December 4, 2016, lasting 10 weeks.

During the trial, fish were hand-fed to apparent satiation four times daily at 08:00, 11:00, 15:00, and 19:00. Water quality was monitored regularly, maintaining dissolved oxygen >7.0 mg/L, total ammonia nitrogen <0.3 mg/L, pH 7.5-8.5, water temperature at (22±1)°C, and natural photoperiod.

1.4 Sample Collection and Analysis

1.4.1 Sample Collection At the start of the experiment, 12 fish were sampled as initial whole-body samples, with every four fish pooled as one composite sample. At the end of the trial, fish in each tank were fasted for 24 hours and weighed. Three fish per tank were sampled as final whole-body samples, and an additional 3-4 fish per tank were randomly selected, anesthetized with 80 mg/L chlorobutanol (Shanghai Guoyao Group Chemical Reagent Co., Ltd.), and measured for body length (from snout to vertebral column end) and body weight. Blood was collected from the caudal vein into tubes containing 30 L/mL anticoagulant (2% NaF + 4% potassium oxalate), centrifuged at 4,000 r/min for 10 min at 4°C, and plasma was stored at -80°C for subsequent analysis. Hepatopancreas (bile duct connection site) and posterior intestine (10 mm from anus) were sampled from two random fish per tank, fixed in 4% paraformaldehyde for 24-48 hours, dehydrated, embedded, sectioned serially at 6 m, dewaxed, stained with hematoxylin-eosin (HE), and examined microscopically.

1.4.2 Analytical Methods Moisture, crude protein, crude lipid, crude ash, and pepsin digestibility in ingredients, diets, and fish were determined using 105°C atmospheric drying (GB/T 6435–2006), Kjeldahl method (GB/T 6432–

1994), acid hydrolysis crude fat determination (GB/T 6433–2006), 550°C incineration (GB/T 6438–2007), and filtration method (GB/T 17811–2008), respectively. Gross energy was measured using an oxygen bomb calorimeter according to ISO-9831:1998 [21]. Amino acid content in diets was analyzed following national standard methods (GB/T 18246–2000). All samples were analyzed in at least duplicate.

Plasma biochemical indexes included total cholesterol (TC), triglycerides (TG), glucose (Glu), immunoglobulin M (IgM), interleukin-8 (IL-8), interleukin-1 (IL-1), malondialdehyde (MDA), and activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total superoxide dismutase (T-SOD). TC and TG assay kits were purchased from Zhejiang Dongou Diagnostic Products Co., Ltd., while other kits were from Nanjing Jiancheng Bioengineering Institute. Analyses were performed using an enzyme-linked immunosorbent assay reader (Bio-Tek, Burlington, USA).

Growth performance and morphological indexes were calculated as follows:

- Survival rate (SR, %) = 100 × Nt/N0
- Weight gain rate (WGR, %) = 100 × (Wt - W0 + Wd)/W0
- Specific growth rate (SGR, %/d) = 100 × [ln(FBW) - ln(IBW)]/t
- Feed conversion ratio (FCR) = C/(Wt + Wd - W0)
- Feeding rate (FR, %) = 100 × C/[(W0 + Wt + Wd)/2]/t
- Protein productive value (PPV, %) = 100 × (Wt × Bpt - W0 × Bp0)/(C × Dp)
- Condition factor (CF, %) = 100 × average body weight (g)/[average body length (cm)]^3
- Viscera-somatic index (VSI, %) = 100 × viscera weight (g)/whole body weight (g)
- Hepato-somatic index (HSI, %) = 100 × liver weight (g)/whole body weight (g)
- Spleen-somatic index (SSI, %) = 100 × spleen weight (g)/whole body weight (g)

Where: Nt = final fish number; N0 = initial fish number; IBW = initial mean body weight (g); FBW = final mean body weight (g); W0 = initial total body weight (g); Wt = final total body weight (g); Wd = total weight of dead fish (g); C = feed intake (g); Bpt = final body protein content (%); Bp0 = initial body protein content (%); Dp = dietary protein content (%); t = feeding days.

