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Effect of dietary chitosan and probiotics on disease resistance and immunity of obscure puffer (*Fugu obscurus*)

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Abstract: Obscure puffer (*Fugu obscurus*) has economic value and aquacultural importance in China. However, it suffers from serious fish disease, particulary bacterial disease in intensive aquaculture. In spite of positive role on immune function of probiotics and chitosan, their influence on immunity and disease resistance of *Fugu obscurus* was unknown. Therefore, the purpose of this experiment was to ascertain the possible role of stimulating and enhancing immune response of probiotics and chitosan on obscure puffer (*Fugu obscurus*). Fish were separately fed on the basal diet as control or on the basal diet with various levels of chitosan(0.2%, 0.5%, 1.0%), probiotics (0.1%, 0.2%, 0.4%), chitosan plus probiotics mixture, and a mixture of mannan oligosaccharide plus probiotics for 8 weeks. The experimental fish fed on the basal diet containing 0.2% chitosan, 0.1% probiotics, the mix of chitosan plus probiotics or mannan oligosaccharide plus probiotics mix with enhanced bactericidal activity were selected to measure immunity. The results showed that T and B lymphocyte proliferation in the head kidney was promoted by feeding on the diet with the mixture of chitosan plus probiotics, while the mixture of mannan oligosaccharide plus probiotics enhanced T lymphocyte proliferation in the head kidney. Furthermore, the mixture of chitosan plus probiotics was more effective in combination than chitosan or probiotics alone according to head kidney lymphocytes proliferation and IgM content in B cell culture supernatant. IgM concentration in spleen under the treatment of LPS stimulation in all four experimental groups was higher than that of the control, however only did the mixture of chitosan plus probiotics have a significant positive effect on IgM secretion($P < 0.05$). IgM level changed greatly between the control and the supplemented chitosan or probiotics group when lymphoid cells were stimulated by LPS in anterior kidney, and all the feed stuff containing chitosan or/and probiotics might result in an increase of IgM concentration($P < 0.05$). However, IFN-α secretion was suppressed by chitosan or probiotics in spleen and head kidney. In the future, we need further research on relationship of several immune parameters, immunity change before and after the tested fish are challenged by *Aeromonas hydrophila* and immunoregulation mechanism of chitosan and probiotics on the fish.

Key words: *Fugu obscurus*; chitosan; probiotics; disease resistibility; immunity

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Fish disease is one of the most serious problems confronted by fish farmers. Once an outbreak of fish disease occurs, it will spread out quickly in an aqueous environment, so the control of fish disease is

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of great concern in aquaculture. A lot of chemicals, either natural or synthetic, are known to stimulate the vertebrate immune system. It is thought that such kind of substances could also be of benefit for stimulating fish immune function that might be useful inpreventing fish disease in aquaculture[1], and some of them have been tested in fish[2]. Several biochemicals have been shown to enhance various facets of the innate immune response, including cellular immunity, humoral immunity and cytokines [3].

Chitin, β - 1,4 - N - acetyl - D - glucosamine, is an abundant polysaccharide in nature found in insect exoskeletons, crustacea shells and fungal cell walls. Its partially deacetylated product is chitosan. Some studies have indicated that chitosan could reduce cholesterol and lipid levels in blood, and there is also some evidence that chitin or chitosan might induce or increase macrophage activity in mammals[4-5]. However, it is just the very beginning that chitosan is used as dietary immunoenhancer in aquaculture. Related papers reported that chitosan could enhance non-specific immunity of brook trout and rainbow trout[6-8]. Probiotics may provide an alternative to the use of antimicrobial compound in some situation, but researches focused on the immunostimulating role of probiotics on fish were rarely reported. Irianto and Austin[9] discovered that there was no indication of serum or mucus antibody to *Aeromonas salmonicida* despite of an increased number of erythocytes, macrophages, lymphocytes and leucocytes, and enhanced serum lysozyme activity by probiotics in the rainbow trout.

Obscure puffer (*Fugu obscurus*) has economic value and aquacultural importance in China. However, it suffers from serious fish disease, particulary bacterial disease in intensive aquaculture. In spite of positive role on immune function of probiotics and chitosan, their influence on immunity and disease resistance of *Fugu obscurus* was unknown. Therefore, the purpose of this experiment was to ascertain the possible role of stimulus and enhancing immune response of probiotics and chitosan on obscure puffer *Fugu obscurus*.

