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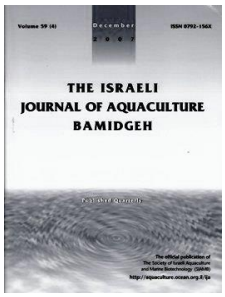


# Effect of Dietary *Ganoderma lucidum* Polysaccharides (GLP) on Cellular Immune Responses and Disease Resistance of Yellow Catfish *(Pelteobagrus fulvidraco)*

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**Abstract**

This study investigated the effects of dietary *Ganoderma lucidum* Polysaccharides (GLP) on cellular immune responses and disease resistance in yellow catfish (*Pelteobagrus fulvidraco*). For 8 weeks, 540 yellow catfish were fed different levels of GLP. The treatments were: 0-control group, 300-$T_1$, 600-$T_2$, 900-$T_3$, 1200-$T_4$, 1500-$T_5$ mg/kg, respectively. After the 8 week feeding trial, the respiratory burst activity of head kidney macrophages, proliferation of peripheral blood leukocytes, and phagocytic activity were assayed. Both the control and treated groups of fish were then challenged with an intraperitoneal injection of *Aeromonas hydrophila*, and cumulative mortality was recorded for 6 days post-infection. Results showed that there was a significant increase in oxygen respiratory burst activity of head kidney macrophages ($p<0.01$), along with significant increases in proliferation of peripheral blood leukocytes ($p<0.01$) in the fish fed dietary GLP. In addition, the 1200-$T_4$and 1500-$T_5$ mg/kg groups also displayed significant increases in nitrogen respiratory burst activity ($p<0.01$). These data indicated that GLP may reduce mortality following *A. hydrophila* infection. The lowest mortality was observed in the $T_5$-treated groups. In conclusion, dietary GLP may effectively enhance cellular immune responses and disease resistance in yellow catfish against *Aeromonas hydrophila*.

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## Introduction

In intensive aquaculture, diseases have seriously hampered rapid and healthy development, resulting in huge economic losses. However, traditional therapeutic agents, antibiotics and chemotherapeutics used to control fish diseases can result in the development of drug-resistant bacteria as well as environmental pollution and other unwanted residues in the environment. Immunostimulants including bacterial products (lipopolysaccharide) (Selvaraj et al., 2009), complex carbohydrates (glucan) (Kamilya et al., 2008), nutritional factors (vitamin E) (Clerton et al., 2001), yeast (Bai et al., 2010), and Astaraglus polysaccharides (Bai et al., 2012), have been studied to prevent diseases. Studies have shown that herbs and herbal extracts could effectively control and prevent aquatic animal diseases (Ganguly, 2010). These are easily obtained, inexpensive, safe, biodegradable, can be fed orally and act against a broad spectrum of pathogens (Bai et al., 2009).

*Ganoderma lucidum* (Leyss. ex Fr.) Karst. (Ling Zhi) (*Aphyllophoromycetideae*) (family *Polyporaceae*) was first indexed in the Shen Nong's Materia Medica as a life extending, tonic herb of the non-toxic superior class, and has been used in traditional Chinese medicine for more than 4000 years to prevent and/or treat various human diseases such as hepatitis, chronic bronchitis, gastritis, tumor growth, and immunological disorders. According to Chinese traditional medicine *Ganoderma lucidum* (GL) is capable of strengthening body resistance and improving constitutive homeostasis in patients. *G.lucidium* polysaccharides (GLP), a glycopeptide isolated from the water-soluble polysaccharides of GL, is a major effective component of GL. Previous studies have demonstrated that GLP has multiple effects on humans and animals. These include modulating immune system activity, anti-tumor effects, or inhibiting tumor growth (Yang et al., 2014), anti-HIV, antimicrobial activity (El-Mekkawy et al., 1998), anti-inflammatory activity (Lin et al., 1993), anti-allergic effect (Tasaka et al., 1988), preventing oxidative damage (You and Lin, 2002), protecting the liver, and reducing serum glucose levels (Zhang et al., 2002). In addition, GLP was found to promote phagocytosis by macrophages in mice immunosuppressed by cyclophosphamide, to stimulate the proliferation of lymphocytes induced by concanavalin A or lipopolysaccharides, and influence the gene expression of cytokines (Wang et al., 1997). GLP can modulate the immune cell function, including promoting the function of macrophages and lysozyme activities, enhancement of B cells, T cells, as well as dendritic cells (Bao et al., 2002). It is also reported that GLP can significantly enhance the leukocyte phagocytic activity of carp and survival after challenging with *Aeromonas hydrophila* (Yin et al., 2009).