1.5 Statistical Analysis

Data are expressed as mean ± standard error (SE). All data were subjected to one-way ANOVA using SPSS 20.0 software, with Duncan' s multiple range test used to examine significant differences. The significance level was set at $P<0.05$.

2. Results

2.1 Effects of Soybean Meal Replacement by *C. autoethanogenum* Protein on Growth Performance and Physical Indices

The effects of soybean meal replacement by *C. autoethanogenum* protein on growth performance and physical indices are presented in Table 3 . Final mean weight, weight gain rate, and specific growth rate in the J5 group were significantly higher than those in the control group ($P<0.05$). The J10 group showed lower values than J5 but higher than J0 for these parameters, though differences were not significant ($P>0.05$). Both J5 and J10 groups had significantly lower feed conversion ratios than the control ($P<0.05$), with J5 showing the lowest value. Although feeding rate did not differ significantly among groups ($P>0.05$), it tended to increase with higher *C. autoethanogenum* protein inclusion. No significant differences were observed in protein productive value among groups ($P>0.05$). Survival rate in the J10 group was significantly lower than in the control group ($P<0.05$). The HSI in J5 was significantly lower than in J10 ($P<0.05$), though neither differed from the control ($P>0.05$). No significant differences were found in viscerosomatic index, condition factor, or splenosomatic index among groups ($P>0.05$).

2.2 Effects of Soybean Meal Replacement by *C. autoethanogenum* Protein on Plasma Biochemical Indexes

Plasma biochemical responses to soybean meal replacement are shown in Table 4 . Plasma TC content in J5 and J10 groups was significantly lower than in J0 ($P<0.05$). Plasma glucose content in J10 was significantly higher than in J0 ($P<0.05$). Plasma AST activity in J10 was significantly higher than in J5 ($P<0.05$), which was significantly higher than in J0 ($P<0.05$). Plasma ALT activity in J10 was significantly lower than in J0 and J5 ($P<0.05$). Although plasma AST and ALT activities in all groups were below normal reference ranges reported in literature, the AST/ALT ratio increased dramatically in J10, being significantly higher than in J0 and J5 ($P<0.05$).

No significant differences were observed in plasma-specific immune indexes IgM, IL-8, and IL-1 among groups ($P>0.05$). Plasma MDA content in J5 was significantly lower than in J0 and J10 ($P<0.05$), while plasma T-SOD activity did not differ significantly among groups ($P>0.05$).

2.3 Effects of Soybean Meal Replacement by *C. autoethanogenum* Protein on Body Composition

Whole-body composition data are presented in Table 5 . Moisture content in J10 was significantly higher than in J5 ($P<0.05$), though no significant differences were detected in whole-body crude ash, crude protein, or crude lipid contents among groups ($P>0.05$).

2.4 Effects of Soybean Meal Replacement by *C. autoethanogenum* Protein on Hepatopancreas and Intestinal Histopathology

Hepatopancreas histology results are shown in Figure 1 [Figure 1: see original paper], with pathological diagnosis analysis summarized in Table 6 . All groups exhibited mild pathological changes in the hepatopancreas. In J0, among 11 samples, 9 appeared normal (Figure 1-a), one showed cellular vacuolation suggestive of fatty liver (Figure 1-b), and one displayed increased nuclear aggregation and hepatocellular nodulation (Figure 1-c). In J5, among 11 samples, 8 were normal, one showed vacuolation, and two exhibited increased nuclear aggregation and nodulation. In J10, among 11 samples, 7 were normal, one showed mild fatty liver, and three displayed hepatocellular nodulation. These results indicate that increasing *C. autoethanogenum* protein inclusion progressively aggravated nuclear aggregation and cellular densification in hepatocytes. Combined with the sharply elevated plasma AST/ALT ratio in J10, this suggests more severe hepatic tissue damage in this group compared to J0 and J5.

