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Diet enriched with mushroom *Phellinus linteus* extract enhances the growth, innate immune response, and disease resistance of kelp grouper, *Epinephelus bruneus* against vibriosis

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ABSTRACT

The effect of diet supplemented with *Phellinus linteus* fed for 30 days was investigated in grouper *Epinephelus bruneus* challenged with *Vibrio anguillarum*, *Vibrio harveyi*, *Vibrio alginolyticus*, and *Vibrio carchariae*; infected and treated fish had a significantly higher percent weight gain and feed efficiency. In groups fed with enriched diet and challenged with *V. anguillarum* and *V. harveyi* the mortality rate declined with a consequent rise in survival rate than with other pathogens. On the other hand, in groups fed with *P. linteus* enriched diet and challenged with *V. anguillarum*, *V. harveyi*, and *V. alginolyticus* the cellular and humoral immune responses, such as the alternative complement activity (ACH_{50}), serum lysozyme activity, phagocytic activity (PA), phagocytic index (PI) significantly higher than in the control group. The respiratory bursts (RB), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities were found significantly enhanced when the groups fed with enriched diet against *V. anguillarum* and *V. harveyi*. The results reveal that kelp grouper fed for 30 days with *P. linteus* enriched diet had higher cellular and humoral immune response and disease protection from vibriosis than the group fed on basal diet with the protection linked to stimulation of immune system.

1. Introduction

The intensification and globalization of the seafood trade have led to remarkable developments in the aquaculture industry. Groupers are popular high priced seafood species cultured widely in Southeast Asian countries. Among more than 150 species of grouper distributed worldwide kelp grouper *Epinephelus bruneus* (Bloch) is one of the commercially important species as luxury protein in Korea and Japan; its culture started recently [1,2]. However, adverse environmental conditions and a host of infectious viral, bacterial, and parasitic pathogens often result in serious economic losses; among these Vibriosis is one of the most widely distributed pathogens that often infect intensive fish farms [3,4].

These pathogens come under the family Vibrionaceae comprise a group of over 80 easily cultured, Gram-negative, heterotrophic bacteria and are most frequently associated with farmed fish and shell fish [5]. Among these pathogens that trigger diseases due to crowded rearing conditions during intensive rearing *Vibrio anguillarum*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio ulnificus*, *Vibrio harveyi*, and *Vibrio carchariae* are known to inflict a most serious disease with fatal haemorrhagic septicaemia and gastroenteritis syndrome that results in swollen intestine containing yellow fluid; this fatal disease has threatened the sustainability of grouper culture and leads to severe economic loss in commercial marine industries throughout the world including South Korea [6–15]. Since little is known about the genetics and immunology of *E. bruneus* it is necessary to establish effective measures for disease control and genetic improvement [16]. Therefore, enhancement of fish's innate immunity that can improve the health fish is a primary concern to ensure viable culture practice.

Extensive chemotherapy with antimicrobial agents used in disease prevention and to promote growth has resulted in the emergence of drug-resistant microorganisms besides leaving antibiotic-residues in the farmed fish and environment [17]. Several studies have shown that different vaccine formulations including

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formalin-killed bacteria, heat-inactivated *V. anguillarum* cells and *V. anguillarum* bacterin provide protection against vibriosis [18,19]. There are currently no effective vaccines available against other species that cause vibriosis, and hence the disease control is still one of the serious problems in marine aquaculture [20].

Mushrooms have been reported as immune potentiators with antibacterial, antifungal, anti-tumor, and immunomodulatory activities which enhance innate immune response, lymphocytes proliferation, and antibody production [21–34]. A variety of mushrooms are distributed in Korea, Japan, and China; among these *Phellinus linteus*, an orange color mushroom in the family of Hymenochaetaceae, has been used as a traditional medicine in South Korea. Recently, *P. linteus* has been reported to possess anti-tumor activity [21–31]. Recent reports indicate that diet containing *P. linteus* or *Coriolus versicolor* enhanced the growth, blood physiology, innate immune response, and disease resistance in olive flounder against *V. anguillarum* [35,36]. To our knowledge this is the first study to assess the effect of feeding *E. bruneus* with diet enriched with *P. linteus* on growth, innate immune response, and diseases resistance against vibriosis caused by *V. anguillarum*, *V. harveyi*, *V. alginolyticus*, and *V. carchariae*.

2. Materials and methods

2.1. Fish

Kelp grouper, *E. bruneus* (average weight 12.4 ± 1.6 g, $n = 600$) were obtained from Dongbok fish farm located in Eastern Jeju Island, Republic of Korea were transported and acclimatized for 2 weeks in a recirculating culture system in 500-L circular cement tanks in the Marine and Environmental Research Institute, Jeju National University. All the tanks were equipped with an air-filter. During the experiment, 50% of the sea water was renewed daily to maintain the water quality monitored as: dissolved oxygen 8.5 ± 0.7 mg l^{-1}, photoperiod 10:14 h (light/dark), temperature 21.3 ± 1.6 °C, salinity 33.3 ± 0.6 ppt, and pH 7.4 ± 0.8. Fish were fed with a basal diet (Table 1) *ad libitum* twice a day at 09:00 and 15:00 h at 5% of their body weight.