1 Materials and methods

1.1 Experimental diets

The basal diet was mainly composed of brown fish meal, soybean meal, rapseed meal and α-starch, which were ground and sieved through 0.442mm mesh befose mixing. The formulation and proximate composition of the basal diet were listed in Table 1. Experimental diets were mixed as control or with various levels of chitosan(c, 0.2%,0.5%,1.0%), probiotics (p, 0.1%,0.2%,0.4%), chitosan plus probiotics mix(c + p),or mannan oligosaccharide plus probiotics mix(m + p). Probiotics contained *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus thermosphaericus*. All diets were prepared by thoroughly mixing the dry ingredients and making soft gobbet form with cold water before feeding.

Tab.1 Formulation and proximate composition of the basal diet

ingredients	percentage by weight(%)
Fish meal	48.0
α - starch	17.0
Soybean meal	11.0
Corn meal	7.0
Rapeseed meal	5.0
Wheat Middling and Reddog	5.7
Fish oil	4.0
Vitamin mixture	0.3
Mineral mixture	0.3
Choline chloride	0.5
cellulose	1.0
others	0.2
Nutrition level(%)	
Crude protein	39.1
Ash	9.9
Crude lipid	8.2
Moisture	11.7

1.2 Fish and rearing conditions

Obscure puffer weighing (3.15 ± 0.05) g were maintained in indoor tanks(5.0 m × 2.0 m × 1.0 m). After 5-day adaptation with the experimental control diet, nine groups of fish (26 individuals each tank) were randomly divided into 27 tanks with triplicate for each group. Each group of fish received one of the diets above mentioned twice a day at 08:30 and 16:00 on the fixed feed dais for 8 weeks. During the

feeding period, the water quality was under control at DO > 7 mg·L^{-1} and NH_3 – N < 0.5 mg·L^{-1}, and water temperature at 23 – 28 ℃.

1.3 Sampling and measurement

Determination of lethal dose of 50 percent fish killed (LD_{50}) After two months feeding, *F. obscurus* fed on the basal diet and on the experimental diets were challenged by intraperitoneal injection of live *Aeromonas hydrophila* suspended in saline ranging from 6 × 10^4 CFU/fish to 6 × 10^7 CFU/fish with tenfold stepwise increase. Five individuals were used in each challenge test. The fish injected with 0.9% NaCl was used as control. After the challenge, the fish were maintained at room temperature of 24.5 – 26.0℃ and the number of dead fish was recorded daily for a period of 5 days. All the challenges were duplicated. LD_{50} was calculated referring to Reed-Muench[10].

Determination of immune parameters The fish, not challenged by Ah, in the groups that had promoted LD50 were selected to analyze immune function. Ten individual fish from each selected group were used and lymphocytes were obtained from the head kidney and spleen.

Cell proliferation The tissues from spleen and head kidney were removed using sterilized tools and pressed gently through metal nets of 0.066 mm and suspended in RPMI 1640 medium, the suspension of which was centrifuged for 10 min at 1000 r·min^{-1}, and the cell pellet was resuspended in RPMI 1640, under which Ficoll solution with density of 1.082 g·mL^{-1} was added, then centrifuged for another 20 min at 2000 r·min^{-1}. Subsequently, the cells at the white interface were collected and washed twice in RPMI 1640, which was diluted to 6 × 10^5 mL^{-1} with RPMI 1640[11].

Cells were seeded in 96 – well tissue culture plate with the volume of 100 μl per well. For the purpose of mitogen stimulation, equal volume of PHA (10 μg·mL^{-1}) or LPS (20 μg·mL^{-1}) was added to the culture which was set up in triplicate. The cells cultures were incubated for 3 or 5 days in a humid atmosphere of 5% CO_2/ 95% air at 29 ℃, and later 0.5 μCi ^{3}H-thymidine was added to each well. After culturing for 16h in a humid atmosphere of 5% CO_2/ 95% air at 29℃, the cells were harvested with a semi – automatic harvestor, transferred onto glass fibre filters and washed with distilled water and the filters were dried. Radioactivity of the filters was counted in a liquid scintillation counter. T or B proliferation was expressed as stimulation index (SI). SI = cpm of stimulated culture/cpm of non – stimulated control culture[12].