Posterior intestine histology is presented in Figure 2 [Figure 2: see original paper], with pathological analysis in Table 7 . All groups showed varying degrees of posterior intestinal damage. In J0, among 11 samples, 6 appeared normal (Figure 2-A), while 4 exhibited microvillus shedding, lamina propria loosening, and reduced or absent goblet cells (Figure 2-B). Intestinal structure improved slightly in J5 and J10, with 8 normal samples in each group. In J5, two samples showed microvillus shedding, lamina propria loosening, and reduced goblet cells, while one displayed shortened villus height and thinned muscularis (Figure 2-C). In J10, three samples showed microvillus shedding, lamina propria loosening, and reduced goblet cells.

3. Discussion

Clostridium autoethanogenum protein is derived from biological fermentation of *C. autoethanogenum*. This bacterium is rod-shaped, Gram-positive, strictly anaerobic, and facultatively chemoautotrophic. Isolated from the environment with stable characteristics, it is sensitive to chloramphenicol, penicillin, ampicillin, and tetracycline [18]. Its relatively complete genome sequence has been obtained with no reports of toxic genes [26-27]. *C. autoethanogenum* utilizes CO and carbon dioxide (CO) as carbon sources, fermenting CO or CO and hydrogen (H) to produce ethanol or acetate. The industrial production process uses CO from steel industry gases (converter gas, blast furnace gas) as carbon source and ammonia as nitrogen source, with a culture medium consisting of phosphoric acid (H PO), potassium hydroxide (KOH), magnesium sulfate (MgSO), ferrous sulfate (FeSO), and trace vitamins (vitamins B1, B2, B5, B6, B12, niacin, folic acid, and biotin). Through gas pretreatment, fermentation, distillation and dehydration, cell separation, spray drying, and wastewater treatment, the process yields clean energy (ethanol) and bacterial protein, with approximately 1,500 tons of bacterial protein obtained per 10,000 tons of ethanol produced, demonstrating enormous development potential. As a byproduct of

steel industry gas fermentation for fuel ethanol production, *C. autoethanogenum* protein promotes industrial waste conversion and reuse while reducing harmful gas emissions. Compared to traditional plant-derived proteins, it contains no antinutritional factors, and compared to animal-derived proteins, it has lower Salmonella and biogenic amine levels. Its production is characterized as green, energy-saving, and environmentally friendly. However, as a microbial fermentation product and with *C. autoethanogenum* not yet included in China' s feed additive directory, comprehensive biological safety assessment of this bacterial strain requires further in-depth research.

Clostridium autoethanogenum protein contains over 80% crude protein that is readily digestible by animals, is rich in various trace elements, free of antinutritional factors, and contains 18 amino acids with total amino acids accounting for 85% of protein content. Lysine and sulfur-containing amino acids are relatively high, while arginine is relatively deficient. Protein is the primary nutrient and energy source for fish, and protein requirements essentially reflect essential amino acid requirements. Protein nutritional balance is actually amino acid balance, and deficiencies in essential amino acids often lead to nutritional diseases and symptoms in fish [28].

Methionine plays a crucial role in metabolism and is the first limiting essential amino acid that is deficient in plant proteins, particularly in legume ingredients. Studies have shown that dietary methionine deficiency significantly reduces plasma insulin-like growth factor 1 (IGF-1) content [29], and animal feeding is regulated by IGF-1 [30]. Therefore, the growth-promoting effect of methionine may be related to increased plasma IGF-1 content in mid-term grass carp, though the specific mechanism requires further investigation. Wang [31] and Tang et al. [32] reported that increasing dietary methionine levels improved weight gain, feed intake, feed efficiency, specific growth rate, and protein efficiency in juvenile and mid-term grass carp. Liao [33] obtained similar results in studies on methionine requirements for juvenile blunt snout bream. Wang [31] also found that weight gain rate, specific growth rate, feed efficiency, and protein efficiency were affected by dietary arginine levels. As soybean meal was the main protein source in this experiment, it is deficient in methionine relative to the ideal protein pattern for fish (methionine/crude protein 2%, arginine/crude protein 5%) [20], while *C. autoethanogenum* protein is deficient in arginine. Therefore, corresponding limiting amino acids were supplemented in this trial. However, as stomachless fish, grass carp may have insufficient utilization capacity for crystalline amino acids, potentially unable to synchronously absorb exogenous crystalline amino acids. Zheng et al. [34] and Tan [35] have reported relatively low utilization efficiency of crystalline amino acids in grass carp. In this experiment, as *C. autoethanogenum* protein inclusion increased and soybean meal decreased, the endogenous bound methionine in the J5 diet was higher than in J0, which may explain the superior weight gain rate, final mean weight, specific growth rate, and feed efficiency in J5 compared to J0. However, the lack of significant difference in growth performance between J10 and J0 may be due to endogenous arginine deficiency causing amino acid imbalance.

