

## Effects of Yupingfeng Polysaccharide on Intestinal Mucosal Morphology and Expression of Major Immune and Absorption-Related Genes in Grass Carp (Postprint)

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### Abstract

grass carp; Yupingfeng polysaccharide; intestinal villi; interleukin-2; interferon- $\gamma$ ; sodium-glucose transporter 1; glucose transporter 2

### Full Text

## Effects of Yupingfeng Polysaccharide on Morphology of Intestinal Mucosa and Expression of Main Immune and Absorption Genes in Grass Carp (*Ctenopharyngodon idellus*)

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### Abstract

This experiment was conducted to investigate the effects of different supplementation levels of Yupingfeng polysaccharide on intestinal mucosal morphology and the expression of key immune and absorption-related genes in grass carp. A total of 750 healthy grass carp with an average body weight of  $(74.50 \pm 2.50)$  g were randomly allocated into five groups, each with six replicates of 25 fish. The control group (Group I) received a basal diet, while the experimental groups

(Groups II-V) were fed diets supplemented with 0.8, 1.2, 1.6, and 2.0 g/kg Yupingfeng polysaccharide, respectively. Following a 7-day acclimation period, the formal feeding trial lasted 28 days.

The results demonstrated several significant effects. First, on day 14 of the trial, Group V showed significantly reduced crypt depth ( $P < 0.05$ ) and significantly increased villus height-to-crypt depth ratio ( $P < 0.05$ ) compared with the control group. Second, on day 7, Groups III and IV exhibited significantly or extremely significantly elevated interleukin-2 (IL-2) mRNA expression in head kidney tissue ( $P < 0.05$  or  $P < 0.01$ ), while all experimental groups showed extremely significant upregulation of interferon- $\gamma$  (IFN- $\gamma$ ) mRNA expression in head kidney ( $P < 0.01$ ). Additionally, Groups III, IV, and V displayed significantly or extremely significantly increased expression of sodium-glucose transporter 1 (SGLT-1) and glucose transporter 2 (GLUT-2) mRNA in the intestine ( $P < 0.05$  or  $P < 0.01$ ).

Third, on day 14, Groups IV and V showed extremely significant elevation of IL-2 mRNA expression in head kidney ( $P < 0.01$ ), while all experimental groups exhibited significant or extremely significant increases in IFN- $\gamma$  mRNA expression in head kidney ( $P < 0.05$  or  $P < 0.01$ ). Groups III, IV, and V also demonstrated extremely significant upregulation of both SGLT-1 and GLUT-2 mRNA in the intestine ( $P < 0.01$ ). Fourth, on day 21, Group IV displayed significantly increased IL-2 mRNA expression in head kidney ( $P < 0.05$ ), while all experimental groups showed extremely significant elevation of IFN- $\gamma$  mRNA expression in head kidney ( $P < 0.01$ ). Concurrently, Groups II-V exhibited significantly or extremely significantly increased expression of both SGLT-1 and GLUT-2 mRNA in the intestine ( $P < 0.05$  or  $P < 0.01$ ).

Finally, on day 28, Groups IV and V showed extremely significant upregulation of IL-2 mRNA in head kidney ( $P < 0.01$ ), while Groups III, IV, and V demonstrated significant or extremely significant increases in IFN- $\gamma$  mRNA expression in head kidney ( $P < 0.05$  or  $P < 0.01$ ). All experimental groups exhibited significantly or extremely significantly elevated SGLT-1 mRNA expression in the intestine ( $P < 0.05$  or  $P < 0.01$ ). In summary, dietary supplementation with Yupingfeng polysaccharide improved intestinal mucosal morphology, enhanced expression of SGLT-1 and GLUT-2 genes in the intestine, and regulated expression of IL-2 and IFN- $\gamma$  genes in head kidney, thereby improving intestinal absorption function and immune capacity. Considering cost-effectiveness, the optimal supplementation level of Yupingfeng polysaccharide in grass carp feed is 1.6 g/kg.