Determination of IFN – α After five days incubation of lymphocytes from spleen and kidney stimulated with LPS, IFN – α in supernatants was harvested and the contents of them were measured in accordance with the method of AB-HRP supplied in IFN – α ELISA kit.

Determination of IgM After five days incubation of lymphocytes from spleen and kidney stimulated with LPS, IgM in supernatants was harvested and the contents of them were measured with a micro-plate modification of a double antibody sandwich enzyme-linked immunosorbent assay (ELISA)[13]. Flat-bottomed 96-well microtitre plates were coated overnight with a mouse anti-human IgM antibody in 0.05M carbonate buffer, pH 9.6. After washing, the plates were saturated with 1% bovine plasma albumin prepared in phosphate buffered saline (PBS, pH 7.4). After washing the plate diluted supernatant samples and standards were added into the wells. The assay was standardized with known concentrations of chromatographically purified human IgM. Next, after washing the plates, the trapped IgM was detected with anti-human IgM antibody-conjugated HRP was added into wells after washing the plates. Washing was performed between each step with PBS containing 0.025% Tween-20. Orthophenylenediamine (OPD) in phosphate-citrate buffered saline (pH 5.0) was used as the substrate, and the optical density was read with a Titertek plate reader at 492.

1.4 Statistical Analysis

After one-way variance analysis, Duncan's multiple range test was conducted to compare the

dietary treatment data using the statistical program (LD_{50} was logarithmically transformed prior to analysis). Statistical analysis was conducted in the same way for all the parameters tested.

2 Results

2.1 Resistibility against *Aeromonas hydrophila*

LD_{50} of the control fish was 1.94×10^5 CFU (Fig.1). The fish fed on the basal diet containing chitosan showed an increase in protection against *Aeromonas hydrophila*, regardless of the chitosan level. LD_{50} in the group containing chitosan was 4.57 – 7.27 times higher than that in the control group. Anti-bacterial ability was also improved by the dietary probiotics and gradually weakened by the increase of probiotics supplementation. LD_{50} in the group containing probiotics was 1.74 – 4.58 times higher than that in the control. When chitosan plus probiotics or mannan oligosaccharide plus probiotics mix was supplemented in the diet, LD_{50} was 15.15 and 9.74 times higher than that in the control ($P < 0.05$), respectively. Four immunoenhancers(0.2% chitosan, 0.1% probiotics, mannan oligosaccharide plus probiotics mix, chitosan plus probiotics mix) were selected according to LD_{50}.

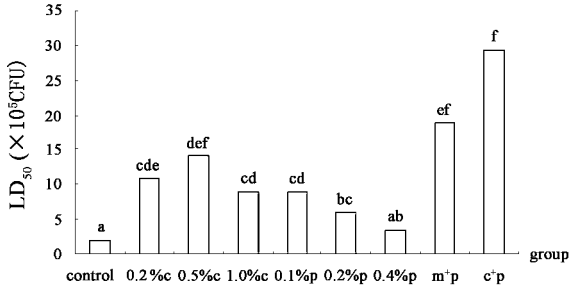

Fig.1 LD_{50} of *Fugu obscurus* against *A. hydrophila*

Different letters at the bar's top indicate significance at $P < 0.05$, and values sharing the same letter are not significantly different ($P > 0.05$)

2.2 Proliferation of lymphocytes from spleen and head kidney

SI of T cells from spleen in control was 1.37 (Fig.2). No enhanced stimulation was observed in any dietary additive. The proliferation of T lymphocytes from spleen was reduced by the addition of chitosan or probiotics or mannan ologosaccharide in the basal diet. However, the SI remained at almost the same level among the treatments of 0.1% probiotics, probiotics plus chitosan mix, probiotics plus mannan oligosaccharide mix. SI of B cells from spleen in control was 0.58 (Fig.2). No marked difference was observed in the B cell proliferation of spleen, indicating that the four immunoenhancers had no effect on the splenic B cell proliferation.