Amino acids are also essential nutrients for maintaining intestinal tight junctions and structural integrity [36], particularly methionine and arginine for intestinal health. Arginine is converted to ornithine via arginase, and ornithine is decarboxylated to produce polyamines (spermine, spermidine, putrescine) via ornithine decarboxylase. Polyamines play crucial roles in intestinal epithelial cell growth, differentiation, and maturation, and are irreplaceable for intestinal mucosal development and repair [37]. Cheng et al. [38-39] confirmed the importance of arginine for intestinal health in hybrid striped bass (*Morone chrysops* × *Morone saxatilis*), red drum (*Sciaenops ocellatus*), and Chi et al. [40] in grouper (*Epinephelus coioides*). Similarly, in intestinal tissue, methionine generates S-adenosylmethionine (SAM) through transmethylation, which decarboxylates to produce 5'-adenosylmethylthiopropylamine that transfers an aminopropyl group to putrescine or spermidine to generate polyamines [41]. Methionine also generates cysteine, a substrate for glutathione, through transsulfuration, thereby regulating intracellular reduced glutathione (GSH) content and cell survival. GSH also plays important protective roles for intestinal epithelial cells [42]. Peng et al. [43] confirmed that methionine promotes intestinal development and increases fold height in juvenile Jian carp. In this experiment, both control and experimental groups showed intestinal damage, possibly due to high soybean meal content in the control diet leading to higher levels of soy antigens [44] and relative endogenous methionine deficiency. Intestinal damage in J10 may be caused by endogenous arginine deficiency, while replacement of soybean meal with *C. autoethanogenum* protein reduced dietary antinutritional factor levels, thereby alleviating posterior intestinal damage in J5 and J10.

However, as *C. autoethanogenum* protein inclusion increased, the proportion of hepatopancreatic lesions in grass carp showed an upward trend, with plasma AST/ALT ratio increasing abnormally in J10. Peng et al. [43] found that methionine promotes hepatopancreatic growth and development in juvenile Jian carp, while Li et al. [45] reported that arginine may protect the liver by reducing pro-inflammatory cytokines and free radical release. In this experiment, due to potentially insufficient absorption and utilization of exogenous amino acids by grass carp [46], actual absorbed methionine in all groups and arginine in J10 were inadequate, leading to hepatic damage in all groups [47-48]. Additionally, as a newly developed protein source, *C. autoethanogenum* protein may contain potential unknown factors during development that contributed to the increased hepatopancreatic lesion proportion with higher inclusion levels. Kiessling et al. [49] reported similar findings when partially replacing fishmeal with bacterial protein in rainbow trout. Currently, relevant reports are limited, and further research combined with continuous process improvement is needed to clarify specific causes. Tang [50] reported that methionine reduces serum ALT activity while increasing hepatopancreatic AST and ALT activities. Wei [51] demonstrated that AST is massively released from mitochondria into blood when liver is damaged. In this experiment, endogenous bound methionine increased progressively from J0 to J5 to J10, which may explain the gradual decrease in plasma ALT activity and the abnormal increase in plasma AST/ALT

ratio in J10, suggesting potential hepatopancreatic functional impairment that may be the primary cause of reduced survival in J10. Additionally, hepatic metabolic disorders may also be related to increased starch content in J10 following replacement of soybean meal with high-protein *C. autoethanogenum* protein.

Furthermore, Liao et al. [52] reported that increasing arginine within an appropriate range enhanced respiratory burst activity of blood cells and reduced cumulative mortality after *Aeromonas hydrophila* infection in juvenile blunt snout bream. Zhao et al. [53] also demonstrated that survival rate of juvenile cobia increased with dietary arginine content within a certain range. This suggests that endogenous arginine deficiency in J10 may be another direct cause of its low survival rate.