Yellow catfish (*Pelteobagrus fulvidraco*), is an important small aquaculture species in China, restricted to freshwater habitats. It is one of the most popular food fish because of its excellent meat quality. It has promising market potential in Japan, South Korea, East and South Asia. The number of yellow catfish exported to other countries is constantly increasing.

There are no reports regarding the effect of dietary GLP as an immunostimulant in yellow catfish. In this study, we investigated the effects of different dietary levels of GLP on the innate cellular immune responses of yellow catfish. We determined the respiratory burst activities of head kidney macrophages, proliferation activities of peripheral blood leukocytes, and protection against *Aeromonas hydrophila.*

## Materials and methods

*Fish and rearing conditions.* 540 healthy juvenile yellow catfish were obtained from the Tianjin Agricultural University pond. The initial mean body weight and initial mean body length was 60.2±5.2 g, and 19.4±3.3 cm, respectively. Six homogeneous triplicate groups of yellow catfish were randomly distributed into 18 cages (1.5 m×1.5 m×1 m). The six different dietary treatments were applied in triplicate. Water temperature was maintained at 26±1℃, pH was maintained at 5.5-6, dissolved oxygen concentration was monitored daily and kept above 3.0 mg/L. During the trials, the fish were fed the experimental diets twice daily at 09:00 am, and 17:00 pm for 8 weeks at the rate of 2% of body weight; the daily exchange rate of water was 30%. The mortality rates per group of the experimental fish were recorded.

At the end of the feeding trial, 18 fish in each group (six fish from each cage) were randomly sampled to assay for respiratory burst of head kidney macrophages, and peripheral blood leukocyte proliferation. The remaining 36 fish in each group were challenged with *Aeromonas hydrophila.*

*Herbal extracts and Feed.* GLPs were obtained by the water-extraction and alcohol precipitation method. The density of polysaccharides was measured by a previously reported method (Dubois et al., 1956) and purity was 90%. Different levels of GLP were added to the basic diet: 0 (control), 300 mg/kg diet ($T_1$), 600 mg/kg diet ($T_2$), 900 mg/kg diet ($T_3$), 1200 mg/kg diet ($T_4$), 1500 mg/kg diet ($T_5$), respectively. The composition of the six diets is shown in Table 1.

**Table 1.** Composition of the basal diet (air-dry basis, %)

| *Ingredients* | *control* | $T_1$ | $T_2$ | $T_3$ | $T_4$ | $T_5$ |
|---|---|---|---|---|---|---|
| Fish meal | 25 | 25 | 25 | 25 | 25 | 25 |
| Soybean meal | 40 | 40 | 40 | 40 | 40 | 40 |
| Shrimp meal | 5 | 5 | 5 | 5 | 5 | 5 |
| Yeast meal | 1 | 1 | 1 | 1 | 1 | 1 |
| Wheat flour | 23.5 | 23.5 | 23.5 | 23.5 | 23.5 | 23.5 |
| Fish oil | 1 | 1 | 1 | 1 | 1 | 1 |
| Soybean oil | 1 | 1 | 1 | 1 | 1 | 1 |
| GLP | 0 | 0.03 | 0.06 | 0.09 | 0.12 | 0.15 |
| Cellulose | 0.5 | 0.47 | 0.44 | 0.41 | 0.38 | 0.35 |
| $Ca(H_2PO_4)_2$ | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| $NaH_2PO_4$ | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Premix[1] | 1 | 1 | 1 | 1 | 1 | 1 |

[1] The premix provided the following components per kg of diet ; Fe 150 mg; Zn 30mg; Mn 13 mg; Cu 3mg; Co 0.1 mg; I 0.6 mg; Se 0.15 mg; VC 100 mg; $VB_1$ 3mg; $VB_2$ 10 mg; $VB_6$ 12 mg; calcium pantothenate 30mg; nicotinic acid 30 mg; biotin 0. 1 mg; folic acid 2 mg; $VB_{12}$ 0.01 mg; inositol 400 mg; choline 1000 mg ; VA 2 000 IU; $VD_3$ 1 000 IU; VE 60 mg; VK 6 mg. All ingredients were diluted with wheat flour.