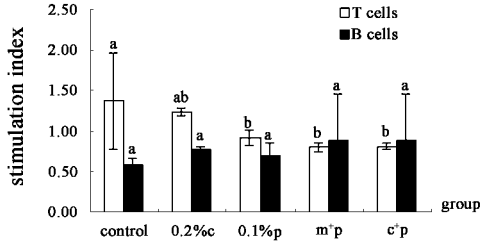

Fig.2 Effect of chitosan and probiotics on transformation of lymphocyte in spleen

Values are expressed as the mean ± SD. Different letters at the bar's top indicate significance at $P < 0.05$, and values sharing the same letter are not significantly different ($P > 0.05$)

SI of T and B cells from head kidney in control was 0.73 and 0.70 respectively. T and B lymphocyte proliferation in head kidney were significantly promoted by probiotics plus chitosan mix (Fig.3). T cell proliferation was also markedly enhanced by mannan oligosaccharide plus probiotics mix, while 0.2% chitosan or 0.1% probiotics alone didn't induce the increment in the proliferation.

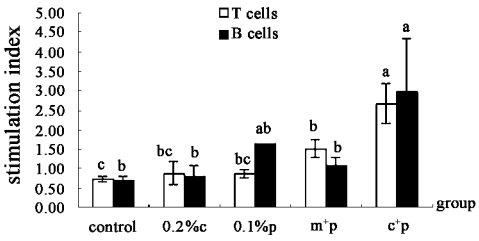

Fig.3 Effect of chitosan and probiotics on transformation of lymphocyte in head kidney

Values are expressed as the mean ± SD. Different letters at the bar's top indicate significance at $P < 0.05$, and values sharing the same letter are not significantly different ($P > 0.05$)

2.3 IgM content in B lymphocyte culture supernatant stimulated with LPS

After B lymphocytes from spleen stimulated with LPS, the level of IgM reached 509.0 $\mu g \cdot mL^{-1}$. Positive response could be seen from B cells treated with the dietary mix of chitosan plus probiotics. In this group, IgM production was 1.94 times higher than that in the control. IgM production secreted by head kidney B lymphocyte was 230.0 $\mu g \cdot mL^{-1}$, and the level of IgM of the other four immunoenhancer groups was statistically enhanced and 1.67 – 2.01 times higher than that in the control group. However, no statistical difference was found among the different treatment groups (Fig.4).

2.4 Content of IFN – α in B lymphocyte culture supernatant stimulated with LPS

IFN – α was also produced by splenic B cells, the level of which was 30.8 $pg \cdot mL^{-1}$ in the control group. The value of IFN – α remains unaffected after 8 weeks feeding of dietary immunoenhancers. Head kidney B cells could secret IFN – α after being stimulated with LPS, which was 20.1 $pg \cdot mL^{-1}$ in the control, noteworthily lower than that in spleen. However, IFN – α secretion in head kidney was merely reduced by 0.2% dietary chitosan and the mix of mannan oligosaccharide plus probiotics (Fig. 5).

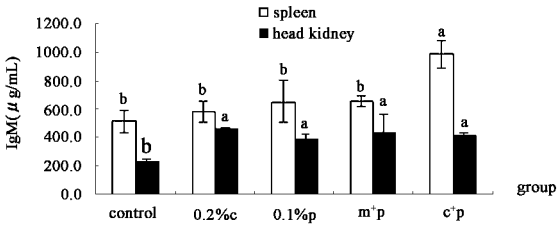

Fig. 4 IgM level of spleen and head kidney in the cultured media under the treatment of LPS stimulation

Values are expressed as the mean ± SD. Different letters at the bar's top indicate significance at $P < 0.05$, and values sharing the same letter are not significantly different ($P > 0.05$)

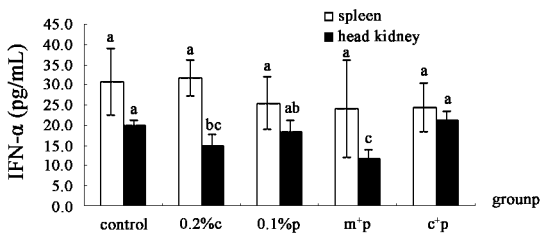

Fig.5 IFN – α concentration of spleen and head kidney in the cultured media with LPS stimulation

Values are expressed as the mean ± SD. Different letters at the bar's top indicate significance at $P < 0.05$, and values sharing the same letter are not significantly different ($P > 0.05$)

3 Discussion

3.1 Effect of chitosan and probiotics on immune function

The non-specific immunity is the first front line of defence against microbial infection in high vertebrate. It involves a multitude of cells and antimicrobial proteins, glycoproteins and peptides in tissues and body fluids. Immunity in fishes is substantially of the same nature.