Table 1
Composition of the basal diet (g/100 g) for kelp grouper.

Ingredients	%
Fish meal	65.0
Squid meal	7.5
Shrimp meal	4.5
α-Starch	18.0
Mineral mixture[a]	2.0
Vitamin mixture[b]	1.0
Skim milk	1.0
Phellinus linteus extract	1.0
Chemical analyses (% DM)	
Crude protein	43.3
Crude carbohydrate	16.8
Crude fat	12.9
Crude ash	7.7

[a] Mineral premix (g kg^{-1} mixture) $MgSO_4 \cdot 7H_2O$, 75.0; $NaH_2PO_4 \cdot 2H_2O$, 350.0; KCl, 125.0; ferric citrate, 50.0; $ZnSO_4 \cdot 7H_2O$, 25.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3 \cdot 6H_2O$, 0.2; $Na_2Se_2O_3$, 0.01; $MnSO_4 \cdot H_2O$, 2.0; and $CoCl_2 \cdot 6H_2O$, 1.0.
[b] Vintamin premix (g kg^{-1} mixture) L-ascorbic acid, 120.0; DL-α tocopheryl acetate, 17.5; thiamin hydrochloride, 2.5; riboflavin, 9.0; pyridoxine hydrochloride, 2.0; niacin, 35.0; Ca-D-pantothenate, 12.5; myo-inositol, 180.0; D-biotin, 0.25; folic acid, 0.65; paminobezoic acid, 18.0; menadione, 2.0; retinyl acetate, 0.70; cholecalficerol, 0.003; and cyanocobalamin, 0.003.

2.2. Preparation of P. linteus extract

Mycelium of mushroom *P. linteus* was purchased locally and extracts were made using ethanol solvent extract fractionation. One hundred grams of dried mushroom mycelium was dissolved 1000-ml of 70% ethanol in 2000-ml conical flasks. The conical flasks were tightly covered with aluminium foil, kept for 7 d at room temperature and agitated daily. Later, the extract was filtered using 3 M filter paper (0.45 μM) to remove debris. The filtrate was collected and the solvent was evaporated (freeze-dried) using rotary vacuum evaporator (Buchi SMP, Switzerland) and stored at −4 °C prior to use.

2.3. Preparation of experimental diets

With basal diet (Table 1) 1 g of *P. linteus* freeze-dried extract was sprayed to the basal diet slowly, mixing evenly in a drum mixer, after which it was air dried under sterile conditions for 12 h. The control basal diet contained the same volume of ethanol without the mushroom extract. The pellets were dried in an oven at 30 °C for 18 h, packed and stored in a freezer at −20 °C until used. The proximate analysis of the basal diet quantified according to the AOAC method comprised 43.3% crude protein, 12.9% crude lipid, 7.7% crude ash, and 16.8% crude carbohydrate.

2.4. Culture of pathogens

In this study, four bacterial pathogens, *V. alginolyticus*, *V. carchariae*, *V. harveyi*, and *V. anguillarum* isolated from infected olive flounder were kindly provided by Dr. Heo M.S, Department of Aquatic Biomedical Science, Jeju National University. The bacterium was cultured and collected by previously described methods [7,10,12,14] except that phosphate buffer saline (PBS) was used to adjust the bacterial suspension approximately to 2.48×10^9 CFU ml^{-1} of each pathogen for the susceptibility study in *E. bruneus*.

2.5. Experimental design

Kelp grouper were divided into two groups of 120 each. One group was fed with *P. linteus* supplementation diet ($n = 120$) and another group on basal diet without supplementation of *P. linteus* ($n = 120$) twice daily for 30 days. On 30th day of feeding each group was further divided into 4 sub groups of 30 fish each; they were randomly collected and challenged intraperitoneally (i.p.) with 0.1 ml phosphate buffer saline (PBS, Sigma–Aldrich) suspensions containing *V. alginolyticus*, *V. carchariae*, *V. harveyi*, or *V. anguillarum* approximately 4.48×10^9 CFU ml^{-1}. Another two groups of 30 fish each were used as control; one group was give with basal diet without mushroom extracts received the same volume of PBS alone and another group given with enriched diet with mushroom extracts. All groups were maintained in replicate as mentioned maintaining water quality. In previous work we determined that these cell numbers inflict death 60–70% mortality of the kelp grouper with either bacterium. After two week post-challenge, six fish were randomly sampled from one of the replicate tanks in each treatment for immunological assay and all the fish ($n = 30$) were used to calculate percent weight gain, feed efficiency, and survival rate [37]. The relative percent survival (RPS) was calculated by the method of Amend [38] as 1-Percent mortality in treated group given enriched diet with mushroom extracts/Percent mortality in control group given enriched diet without mushroom extracts × 100. Individual fish were sampled once to avoid multiple bleeding and/or handling stress.