**Keywords:** grass carp; Yupingfeng polysaccharide; intestinal villus; interleukin-2; interferon- $\gamma$ ; sodium-glucose transporter 1; glucose transporter 2

## Introduction

Grass carp, together with silver carp, black carp, and bighead carp, constitute the four major Chinese domestic fish species. Due to its delicious taste and high nutritional value, grass carp is widely favored by consumers and extensively cultured. However, the continuous expansion of aquaculture in China and the adoption of high-density, intensive farming models have led to increased disease outbreaks in grass carp. Over the past two decades, large quantities of veterinary drugs have been used as prophylactic antibiotics in aquatic environments. This misuse of antibiotics has resulted in the emergence of antibiotic-resistant bacteria in water environments, enhanced drug resistance in fish pathogens, and facilitated the transfer of resistance genes among soil bacteria, animal pathogens, and human pathogens, ultimately altering bacterial communities in sediments and water columns. Consequently, enhancing grass carp immunity and reducing antibiotic usage have become primary research objectives.

Recent studies have demonstrated that traditional Chinese medicine polysaccharides, as feed additives, offer advantages such as low toxicity, minimal side effects, and no residues, showing promising applications for improving fish growth, immunity, and digestive performance. Yupingfeng formulation consists of three medicinal herbs: *Astragalus membranaceus*, *Atractylodes macrocephala*, and *Saposhnikovia divaricata*. This traditional formula functions to replenish qi, consolidate the exterior, tonify the spleen, fortify defensive qi, and arrest sweating. Yupingfeng polysaccharide is extracted from these three herbs through a process of soaking, aqueous extraction, heating and concentration, cooling and centrifugation, followed by alcohol precipitation. The polysaccharide components from each herb in Yupingfeng formulation have been confirmed to influence the immune system. Yuan et al. reported that injecting different doses of *Astragalus* polysaccharide in carp increased interleukin expression in head kidney in a dose-dependent manner. Mao's experiments demonstrated that *Atractylodes* polysaccharide could promote IL-2 secretion from mouse lymphocytes. Zhu et al. found that *Atractylodes* polysaccharide enhanced interferon- $\gamma$  secretion in porcine blood lymphocytes and corrected disturbances in T-cell subset secretion. Gao et al. hypothesized that the mechanism by which *Saposhnikovia* polysaccharide enhances natural killer cell activity involves promoting IL-2 activity and inducing high expression of IL-2 in lymphocytes. These studies collectively indicate that Yupingfeng polysaccharide can improve immune function.

Building upon previous research, this experiment investigated the effects of different supplementation levels of Yupingfeng polysaccharide on intestinal villus morphology and the mRNA expression of immune-related genes (IL-2 and IFN- $\gamma$ ) in head kidney and absorption-related genes (SGLT-1 and GLUT-2) in the intestine of grass carp, thereby providing experimental evidence for the application of Yupingfeng polysaccharide in aquaculture.

## Materials and Methods

**1.1 Experimental Animals and Diets** Healthy grass carp were obtained from Nanhai Bairong Aquatic Breeding Co., Ltd. in Foshan. Yupingfeng polysaccharide was purchased from Foshan Dezhong Pharmaceutical Co., Ltd., with laboratory analysis indicating contents of total reducing sugar (34.2%), crude protein (18.52%), crude fat (0.71%), crude ash (8.86%), moisture (8.21%), and crude fiber (0.67%). The composition and nutrient levels of the basal diet are presented in Table 1 .

**1.2 Experimental Design and Management** A total of 750 healthy grass carp with an average body weight of  $(74.50 \pm 2.50)$  g were randomly divided into five groups, each consisting of six replicates of 25 fish. The control group (Group I) received the basal diet, while experimental groups (Groups II-V) were fed diets supplemented with 0.8, 1.2, 1.6, and 2.0 g/kg Yupingfeng polysaccharide, respectively. The trial utilized 200 L culture barrels (51 cm diameter, 40 cm opening, 98 cm height) with continuous aeration and water exchange every three days (one-third of total volume). Following a 7-day acclimation period, the formal 28-day trial commenced with feeding at 09:00 and 17:00 daily at 4% of total fish body weight.