Lymphocytes from spleen and head kidney showed responses to LPS and PHA, which demonstrated that there existed T and B cell lines both in spleen and head kidney. The different responses between spleen and head kidney lymphocyte proliferation were similar to the research of lymphocyte from thymus, anterior kidney and peripheral blood of common carp[14]. The difference may result from different total counts or differentiation degree of T and B cells[15]. In our study, different response was found in various groups or different organs of the same group, for instance, the mix of chitosan plus probiotics stimulated lymphocyte proliferation in head kidney, but not in spleen that indicated the selective feature of the mix on immune organs. Jang *et al*[16] also ascertained that glycyrrhizin served as immune stimulant affected leucocytes response to PHA but not LPS in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Accordingly, it is necessary to conduct further research on whether immunohancers have selective effect on immune organs or lymphocytes.

Immunoglobulin is the most important medium in humoral response. Up to now, it is commonly known

that bony fish has no other immunoglobulin class than IgM class. For this reason, the quantitative determination of IgM can reflect fish immune function to a considerable extent. Commonly, bony fish has IgM similar to that of mammals. Because there is no enough serum in the tested fish and consequently lack of purified pufferfish IgM, so mouse anti-human IgM was used in the quantitative determination instead of antiserum against IgM of the tested fish. Naturally, a certain mount of IgM exists in fish body, and furthermore, the variation in level and proportion of IgM was reported in different fish species. The IgM concentration in serum of *Salmo salar* and *Oncorhynchus keta* is generally lower than 1 mg · mL^{-1}[17-18], and that of paddlefish, *Polyodon spathula* is up to 17 mg · mL^{-1}[19]. The IgM concertration of *Fugu obscurus* produced by B lymphocytes stimulated with LPS was 0.23 – 0.52 mg · mL^{-1}, comparable to the IgM level found in *Salmo salar*. Ig expression and secretion was affected by stress, temperature, antigen route of administration as well as immunoenhancers. It has shown an increase of level of gamma-globulin fraction in plasma of Russian sturgeon (*Acipenser gualdenstaedti* Brandt) when immersed with chitosan[20]. Healthy and cortisol treated rohu (*Labeo rohita*) after single intraperitoneal injection of chitosan had significantly higher responses in serum albumin and serum globulin[21]. Rainbow trout treated with chitosan by injection or immersion or oral intake showed the increment in total Ig concentration in blood[7-8]. In this experiment, the oral administration of chitosan could increase IgM concentration in B cells culture supernatant, not of spleen, but of head kidney. It is the same as that treated with 0.1% probiotics and the mix of mannan oligosaccharide plus probiotics, while the mix of chitosan plus probiotics enhanced IgM concentration both in spleen and head kidney cells. In conclusion, immunoenhancers play a more important enhancing role on immune function of head kidney cells (B cells proliferation and IgM level). The following fact or suspect might be the reasons that are responsible for the above results. Firstly, head kidney is an important antibody production organ and has a superior role in humoral response in comparison with spleen[22]. Secondary, chitosan or other immunoenhancers can enhance an increase in the number of antibody formation cells and Ig production, particularly in head kidney.

The interferon system is an important constituent of the cellular non-specific defence mechanisms. Type Ⅰ IFNs(IFN – α and β) activity has been detected in cell supernatants, serum and organs from fish induced by virus, their characterization was consistant with that of high vertebrate α/β interferon, and type Ⅱ IFN (IFN – γ) activity similar to mammal has been also measured from trout and grass carp induced by ConA or PMA or PHA[23-24]. Graham and Secombas believed that there was an increased level of IFN with the increased ratio of the number of macrophages to the same total number of leucocytes[23]. In our study, human IFN – α – like was quantitatively determined firstly in B cells supernatants of spleen and head kidney induced by LPS. The level of human IFN – α – like differed between spleen and head kidney, also among different immunoenhancers groups, but it is in need of further study to determine whether it is related to the number of macrophages. Except the groups treated with 0.1% probiotics and the mix of probiotics plus chitosan, IFN – α – like concentration in head kidney B cells supernatant of other two test groups was lower than that of control group, which might be with mutual coordination and restriction among immune cells or cytokines. Due to low homology between some species of fishes immune system genes and their orthogene in mammals and lack of an effective system for studying these genes in previous researches, little has been known about fish IFN gene[25-26]. However, pufferfish has high degree of genic homology with mammals, and the great majority of human genes have counterparts in *Fugu* (http://genome.jgi – org/fugu6/fugu6.info.html), and has been proposed as a model vertebrate genome to study human gene[27], accordingly, ELISA kit for human IFN – α assay was used to determine IFN – α content in fish in present experiment, which was

