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Enhanced immune response and resistance to edwardsiellosis following dietary chitooligosaccharide supplementation in the olive flounder (*Paralichthys olivaceus*)

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ABSTRACT

This study was conducted to evaluate the effects of dietary chitooligosaccharide (COS) supplementation on peripheral leukocyte count, head kidney leukocyte phagocytic rate, phagocytic index, respiratory burst activity, serum lysozyme activity, and immune protection in *Paralichthys olivaceus*. A total of 300 flounder with an average body weight of 80–100 g were randomly assigned into four dietary groups: (I) basic diet (control), basic diet containing (II) 0.5% COS, and (III) 1% COS, fed continuously for 28 d, and (IV) basic diet containing 1% COS fed in 14 d intervals. Continuous feeding of 0.5% and 1% COS diets for 28 d significantly increased the number of peripheral leukocytes, head kidney leukocyte phagocytic rate, phagocytic index, respiratory burst activity, and serum lysozyme activity ($P < 0.05$ or $P < 0.01$). After a 10 d *Edwardsiella tarda* challenge, the immune protection rates in the 0.5% and 1% COS groups were 30% and 60%, respectively. No control fish survived the *E. tarda* challenge treatment. Most immune indices were slightly lower after removal of COS from the diet for 14 d, but all immune indices were observed to recover after another 14 d of COS supplementation. This study demonstrates that supplementation of a basic diet with COS enhances the non-specific immune response and improves survival rates following infection with *E. tarda* in *P. olivaceus*. An optimized interval feeding strategy with diets containing 1% COS may have potential applications in the prevention of disease in aquacultured *P. olivaceus*.

1. Introduction

Olive flounder (*Paralichthys olivaceus*) is one of the most important maricultured fish species in several Eastern Asian countries, including China, Japan, and Korea. In recent years, the rapidly expanding olive flounder aquaculture industry has experienced substantial economic losses due to bacterial pathogens, particularly *Streptococcus iniae*, *Streptococcus parauberis*, *Lactococcus garvieae*, and *Edwardsiella tarda* [1–4]. *E. tarda* is one of the most notorious pathogenic bacteria in aquaculture and the mechanisms that enable it to survive host defense systems remains unclear. Prevention and treatment of edwardsiellosis is very difficult and typically depends upon the use of pharmaceuticals in flounder. Substance abuse has resulted in serious food safety problems for humans. Therefore, there is an urgent need to find safe and effective ways to prevent and treat edwardsiellosis.

Immune boosters have been widely used in fish and have proven effective in enhancing immunity and resistance to aquatic diseases. Chitooligosaccharide (COS) can be efficiently derived by chemical and enzymatic hydrolysis of polychitosan, which is the second most abundant carbohydrate polymer in nature [5–7]. More recently, COS has been shown to have immune-enhancing characteristics in livestock and fish, including *Trachinotus ovatus* and *Oncorhynchus mykiss* [8–10]. However, there are no relevant studies on the effects of COS supplementation on the enhancement of the immune response and protection from disease in flounder. In addition, the appropriate use of COS, which includes the correct dosage and route of administration, could make a difference in the effectiveness of the treatment.

In this study, a basic diet was supplemented with different concentrations of COS and fed continuously for 28 d or fed in 14 d intervals (COS diet for 14 d, basal diet for 14 d, COS diet for 14 d). Peripheral leukocyte count, head kidney leukocyte phagocytic rate, phagocytic index, respiratory burst activity, serum lysozyme activity, and immune protection in flounder were assessed throughout the feeding trial. Information regarding the effects of

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COS supplementation on the enhancement of immunity and disease resistance in flounder will have potential applications in the aquaculture industry.

2. Materials and methods

2.1. Experimental diets

The basic feed used in the experiments was purchased from Shandong Shengsuo Fish-feed Research Center (Shandong, China). The COS used in the current study was provided by the Dalian Institute of Chemical Physics, Chinese Academy of Sciences. Experimental feed was prepared by evenly spraying COS, dissolved in water, onto the basic feed at a rate of 0%, 0.5%, and 1% of diet weight. The diets were then dried in an oven for 12 h and stored in vented containers in a dry area at room temperature.

2.2. Fish and experimental design

A total of 300 apparently healthy olive flounder with a length of 19 ± 2 cm and a weight of 90 ± 10 g were purchased from He Shengfeng marineland (Dalian, China), transferred into the school laboratory (Mariculture & Stock Enhancement in North China's Sea, Ministry of Agriculture), and acclimated for 10 d with re-circulated seawater at 16 °C before the initiation of the feeding trial. Fish were fed at 3% BW d^{-1} twice daily (9 am and 6 am).