4. Conclusion

Dietary supplementation with 5% *C. autoethanogenum* protein to replace 27.5% soybean meal effectively improved feed efficiency, weight gain rate, and specific growth rate, reduced plasma TC and MDA contents, improved lipid metabolism and antioxidant capacity, and benefited intestinal health, thereby enhancing growth performance with proven safety and efficacy within this range. However, further increasing the inclusion level to 10% caused hepatocellular damage, impaired liver function, and reduced survival rate. Based on these results, the recommended inclusion level of *C. autoethanogenum* protein in grass carp diets is 5%.

References

[1] Ministry of Agriculture and Rural Affairs, Bureau of Fisheries. China Fisheries Statistical Yearbook-2016[M]. Beijing: China Agriculture Press, 2016.

[2] Ye YT, Xue M, Lin SM, et al. Comparison of protein enzymatic hydrolysis rates between intestine and hepatopancreas of grass carp to dietary protein[J]. Journal of Fishery Sciences of China, 2003, 10(2): 173-176.

[3] Liu LX, Huang ZW, Xu XY, et al. Research progress on effects of microbial fermentation on antinutritional factors and nutritional value of soybean meal[J]. Henan Journal of Animal Husbandry and Veterinary Medicine (Comprehensive Edition), 2014, 35(4): 8-10.

[4] Mambrini M, Roem AJ, Carvèdi JP, et al. Effects of replacing fish meal with soy protein concentrate and of DL-methionine supplementation in high-energy, extruded diets on the growth and nutrient utilization of rainbow trout, *Oncorhynchus mykiss*[J]. Journal of Animal Science, 1999, 77(11): 2990-2999.

[5] Yang YJ, Yao YS, Qin YC, et al. Investigation and analysis of major antinutritional factors in soybean meal and fermented soybean meal[J]. Scientia Agricultura Sinica, 2016, 49(3): 573-580.

[6] Gatlin III DM, Barrows FT, Brown P, et al. Expanding the utilization of sustainable plant products in aquafeeds: a review[J]. Aquaculture Research, 2007, 38(6): 551-579.

[7] Zhu CS, Yin RH, Zhao YP. Research progress on relationship between production process and product quality of fermented soybean meal[J]. Guangdong Feed, 2014, 23(5): 35-37.

[8] Fu TT, Li AK, Liang XX, et al. Research progress on detection techniques for antinutritional factors in soybean meal[J]. Science and Technology of Cereals, Oils and Foods, 2014, 22(1): 85-90.

[9] Chen GX, Cao Z, Gao ZH. Nutritional value and application of different fermented soybean meals[J]. China Animal Husbandry and Veterinary Medicine, 2014, 41(2): 111-114.

[10] Xue M, Xie SQ, Cui YB. Effect of a feeding stimulant on feeding adaptation of gibel carp *Carassius auratus gibelio* (Bloch), fed diets with replacement of fish meal by meat and bone meal[J]. Aquaculture Research, 2004, 35(5): 473-482.

[11] Luo L, Xue M, Wu XF, et al. Partial or total replacement of fishmeal by solvent-extracted cottonseed meal in diets for juvenile rainbow trout (*Oncorhynchus mykiss*)[J]. Aquaculture Nutrition, 2006, 12(6): 418-424.

[12] Xiang X, Zhou XH, Chen J, et al. Effects of dietary soybean meal protein replacing fish meal protein on growth performance, body composition and blood biochemical indexes of juvenile *Schizothorax prenanti*[J]. Journal of Fisheries of China, 2012, 36(5): 723-731.

[13] Yu XT. Effects of different protein sources on feeding response, immune response and flesh quality of grass carp[D]. Master' s thesis. Beijing: Chinese Academy of Agricultural Sciences, 2016: 28-32.

[14] Xiong ZH. Research progress on single-cell protein in feed industry[J]. Animals Breeding and Feed, 2006(7): 11-15.