undoubtedly helpful for future study on fish IFN.

3.2 Effect of chitosan and probiotics on antimicrobial activity

Chitosan has antimicrobial activity characteristics. Previous papers showed that antimicrobial activity of chitosan for *Esherichia coli* would be strengthened with the increase of deacetylation degree[28]. The deacetylation degree of chitosan used in this experiment was higher than 90% with enough NH3 + existing in molecular chain, by which a lot of *E. coli* and *Aeromonas* sp. would be deformed and autolysed. At the same time, the molecular weight of chitosan also affected the antibacterial activity. It became strengthened with the decrease of molecular weight [29]. Although the molecular weight of chitosan used in this study was less than 300 000, chitosan with high deacetylation degree might be degraded to smaller molecules by chitosanase generated by *B. subrilis* and *B. licheniformis*[30-31]. Moreover, chitosan could induce and enhance expression of chitosanase gene [32]. Thus, different chitosans with various molecular weights played a more effective role against pathogen together. Some probiotics, such as *B. subrilis* and *B. licheniformis*, showed restraining effect on pathogenic microorganism[33-36]. Both antimicrobial activity and immune role of chitosan and probiotics may be attributed to the improved ability of fish against Ah infection.

Theoretically, mechanism of immunoenhancers differed in various species. So synergic effect or counteraction will be emerged when they are mixed. No synergic or cumulative effects were achieved by combining lactic bacteria and immuno - stimulating peptides on Atlantic cod (*Gadus morhua*) challenged with *Vibrio anguillarum*[37]. The efficacy of the mixture of selenoyeast and *Bacillus* sp. on resistance against *A. hydrophila* was better than that of selenoyeast or *Bacillus* sp. did alone[38]. In this experiment, the mix of chitosan plus probiotics was more effective than chitosan or probiotics did alone on disease resistibility and non-specific immunity (LD_{50}, IgM content in B cell culture supernatant of spleen and cell proliferation from head kidney) as well as the mixture of mannan oligosaccharide plus probiotics (disease resistibility, T cells proliferation from head kidney). It seemed that the interaction of chitosan and probiotics perhaps led to the enhanced resistibility against Ah in the mixture groups compared with the single chitosan or probiotics group, and the resistibility against infection of pathogen might be dependent on head kidney cells proliferation much more in *Fugu obscurus*.

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壳聚糖和益生菌对暗纹东方鲀 抗病力和免疫功能的影响

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摘要: 为了解壳聚糖和益生菌刺激和增强免疫应答的可能作用,本试验在基础饲料中添加0.2%、0.5%、1.0%的壳聚糖,0.1%、0.2%、0.4%的益生菌,甘露聚糖与益生菌混合物,壳聚糖与益生菌的混合物,在室内水泥池中喂养暗纹东方鲀(3.15±0.05) g 2个月,根据抗菌活力筛选出免疫增强饲料组(0.2%壳聚糖,0.1%、益生菌,甘露聚糖与益生菌混合物,壳聚糖与益生菌混合物)测定免疫功能。结果表明,甘露聚糖与益生菌混合物仅提高头肾T淋巴细胞转化,壳聚糖与益生菌混合物显著增强头肾T、B淋巴细胞转化和脾脏B淋巴细胞体外培养时分泌的IgM。筛选出的四种免疫增强饲料均能使头肾B淋巴细胞体外培养时 分泌的IgM显著增加。无论是脾脏B淋巴细胞还是头肾B淋巴细胞 ,体外培养时所分泌的IFN – α都不同程度地受抑制。

关键词: 暗纹东方鲀; 壳聚糖; 益生菌; 抗病能力; 免疫功能

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