### 1.3 Sample Collection and Processing

**1.3.1 Intestinal Histological Samples** On days 13 and 27 of the formal trial, feeding was suspended at 08:00. On days 14 and 28 at 08:00, one fish per replicate (six fish per group) was randomly selected, anesthetized on ice, and dissected to rapidly remove the intestine. After trimming excess adipose tissue, approximately 1 cm of anterior intestine was excised, rinsed with phosphate buffer (pH 7.2-7.4), and fixed in Carnoy's solution (anhydrous ethanol 60 mL, chloroform 30 mL, glacial acetic acid 10 mL). Samples were paraffin-embedded, sectioned, and stained with hematoxylin-eosin (HE). Microscopic examination revealed intestinal mucosal morphology, and muscularis thickness was measured. Five straight, well-extended villi were selected per section to measure villus height and crypt depth using Image-Pro Plus 6.0 software, from which the villus height-to-crypt depth ratio (V/C) was calculated.

**1.3.2 Samples for Gene Expression Analysis** On days 7, 14, 21, and 28 of the trial, five fish per group were randomly selected at 08:00, fed, and then sampled at 09:00. After ice anesthesia and dissection, intestinal tissue was collected by trimming adipose tissue and rinsing with phosphate buffer (pH 7.2-7.4). Approximately 0.2 g of anterior intestine was rapidly weighed and preserved in RNA fixative at  $-80^{\circ}\text{C}$  after 24 hours. Head kidney samples were collected similarly.

Total RNA extraction and reverse transcription were performed using the PrimeScript<sup>TM</sup> RT Reagent Kit according to manufacturer in-

structions. Reference gene primers for grass carp \$2m\$ (GenBank : AB190815.1) were redesigned based on NCBI sequences, along with primers for IL-2 (GenBank : AF486820.1), IFN-\$\gamma\$ (GenBank: FJ695519.1), SGLT-1 (GenBank: JN867793.1 from common carp), and GLUT-2 (GenBank: NM\_{001042721}.1 from zebrafish) (Table 2 ). All primers were synthesized by Shanghai Biological Engineering Co., Ltd. (Guangzhou Branch).

Real-time quantitative PCR was conducted using SYBR® Premix Ex Taq™ II with the following protocol: initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 55°C for 20 s, and 72°C for 34 s, concluding with a melting curve analysis. The 20 µL reaction mixture contained 10 µL 2×SYBR® Premix Ex Taq™ II, 0.8 µL each of forward and reverse primers (10 µmol/L), 0.4 µL 50×ROX Reference Dye II, 2 µL cDNA template, and 6 µL ddH<sub>2</sub>O.

Relative mRNA expression was calculated using the  $2^{-\Delta\Delta Ct}$  method, where Ct represents the cycle number at which fluorescence crosses the threshold. The formulas were:  $\Delta Ct(\text{treatment}) = Ct(\text{target gene}) - Ct(\text{reference gene})$  for treatment groups;  $\Delta Ct(\text{control}) = Ct(\text{target gene}) - Ct(\text{reference gene})$  for the control group;  $\Delta\Delta Ct = \text{mean } \Delta Ct(\text{treatment}) - \text{mean } \Delta Ct(\text{control})$ . The relative expression level (treatment/control) =  $2^{-\Delta\Delta Ct}$ .

**1.4 Statistical Analysis** Data were initially processed using Excel 2010 and analyzed using one-way ANOVA in SPSS 19.0 software. Duncan's multiple range test was applied for post-hoc comparisons. Results are expressed as mean  $\pm$  standard deviation.