[15] Hu MH, Wang YJ. Current status of bacterial protein development and its application prospects in aquafeeds[J]. Beijing Fisheries, 2006(3): 42-45.

[16] Li TT, Deng XJ. Research progress on single-cell protein feed and its application in animals[J]. Feed and Animal Husbandry, 2015(5): 57-61.

[17] Zhao XF. Investigation and nutritional evaluation of monosodium glutamate bacterial protein resources[D]. Master' s thesis. Tai' an: Shandong Agricultural University, 2003.

[18] Abrini J, Naveau H, Nyns EJ, et al. *Clostridium autoethanogenum*, sp. nov., an anaerobic bacterium that produces ethanol from carbon monoxide[J]. Archives of Microbiology, 1994, 161(4): 345-351.

[19] NRC. Nutrient requirements of fish and shrimp[M]. Washington, D.C: The National Academics Press, 2011.

[20] Kaushik SJ, Seiliez I. Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs[J]. Aquaculture Research, 2010, 41(3): 322-332.

[21] ISO. ISO-9831:1998 Animal feeding stuffs, animal products, and faeces or urine—determination of gross calorific value—bomb calorimeter method[S]. Berlin: Deutsches Institut für Normung, 1998.

[22] Zhang YR. Determination of blood indexes in grass carp[C]//Proceedings of the 2012 Academic Symposium of the Ichthyology Branch of the Chinese Society of Oceanology and Limnology and the Ichthyology Branch of the Chinese Zoological Society. Lanzhou: Chinese Society of Oceanology and Limnology, 2012.

[23] Wen AX, Zhou DG. Comparative study on some blood biochemical indexes of several fish species with different feeding habits[J]. Sichuan Journal of Zoology, 2005, 24(4): 80-84.

[24] Mi RF. Determination of hematological indexes in grass carp, common carp and silver carp[J]. Freshwater Fisheries, 1982(4): 10-16.

[25] Zhou Y, Guo WC, Yang ZG, et al. Research progress on hematological indexes of fish[J]. Journal of Shanghai Fisheries University, 2001, 10(2): 163-165.

[26] Utturkar SM, Klingeman DM, Klingeman JM, et al. Sequence data for *Clostridium autoethanogenum* using three generations of sequencing technologies[J]. Scientific Data, 2015, 2: 150014.

[27] Humphreys CM, McLean S, Schatschneider S, et al. Whole genome sequence and manual annotation of *Clostridium autoethanogenum*, an industrially relevant bacterium[J]. BMC Genomics, 2015, 16: 1085.

[28] Zhou F, Shao QJ. Research progress on essential amino acid nutrition in fish[J]. Feed and Animal Husbandry, 2010(8): 20-26.

[29] Takenaka A, Oki N, Takahashi SI, et al. Dietary restriction of single essential amino acids reduces plasma insulin-like growth factor-I (IGF-I) but does not affect plasma IGF-binding protein-1 in rats[J]. The Journal of Nutrition, 2000, 130(12): 2910-2914.

[30] Tatar M, Bartke A, Antebi A. The endocrine regulation of aging by insulin-like signals[J]. Science, 2003, 299(5611): 1346-1351.

[31] Wang S. Studies on protein and major essential amino acid requirements of juvenile grass carp (*Ctenopharyngodon idellus*)[D]. PhD thesis. Guangzhou: Sun Yat-sen University, 2006.

[32] Tang BR, Feng L, Liu Y, et al. Methionine requirement of mid-term grass carp[J]. Chinese Journal of Animal Nutrition, 2012, 24(11): 2263-2271.

[33] Liao YJ. Studies on methionine, lysine and arginine requirements of juvenile blunt snout bream (*Megalobrama amblycephala*)[D]. Master' s thesis. Nanjing: Nanjing Agricultural University, 2014.

[34] Zheng ZL, Song HB, Li HQ, et al. Study on utilization efficiency of different slow-release amino acids in grass carp fingerling feeds[J]. Cereal & Feed Industry, 2009(6): 42-45.