For all the feeds, the moisture composition was 12.36%, crude ash 12.37%, crude protein 38.14%, and crude lipid 3.08%.

*Isolation of the head kidney macrophages and peripheral blood leukocytes.* Measurements of immune parameters in fish, from the six different dietary treatments were taken after 8 weeks. Macrophages were isolated from each head kidney sample (12 fish/group) based on the method described (Bayne, 1986). Head kidney was excised and ground on ice with a mortar and pestle with the addition of L-15 medium. Cell suspensions were obtained by pressing samples through a 150 μm stainless steel mesh with a syringe rod, layered carefully onto a histopaque 1.077 g/cm$^3$ (Sigma), and centrifuged at 400×g for 30 min at 4℃. Isolated macrophages were gently removed, and then washed twice using Hanks solution (1% penicillin and streptomycin, 0.2% heparin, and 10% FCS). Purified macrophages were counted using a hemocytometer and cell viability was determined by the

trypan blue exclusion method. The macrophages were resuspended in L-15 growth medium and adjusted to $1\times10^7$ cells/mL for further analysis.

Blood samples (12 fish per group) were collected from the caudal vein 8 weeks after onset of feeding. Heparin was used as an anticoagulant. Blood and L-15 medium were mixed at a rate of 1:1. Peripheral blood leukocytes of yellow catfish were separated by Percol (1.077 g/cm$^3$/1.119 g/cm$^3$) continuous density gradient centrifugation from the head kidney macrophages.

*Respiratory burst activity.* The oxygen respiratory burst activity of head kidney macrophages was evaluated with nitroblue tetrazolium (NBT, Sigma) reduction that measures intracellular oxidative free radicals (Secombes, 1990) with slight modifications. One hundred μL of macrophages ($1\times10^7$) were plated into the wells of U-bottom microtitre plates and incubated at 27℃ for 3 h to allow adhesion of cells. After incubation, the 96-well plates were centrifuged three times at 400 ×g for 10 min. The supernatant was then removed and the wells were washed three times with Hanks' balanced salt solution (HBSS). After washing, 100 μL 0.1% NBT (sigma, USA) and 1 μg/mL Phorbol 12-myristate 13-acetate (PMA, sigma, USA) were added for 1 h (positive control containing 100 μg/mL LPS). Absolute methanol was added to terminate the staining. Each tube was washed three times with 70% methanol and air-dried. One hundred twenty μL 2 M KOH and 140 μL dimethyl sulfoxide (DMSO, sigma, USA) were then added and the color was subsequently measured at 620 nm. In order to detect cell activity during culture, 500 μg/mL LPS was added to the cell solution and used as a positive control. The optical density was recorded in a microplate reader (Therme) at 620 nm.

The nitrogen respiratory burst activity was evaluated using Griesse reagent coloration (Bai et al., 2012). The incubation and washing of macrophages was similar to the described oxygen respiratory burst above. After washing, 100 μL L-15 medium (1% penicillin and streptomycin, 0.2% heparin, and 10%FCS) was added and incubated for an additional 24 h at 27℃ (positive control group containing 100 μg/mL LPS). Cells were washed twice with HBSS and 100 μL of Griesse (Beyotime Institute of Biotechnoligy) was added and incubated for 10 min. The optical density was recorded at 540 nm.

*Proliferation of peripheral blood leukocytes.* The proliferation of peripheral blood leukocytes was measured by MTT (Mosmann, 1983). The incubation and washing of leukocytes was similar to the oxygen respiratory burst of head kidney macrophages as described above. After washing, 100 μL L-15 media (1% penicillin and streptomycin, 0.2% heparin, and 10% FCS) was added and incubated for an additional 24 h at 27℃ (positive control group containing 100 μg/mL LPS). One hundred μL of L-15 media and 20 μL MTT was added to each well and incubated for another 4 h at 27℃. After incubation, plates were centrifuged to remove the L-15 medium, washed twice with HBSS, and 150 μL DMSO was added to each well. The optical density was measured at 570 nm.

