Fish & Shellfish Immunology 32 (2012) 339–344

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Effect of *Coriolus versicolor* supplemented diet on innate immune response and disease resistance in kelp grouper *Epinephelus bruneus* against *Listonella anguillarum*

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ARTICLE INFO

Article history:
Received 13 July 2011
Received in revised form
21 November 2011
Accepted 22 November 2011
Available online 30 November 2011

Keywords:
Coriolus versicolor
Epinephelus bruneus
Innate immune response
Listonella anguillarum
Supplementation diet

ABSTRACT

The effect of *Coriolus versicolor* extract supplemented diets on innate immune response and disease resistance in kelp grouper, *Epinephelus bruneus* against *Listonella anguillarum*, is reported. Kelp grouper were divided into four groups of 25 each and fed with *C. versicolor* enriched diets at 0% (control), 0.01%, 0.1%, and 1.0% level. After 30 days of feeding, all fish were injected interaperitoneally (i.p.) with 50 μl of *L. anguillarum* (4.7×10^7 CFU) to investigate the immune parameters at weeks 1, 2, and 4. The reactive oxygen species and reactive nitrogen species production were significantly enhanced in fish fed with 0.1% and 1.0% supplementation diets from weeks 1–4 when compared to the non enriched diet fed and infected control. The phagocytic activity significantly increased with 0.1% and 1.0% diets on weeks 2 and 4. The leucocyte myeloperoxidase content, lysozyme activity, and total protein level significantly increased when fed with 0.1% and 1.0% supplementation diets from weeks 1–4. The cumulative mortality was 35% and 45% in 1.0% and 0.1% enriched diet fed groups whereas it was 55% and 80% in 0.01% and 0% groups respectively. The present results suggest that diets enriched with *C. versicolor* at 0.1% or 1.0% level positively enhance the innate immune system and affords protection from *L. anguillarum*.

1. Introduction

Groupers belong to the genus *Epinephelus* and are widely distributed in subtropical and temperate waters. Recently there has been a considerable interest in the development of grouper aquaculture particularly in the Asia-Pacific region because of their fast growth, efficient feed conversion, and high market value [1]. There is a corresponding spurt in the annual market for grouper larvae to the tune of more than 300 million US dollars [2]. However to meet the increasing demand and limited supply of farm reared larvae still fry are still collected from the wild to supplement the demand [3]. Added to this is the limited supply of trash fish which is the main feed source during grow-out phase [1,4]. Besides grouper aquaculture has incurred severe economic loss due to viral [5–7] and bacterial [8–10] diseases. Among the bacterial disease, *Listonella (Vibrio) anguillarum* is an importance in grouper culture.

In aquaculture immunostimulation is one of the promising tools in fish disease management since vaccination processes are often laborious and expensive; repeated administration of chemotherapeutic agents also pose the problem of emergence of drug resistant strains of pathogens. A promising alternative is the immunostimulants which activate non-specific defense mechanisms thereby protecting the fish against infectious pathogens. For example FK-565 [11] and MDP (muramyl dipeptide) derived from *Mycobacterium* [12], lipopolysaccharide (LPS) of *Vibrio anguillarum* bacterin [13], glucan [14], chitin [15], levamisole [16], lactoferrin [17], nisin [18], recombinant transferrin [19], yeast RNA, and modified carbohydrate [20] have been reported to enhance non-specific immune system and protect from diseases in cultured fish.

In traditional Chinese medicine mushrooms have been highly valued for their energizing and healing properties for thousands of years. One among these is *Coriolus versicolor* an extremely common polypore mushroom of the Basidiomycetes family with global distribution. Recent research findings show that *C. versicolor* stands out above the rest for improving the immune system. In China, Japan, and Korea *C. versicolor* is used as an immunoadjuvant in the treatment of cancer and boost the immune system (www.cancer.

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doi:10.1016/j.fsi.2011.11.026

com); it exhibits anti-cancer activity both *in vitro* [21] and *in vivo* [22]. Polysaccharopeptides (PSPs), a protein-bound polysaccharide isolated from *C. versicolor*, is considered as a strong potential candidate for drug development in treatment and prevention of human cancers [23,24], suppression on DNA and RNA synthesis [25], and enhance immune functions [23,24]. Therefore, the present study was performed to investigate the effect of *C. versicolor* supplemented diets on innate immune response and disease resistance in kelp grouper *Epinephelus bruneus* against *Listonella anguillarum*.