[35] Tan FF. Study on supplementation of microencapsulated and crystalline amino acids in grass carp (*Ctenopharyngodon idellus*) feeds[D]. Master' s thesis. Suzhou: Soochow University, 2010.

[36] Zhang J, Dai ZL, Zhu WY. Research progress on intestinal essential amino acid metabolism and its functions[J]. Parenteral & Enteral Nutrition, 2010, 17(1): 55-59.

[37] Johnson LR. Regulation of gastrointestinal mucosal growth[J]. World Journal of Surgery, 1979, 3(4): 477-486.

[38] Cheng ZY, Gatlin III DM, Buentello A. Dietary supplementation of arginine and/or glutamine influences growth performance, immune responses and intestinal morphology of hybrid striped bass (*Morone chrysops* × *Morone saxatilis*)[J]. Aquaculture, 2012, 362-363: 39-43.

[39] Cheng ZY, Buentello JA, Gatlin III DM. Effects of dietary arginine and glutamine on growth performance, immune responses and intestinal structure of red drum, *Sciaenops ocellatus*[J]. Aquaculture, 2011, 319(1/2): 247-252.

[40] Chi SY, Han FL, Tan BP, et al. Effects of dietary arginine levels on growth and intestinal morphology of juvenile grouper (*Epinephelus coioides*)[J]. Acta Hydrobiologica Sinica, 2016, 40(2): 388-394.

[41] Seiler N, Raul F. Polyamines and the intestinal tract[J]. Critical Reviews in Clinical Laboratory Sciences, 2007, 44(4): 365-411.

[42] Wang JJ, Chen LX, Li P, et al. Gene expression is altered in piglet small intestine by weaning and dietary glutamine supplementation[J]. The Journal of Nutrition, 2008, 138(6): 1025-1032.

[43] Peng Y, Tang L, Shuai K, et al. Effects of methionine on growth and digestive-absorptive function of juvenile Jian carp[J]. Chinese Journal of Animal Science, 2009, 45(13): 33-38.

[44] Wu LF, Xing XP, Lai HE, et al. Effects of soybean antigen protein glycinin on intestinal tissue of juvenile and young common carp (*Cyprinus carpio*)[J]. Journal of Northwest A&F University (Natural Science Edition), 2014, 42(10): 7-14.

[45] Li Q, Liu YL, Che ZQ, et al. Dietary L-arginine supplementation alleviates liver injury caused by *Escherichia coli* LPS in weaned pigs[J]. Innate Immunity, 2012, 18(6): 804-814.

[46] Chen HM, Zhong WB, Xu HJ, et al. Research progress on utilization of exogenous amino acids by fish[J]. Feed and Animal Husbandry, 2015(1): 29–32.

[47] Guo JS, Zhao FJ. Research progress on amino acid preparations for liver diseases[J]. New Drugs and Clinical Remedies, 1990, 9(5): 293–295.

[48] Tang XF. L-arginine: nitric oxide pathway and research progress on liver diseases[J]. Journal of Chongqing Medical University, 1994, 19(2): 157–160.

[49] Kiessling A, Askbrandt S. Nutritive value of two bacterial strains of single-cell protein for rainbow trout (*Oncorhynchus mykiss*)[J]. Aquaculture, 1993, 109(2): 119–130.

[50] Tang BR. Effects of methionine on digestive-absorptive capacity and antioxidant capacity of mid-term grass carp[D]. Master' s thesis. Ya' an: Sichuan Agricultural University, 2012.

[51] Wei XY. Current status and research progress of liver function tests[J]. Foreign Medical Sciences (Hygiene Fascicle), 1992(3): 141–146.

[52] Liao YJ, Liu B, Ren MC, et al. Effects of arginine on growth, serum free arginine and lysine, blood biochemistry and immune indexes of juvenile blunt snout bream (*Megalobrama amblycephala*)[J]. Journal of Fishery Sciences of China, 2014, 21(3): 549–559.

[53] Zhao HX, Cao JM, Wu JK, et al. Arginine requirement of juvenile cobia (*Rachycentron canadum*)[J]. Journal of South China Agricultural University, 2007, 28(4): 87–90.

Note: Figure translations are in progress. See original paper for figures.