*Infection challenge using A. hydrophila.* At the end of each experiment, the treatment groups were challenged with *A. hydrophila* that was isolated from yellow catfish obtained from the Fishery Institute of Tianjin, China. Bacteria were inoculated into 10 mL of liquid tryptic soy broth (TSB, Sigma) medium and incubated overnight at 28°C. Cultures were centrifuged at 850×g for 15 min. Supernatants were removed and the pelleted bacteria washed twice in sterile phosphate buffered saline (PBS) solution. The concentration of bacteria was adjusted to $1\times10^6$ by the optical density of suspension.

0.1 mL of suspended bacteria was injected into the peritoneal cavity of fish (24

fish/group). Mortality was recorded during the 6 days following infection.

*Statistical analysis.* Results of the assays are presented as average (± standard error) for six fish and were compared at each time of sampling using one way analysis of variance (ANOVA) in SPSS (Statistical Products Service & Solutions) version 17.0 for Microsoft windows. Significant differences between experimental groups were expressed at a significance level of 0.05 and 0.01.

## Results

Oxygen respiratory burst activity of macrophages in head kidney of yellow catfish fed with GLP for 8 weeks was determined. The increase in oxygen respiratory burst activity of head kidney macrophages in the positive control (LPS group) for catfish fed GLP for 8 weeks was highly significant ($p<0.01$) compared to the control group. Enhancement of the oxygen respiratory burst activity of yellow catfish fed with GLP at 300-1500 mg/kg diet was highly significant ($p<0.01$), and the oxygen respiratory burst activity increased dietary GLP levels from 300 mg/kg to 1200 mg/kg. The highest oxygen respiratory burst activity of head kidney macrophages was found in the 1200 mg/kg GLP group ($P<0.01$). Although the oxygen respiratory burst activity of 1500 mg/kg GLP group was lower than that of 1200 mg/kg GLP group, there was no significant difference between the treatments ($P>0.05$).

Nitrogen respiratory burst activities of head kidney macrophages in yellow catfish fed with GLP for 8 weeks are shown in Fig 2. As in Fig 1, positive control (LPS group) the increase in oxygen respiratory burst activity of head kidney macrophages was highly significant ($p<0.01$). Although the nitrogen respiratory burst activities of yellow catfish fed with GLP at 300-1500 mg/kg diet were higher than that of control group, only those of the 1200-1500 mg/kg GLP groups improved significantly ($p<0.01$). Both higher level groups were not significantly different ($p >0.05$).

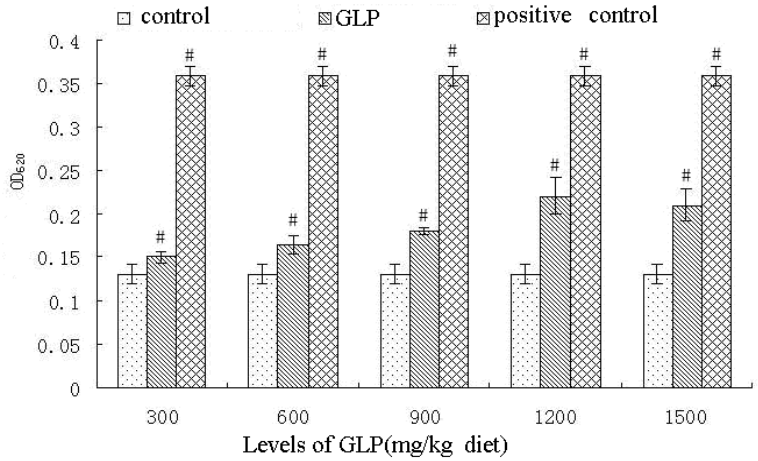


**Fig.1.** The effects of *Ganoderma lucidum* Polysaccharides on oxygen respiratory burst activity of yellow catfish head kidney macrophages.
Significant difference from the control are indicated by asterisks (* $p<0.05$, # $p<0.01$).