2. Material and methods

2.1. Supplementation diets

Mycelium of mushroom *C. versicolor* was purchased locally and its extracts were made using ethanol solvent following Harikrishnan et al. [26]. One hundred grams of dried mushroom mycelium was dissolved in 1000-ml of 70% ethanol in 2000-ml conical flasks. The conical flasks were tightly covered with aluminum foil, kept for 7 d in room temperature and agitated daily. Then extract was then filtered using 3 M filter paper (0.45 mM) 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. The basal diet containing 55% fish meal and 14% krill meal were used as the protein sources. The 8% fish oil, 2% guar gum, 4% CMC Na, 11% cellulose, and 6% vitamin and mineral mixture was used as the carbohydrate and lipid sources. The ingredients of the experimental diet were well mixed and extruded using a pellet extruder (EX 920, Matador, Denmark). Four experimental diets were prepared with 0% (control) 0.01%, 0.1%, and 1.0% of *C. versicolor*; the extracts were sprayed to the basal diet slowly and mixed evenly in a drum mixer, after which the feeds were air dried under sterile conditions for 12 h. The pellets were oven dried at 30 °C for 18 h, packed, and stored in a freezer at −20 °C until used. The proximate composition (g/kg) of the diet comprised crude protein 46.3%, crude fat 12.6%, crude moisture 11.8%, and crude ash 9.2%.

2.2. Fish

Kelp grouper, *E. bruneus* (weight 25.4 ± 1.3 g) obtained from Dongbok fish farm located in Eastern Jeju Island, South Korea were transported to the laboratory and acclimated in the Marine and Environmental Research Institute, Jeju National University indoor tanks (capacity: 500-L) with recirculating aerated seawater. Continuous aeration was also provided to maintain dissolved oxygen levels at 8.5 ± 0.5 mg l^{-1}; 50% of the seawater was exchanged with sand-filtered seawater daily. During the experimental period water temperature, pH, and salinity were 26 ± 1 °C, at 7.7 ± 0.8, and at 31.2 ± 1.3‰. The photoperiod of 10:14 h (light/dark cycle) was provided by fluorescent light throughout the experimental period. During the acclimatization period fish were provided with a basal diet without *C. versicolor* extract *ad libitum* twice a day at 09:00 and 15:00 h at a rate of 5% of their body weight.

2.3. Listonella anguillarum

L. anguillarum was isolated from diseased olive flounder and maintained in the Department of Aquatic Biomedical Science, Jeju National University. The bacterium was cultured and characterized by previously described method [26]. For challenge study the phosphate buffer saline (PBS) was used after adjusting the bacterial suspension to 4.7 × 10^7 CFU ml^{-1}.

2.4. Experimental design and challenging study

The kelp grouper were divided into four groups of 25 each in triplicate in 100-L tanks and fed with 0% (control), 0.01%, 0.1%, and 1.0% *C. versicolor* supplementation diets as described above. Diets were provided twice a day at 5% of their body weight until the end of the experiment. After 30 days of feeding, each fish was injected intraperitoneally (i.p.) with 50 μl of PBS suspension containing *L. anguillarum* at 4.7 × 10^7 CFU ml^{-1}; the control group was injected only with PBS. The cumulative mortality was calculated following Harikrishnan et al. [26] as 1-Percent mortality in treated group given diet supplementation diet/Percent mortality in control group given without supplementation diet × 100.

2.5. Sampling

The head kidney (HK) leucocytes and blood were collected from six fish in each experimental group at weeks 1, 2, and 4 for immunological and biochemical assays. Blood was collected from the dorsal aorta and allowed to clot at 20 °C for 30 min and cooled at 0 °C for 1 h. The serum was obtained by centrifugation at 2000 g for 5 min and stored in frozen at −20 °C until used.

2.6. Preparation of head kidney (HK) leucocytes

The HK leucocytes were prepared by a modified method of Santarem et al. [27]. The grouper HK was dissected out by a ventral incision, cut into small fragments, and transferred to 5-ml Hanks' balanced salt solution (HBSS). The HK leucocytes cell suspensions were obtained by teasing the HK tissues with 2 glass slides in HBSS in a Petri dish (Corning, USA). The supernatants were removed after sedimentation of tissue debris at 4 °C for 1 min. The HK cell suspensions were layered over a 34–51% percoll gradient and centrifuged at 800 g for 30 min at 14 °C. Then the bands of leucocytes above 34–51% interfaces were collected with a Pasteur pipette and washed twice at 125 g for 8 min in HBSS. The concentration of viable cells was determined by trypan blue exclusion.

2.7. Immunological and biochemical assays

The reactive oxygen species (ROS) production was measured from grouper HK leucocytes by monitoring their ability to reduce nitroblue tetrazolium (NBT, Sigma, USA) according to the method of Secombes et al. [28]. Reactive nitrogen species (RNS) production was measured by peripheral blood leucocytes using Griess reagent [29]. Phagocytic activity was measured from HK leucocytes according to Matsuyama et al. [30]. Total myeloperoxidase content in peripheral blood leucocytes was measured following Palic et al. [31]. Serum lysozyme activity was measured using turbidimetric microtitre plate technique following Ellis [32]. The serum total protein was determined following Bradford method [33].

2.8. Statistical analysis

The statistical significance as differences between groups was calculated by applying Student's *t*-test.

3. Results

3.1. Reactive oxygen species (ROS) production

The ROS production did not get enhanced significantly in fish fed with 0.01% and 0.1% diet fed groups on week 1 against *L. anguillarum* when compared to control. However, 1.0% supplementation diet significantly enhanced the ROS production against