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## Results

**2.1 Effects of Yupingfeng Polysaccharide on Intestinal Mucosal Morphology** As shown in Table 3 , dietary supplementation with different levels of Yupingfeng polysaccharide did not significantly affect villus height ( $P > 0.05$ ). On day 14, crypt depth in all treatment groups was lower than the control, with Group V showing a significant reduction ( $P < 0.05$ ) that was also significantly lower than Groups II, III, and IV ( $P < 0.05$ ). The villus height-to-crypt depth ratio was higher in all treatment groups on day 14, with Group V significantly exceeding the control ( $P < 0.05$ ) and Group II ( $P < 0.05$ ). On day 28, this ratio was slightly lower in treatment groups but differences were not significant ( $P > 0.05$ ). Muscularis thickness was greater in all treatment groups on both days 14 and 28, though differences were not statistically significant ( $P > 0.05$ ).

**2.2 Effects of Yupingfeng Polysaccharide on mRNA Expression of Immune Genes IL-2 and IFN- $\gamma$  in Head Kidney** Table 4 demonstrates that Yupingfeng polysaccharide influenced the temporal mRNA expression of IL-2 and IFN- $\gamma$  in head kidney, with all treatment groups showing enhanced expression relative to the control.

For IL-2 mRNA expression, Group III was significantly higher than the control on day 7 ( $P < 0.05$ ), while Group IV was extremely significantly elevated ( $P < 0.01$ ); Groups II and V did not differ significantly from the control ( $P > 0.05$ ). On days 14 and 28, Groups IV and V showed extremely significant upregulation ( $P < 0.01$ ), while Groups II and III remained similar to the control ( $P > 0.05$ ). On day 21, only Group IV exhibited significantly increased expression ( $P < 0.05$ ).

For IFN- $\gamma$  mRNA expression, all treatment groups showed extremely significant differences from the control on days 7 and 21 ( $P < 0.01$ ). On day 14, Groups II and IV differed extremely significantly ( $P < 0.01$ ), while Groups III and V differed significantly ( $P < 0.05$ ). On day 28, Group II was not significantly different from the control ( $P > 0.05$ ), whereas Groups III and IV differed significantly ( $P < 0.05$ ) and Group V differed extremely significantly ( $P < 0.01$ ).

**2.3 Effects of Yupingfeng Polysaccharide on mRNA Expression of Absorption Genes SGLT-1 and GLUT-2 in Intestine** Yupingfeng polysaccharide also affected the temporal mRNA expression of SGLT-1 and GLUT-2 in the intestine (Table 5).

For SGLT-1 mRNA expression, all treatment groups exceeded the control on day 7, with Groups III and V significantly higher ( $P < 0.05$ ) and Group IV extremely significantly higher ( $P < 0.01$ ); Group II was not significantly different ( $P > 0.05$ ). On day 14, Groups III, IV, and V were extremely significantly elevated ( $P < 0.01$ ), while Group II remained similar to the control ( $P > 0.05$ ). On day 21, all treatment groups were extremely significantly higher than the control ( $P < 0.01$ ). On day 28, Groups II and IV were significantly elevated ( $P < 0.05$ ), while Groups III and V were extremely significantly higher ( $P < 0.01$ ).

For GLUT-2 mRNA expression, Group II did not differ from the control on day 7 ( $P > 0.05$ ), Group III was significantly higher ( $P < 0.05$ ), and Groups IV and V were extremely significantly elevated ( $P < 0.01$ ). On day 14, Groups III, IV, and V were extremely significantly higher than the control ( $P < 0.01$ ), while Group II remained similar ( $P > 0.05$ ). On day 21, Groups II and III were significantly elevated ( $P < 0.05$ ) and Groups IV and V were extremely significantly higher ( $P < 0.01$ ). On day 28, all treatment groups showed higher expression than the control, but differences were not statistically significant ( $P > 0.05$ ).