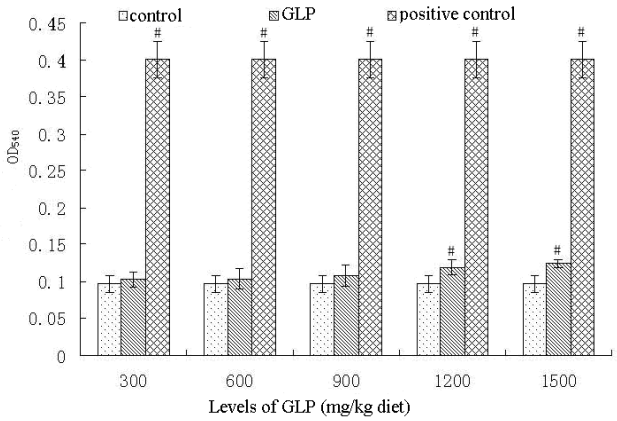


**Fig.2.** The effects of *Ganoderma lucidum* Polysaccharides on nitrogen respiratory burst activity of yellow catfish head kidney macrophages. Significant difference from the control are indicated by asterisks (* P<0.05; # P<0.01).

After 8 weeks of the experiment, dietary LPS (position control) significantly increased proliferation of peripheral blood leukocytes of yellow catfish. The increase in proliferation of peripheral blood leukocytes of yellow catfish was highly significant (p<0.01), in catfish fed with GLP levels of 300 -1500 mg/kg and the activities of those fed with 1200-1500 mg/kg GLP group were highly significantly greater than those of the positive control (p<0.01), the highest of which were with those fed 1200 mg/kg GLP (Fig. 3)

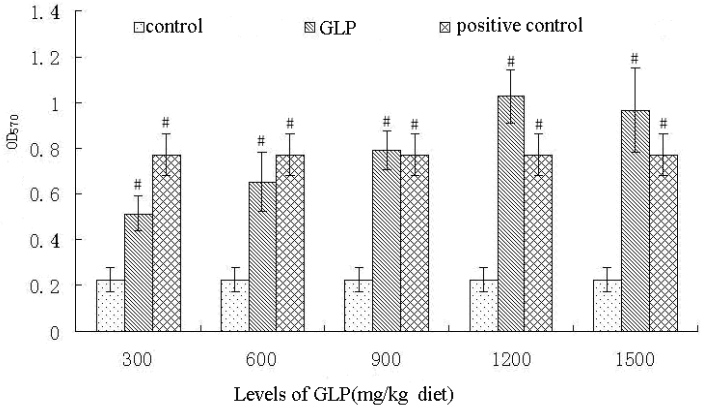


**Fig.3.** The effects of *Ganoderma lucidum* Polysaccharides on proliferation of peripheral blood leukocytes of yellow catfish. Significant difference from the control are indicated by asterisks (* P<0.05; # P<0.01).

After 8-weeks of feeding the experimental diets, yellow catfish were challenged with *A. hydrophila* and the cumulative mortality was recorded daily for 6 days (Fig. 4).

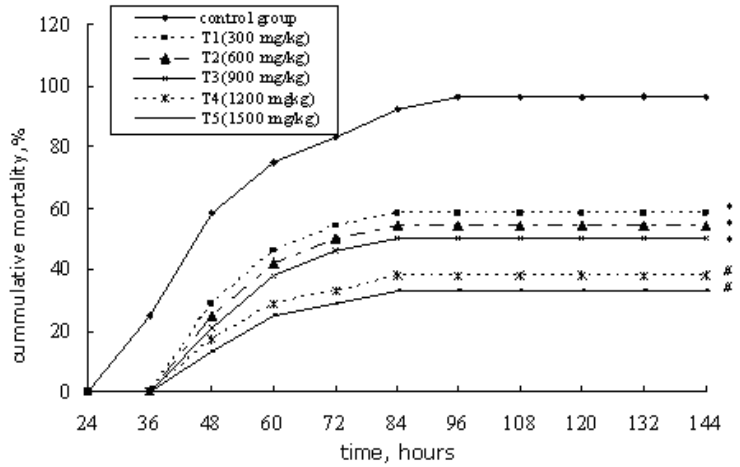


**Fig. 4.** Cumulative mortalities after challenging with *Aeromonas hydrophila* in yellow catfish. Significant difference from the control are indicated by asterisks (* P<0.05; # P<0.01).