At the beginning of the experiment, flounder were randomly divided into four groups: basal diet (group I; control), basal diet supplemented with 0.5% (group II) or 1% COS (group III) fed continuously for 28 d, and basal diet supplemented with 1% COS fed in 14 d intervals (group IV; 1% COS diet for 14 d, basal diet for 14 d, and 1% COS diet for 14 d). Each experimental and control group consisted of three replicates with 25 fish in each tank.

2.3. Sample collection

Samples were collected at 7 d intervals throughout the experiment. In order to obtain the serum and whole blood, respectively, blood was collected into both nonheparinized and K_3EDTA vacuum tubes by caudal venipuncture with a 27 gauge needle and 1 mL syringe. The whole blood from nonheparinized tubes was allowed to clot at room temperature for 4 h after collection. Following centrifugation (3000× *g*, 10 min, 4 °C), the serum was removed and frozen at −80 °C until further use.

Head kidney leukocyte from olive flounder in each tank were isolated as described by Secombes with some modifications [11,12].

2.4. Immunological assays

2.4.1. Total leukocyte count (TLC)

The TLC was calculated using a Neubauer chamber, according to Ranzani-Paiva et al. [11]. The leukocytes were counted manually in all 25 squares (=0.1 mm^3). Differential leukocyte counts (neutrophils, lymphocytes, monocytes) were determined using blood smears under a light microscope.

2.4.2. Phagocytic activity

A 100 μL cell suspension of head kidney leukocytes (1×10^7 cells/mL) was placed onto a sterile slide and the cells were allowed to adhere for 30 min at 25 °C. Following attachment, 100 μL of yeast suspension (Baker's yeast, Type II, Sigma, USA, 1×10^8 cells/mL) was added to the cell monolayer and the slide was incubated for 45 min at 25 °C. The unattached cells were washed off with PBS and the slides were air-dried prior to being fixed in methanol, dried, and stained with Giemsa solution (Sigma–Aldrich, St. Louis, MO, USA). Slides were viewed under oil immersion at 100×. Approximately 100 cells were counted in random fields of view and phagocytic percentage (PP) and phagocytic index (PI) were determined as follows:

PP (%) = (number of cells involved in phagocytosis ÷ total duration head kidney cells) × 100

PI (%) = (number of phagocytosed bacteria ÷ number of cells involved in phagocytosis) × 100

2.4.3. Respiratory burst activity

The respiratory burst activity of head kidney leukocyte was measured using nitroblue tetrazolium chloride (NBT, Sangong Biotech Corp., China) following the method of Secombes et al. [11]. The absorbance at 630 nm was measured with a Model Multiskan spectrum (Thermo, USA) using KOH/DMSO alone as a blank. Respiratory burst activity was expressed as NBT-reduction in 100 μL of cell suspension.

2.4.4. Lysozyme activity

Serum lysozyme level was determined using a turbidimetric assay according to the method described by Ellis et al. [13]. Briefly, test serum (0.1 mL) was added to 1.9 mL of 0.05 M sodium phosphate buffer (pH 6.2) containing 0.2 mg/mL *Micrococcus lysodeikticus* (Sigma). The reaction was performed at 25 °C and the absorbance was measured at 530 nm after 0.5 and 4.5 min in a spectrophotometer. One unit of lysozyme activity was defined as the amount of sample causing a reduction in absorbance of 0.001 min^{-1}.

2.5. Challenge

The *E. tarda* strain (stored in our lab) was originally isolated from infected *P. olivaceus* and identified using morphological, physiological, and biochemical characteristics, as well as sequence analysis of the 16S rRNA gene. At the end of 28 d feeding experiment, ten fish from each replicate of I, II and III group were individually injected intraperitoneally with 0.2 mL of aseptic seawater containing 2.2×10^8 live *E. tarda*. The water temperature was maintained at approximately 13 °C–19 °C. No food was given to the animals during the *E. tarda* challenge experiment. The cumulative mortality was counted over 10 d and a total of 10 flounder in each tank were used to express cumulative mortality and relative percent survival (RPS) values as follows.

2.6. Statistical analysis

All data were analyzed with SPSS software (SPSS Inc., Chicago, IL, USA), and the results are presented as mean ± standard error (SE). Differences between groups were determined using Duncan's test.

3. Results

3.1. TLC

The TLC was significantly improved in all *P. olivaceus* fed diets containing COS in comparison to the controls. In the groups continuously fed with 0.5% and 1% COS for 28 d, the TLC first increased and then decreased. In fish fed diets containing COS in 14 d intervals (group IV), the TLC decreased slightly after the removal of treated food, but after another 14 d of continuous feeding with the COS diet, the TLC recovered to the previous high level (Table 1).