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## Discussion

**3.1 Effects of Yupingfeng Polysaccharide on Intestinal Mucosal Morphology** The results indicate that after 14 days of feeding, Yupingfeng polysaccharide reduced crypt depth and increased the villus height-to-crypt depth ratio, thereby enhancing intestinal digestive and absorptive capacity. The intestine serves as both a primary site for nutrient absorption and an important immune organ. Intestinal mucosal morphology parameters including villus height, crypt depth, villus-to-crypt ratio, and muscularis thickness are closely associated with

digestive and immune functions. Research has shown that greater villus height increases the surface area for nutrient contact and utilization. Crypt depth reflects the proliferation rate and developmental status of crypt cells, while the villus-to-crypt ratio comprehensively indicates nutrient absorption capacity. Studies have demonstrated that muscularis thickness correlates positively with nutrient digestibility. Previous research found that Astragalus polysaccharide increased villus height, villus-to-crypt ratio, and muscularis thickness while decreasing crypt depth in broiler chickens. Similar effects were observed with Astragalus polysaccharide in tilapia and *Atractylodes* polysaccharide in weaned piglets. Since Astragalus and *Atractylodes* polysaccharides are major components of Yupingfeng polysaccharide, it can be inferred that Yupingfeng polysaccharide similarly promotes crypt cell maturation, accelerates villus cell renewal, and enhances nutrient utilization.

**3.2 Effects of Yupingfeng Polysaccharide on IL-2 and IFN- $\gamma$  mRNA Expression in Head Kidney** Quantitative PCR analysis revealed that dietary supplementation with 1.6 g/kg Yupingfeng polysaccharide significantly increased IL-2 mRNA expression in head kidney, while 1.2, 1.6, and 2.0 g/kg supplementation significantly elevated IFN- $\gamma$  mRNA expression. Previous studies have reported immunomodulatory effects of component polysaccharides. Li et al. found that Astragalus polysaccharide injection in nephritic mice normalized elevated serum IL-2 levels without affecting healthy mice, suggesting a regulatory role. Chen et al. demonstrated that Astragalus polysaccharide enhanced Newcastle disease vaccine antibody titers and IL-2 content in roosters, with synergistic effects when combined with oxymatrine. Yuan et al. observed that dietary Astragalus polysaccharide promoted IL-2 and IFN- $\gamma$  expression in weaned piglets. Lu et al. reported that *Atractylodes* polysaccharide significantly increased serum IFN- $\gamma$  in mice. Deng et al. showed that Yupingfeng polysaccharide enhanced erythrocyte immune function and increased IL-2 and IFN- $\gamma$  levels in immunosuppressed mice. These findings collectively support that appropriate dietary levels of Yupingfeng polysaccharide can promote IL-2 and IFN- $\gamma$  production in grass carp head kidney, thereby exerting immunoenhancing effects.

**3.3 Effects of Yupingfeng Polysaccharide on SGLT-1 and GLUT-2 mRNA Expression in Intestine** Quantitative PCR results showed that dietary supplementation with 1.2, 1.6, and 2.0 g/kg Yupingfeng polysaccharide increased SGLT-1 and GLUT-2 mRNA expression in grass carp intestine. Glucose is a crucial energy substrate and metabolic intermediate primarily absorbed in the small intestine through active transport requiring carrier proteins. The main intestinal glucose transporters are SGLT-1 and GLUT-2, whose expression can be regulated to influence glucose absorption. Research indicates a mutual promotion between glucose and these transporters, where increased glucose and glucose-like substances in the intestine can upregulate transporter expression, thereby enhancing glucose absorption. Yupingfeng polysaccharide contains abundant glucose as well as arabinose, galactose, and other glucose-structured

monosaccharides. Therefore, it can be hypothesized that Yupingfeng polysaccharide enhances nutrient absorption by upregulating SGLT-1 and GLUT-2 gene expression in grass carp intestine.

## Conclusion

Dietary supplementation with Yupingfeng polysaccharide improves intestinal mucosal morphology, promotes SGLT-1 and GLUT-2 gene expression in the intestine to enhance absorption function, and regulates IL-2 and IFN- $\gamma$  gene expression in head kidney to strengthen immune capacity. From a cost-saving perspective, the optimal supplementation level in grass carp feed is 1.6 g/kg.

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