Fish challenged with *A. hydrophila* started to die 36 h post-infection. Fifty-eight percent of control fish survived at 48 hours post-infection and the cumulative mortality rate over a 6-day treatment period was 96%. In all of the treated groups, fish mortality occurred 48 hours

post-infection. Cumulative mortality rates of the 300, 600, 900 mg/kg GLP treatment groups were significantly reduced compared to control by 58%, 54% and 50%, respectively ($p<0.05$). Furthermore, the rates of 1200 mg/kg and 1500 mg/kg GLP groups were highly significantly lower by 38% and 33%（$p<0.01$）. The lowest mortality rate was observed in the 1500 mg/kg GLP group.

## Discussion

The results of our experiment show that GLP can effectively enhance the activation of immunologically active cells of yellow catfish, and can be used to enhance immune response and disease resistance of cultured yellow catfish. The appropriate level of GLP to be supplemented was 1200 mg/kg.

As a primitive defense mechanism in fish, phagocytosis has been recognized as an important cellular component of the innate immune system against invading microorganisms of fish (MacArthur and Fletcher, 1985). Phagocytes can engulf microorganisms and kill them principally by production of reactive oxygen species (Kozarski et al., 2012) during the so-called respiratory burst. Fish treated with polysaccharides like chitosan (Siwicki et al., 1994) showed increased phagocytosis as well as respiratory burst activity. Our results indicate that suitable levels of dietary GLP significantly enhanced oxygen and nitrogen respiratory burst in head kidney macrophages in yellow catfish. Similar effects were found in *Cyprinus carpio* (Yin et al., 2009). This suggests that GLP has a priming effect that responds to stimulation. Some of the herbal ingredients that prove effective in mammals are also used in aquatic species. *Ganoderma lucidum* polysaccharides used as feed additives in this experiment elevated oxygen respiratory burst activity of mouse head kidney macrophages (Li, 2007b). Additionally, feeding tilapia with two herbs (*Astragalus membranaceus and Lonicera japonica*) individually or in combination significantly enhanced phagocytic and respiratory burst activity of blood phagocytic cells (Ardó et al., 2008). The polysaccharides in *Lonicera japonica*, as *Astragalus membranaceus* also had a positive effect on the respiratory burst activity of blood phagocytic cells.

Proliferation of white blood cells is also considered an important immunostimulant. In this study, feeding yellow catfish suitable levels of GLP effectively enhanced the proliferation of peripheral blood leukocytes. A similar result was observed (Li, 2007a) in GLP-treated mice which all had greater total counts of leukocytes and lymphocytes than the control mice. The most highly significant increase in the number of immune cells ($P<0.01$) resulted from 200 mg/kg GLP. In other reports, the polysaccharides in GLP at concentrations ranging from 50 to 400 μg/mL, promoted the proliferation of tumor-bearing mouse spleen cells and the amount of IFN-γ in the supernatant of tumor-bearing mouse spleen cell cultures (Zhang, 2006). Both these results show that GLP can enhance the proliferation of peripheral blood leukocytes not only in mammals (such as mice) but also in poikilothermic organisms (such as yellow catfish). Mortality was 33% and 38% in the 1200 mg/kg, and 1500 mg/kg GLP fed groups in this experiment, respectively. Similar results were also found in the resistance of yellow catfish fed with the same concentrations of *astragalus* root polysaccharides which could significantly increase leukocyte proliferation and decrease the mortality to 55% and 53%, respectively (Bai et al., 2012). This indicates that the effectiveness of GLP is higher than the same dose of *astragalus* root polysaccharides in decreasing mortality of yellow catfish infected with *Aeromonas hydrophila*.

Results obtained in this study showed that the appropriate supplemental levels of GLP range between 1200-1500 mg/kg and these doses significantly enhance oxygen and nitrogen respiratory burst of head kidney macrophages and proliferation of peripheral blood leukocytes of fish. They can be used as an immunostimulant to enhance cellular immune response and disease resistance of cultured yellow catfish.

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