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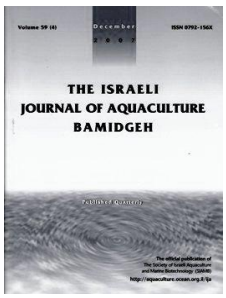
ISSN 0792 - 156X

PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il



Effect of *Coriolus versicolor* Supplementation on Hematology and Innate Immune Response in Olive Flounder against *Pseudocohnilembus persalinus*

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(Received 4.8.11, Accepted 13.7.11)

Key words: hematological and biochemical profile, innate immune response, *Coriolus versicolor, Pseudocohnilembus persalinus, Paralichthys olivaceus*

Abstract

The effect of dietary *Coriolus versicolor* supplementation at 0%, 0.01%, 0.1%, and 1.0% on the hematology and innate immune response of olive flounder (*Paralichthys olivaceus*) against the ciliate *Pseudocohnilembus persalinus* was studied after one, two, and four weeks of feeding. White blood cells were significantly higher in fish fed the 0.1% and 1.0% diets than in those fed the control and 0.01% diets from week two onwards. Similarly, red blood cells were significantly higher in the 0.1% and 1.0% diets than the other diets at week 4. Hemoglobin was significantly highest in the 1.0% diet and hematocrit significantly highest in the 0.1% diet at weeks 2 and 4. Total protein, glucose, and calcium were significantly higher in fish fed the 0.1% and 1.0% supplementation diets from weeks 1 to 4. Scuticocidal and lysozyme activity were significantly enhanced in fish fed the 0.1% and 1.0% diets from week 1 to 4 while respiratory burst activity was significantly higher in fish fed the 0.1% and 1.0% diets from week 2 to 4. Cumulative mortality was lower in groups fed the 0.1% and 1.0% diets than in groups fed the control or 0.01% diets. The present study suggests that dietary supplementation with 0.1% or 1.0% *C. versicolor* protects hematological parameters, improves innate immune response, and affords protection from *P. persalinus* infection in olive flounder.

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Introduction

Scuticociliatosis caused by histophagous opportunistic ciliate pathogens is an important parasite in farmed marine fish, including *Philasterides dicentrarchi* infecting turbot (Iglesias et al., 2003) and *Uronema marinum* in olive flounder (Jee et al., 2001). *Pseudocohnilembus persalinus* is an important scuticociliate responsible for scuticociliatosis in cultured olive flounder in Korea (Kim et al., 2004). Scuticociliates are characterized by high potential for systemic invasion, destroying tissues and leading to high mortality of the host.

Attempts have been made to treat scuticociliatosis with chemotherapeutants (Iglesias et al., 2002), however development of an effective vaccine may be the best way to control scuticociliatosis. Surface immobilization antigens (i-antigens) have been studied as the principal target antigens for developing protective vaccines against ciliates such as *P. dicentrarchi* and *Ichthyophthirius multifiliis*, an obligate parasitic ciliate of freshwater and marine fish (Iglesias et al., 2003). Since different serotypes of the ciliates exist in nature on the basis of immobilization (Dickerson and Clark, 1996), protective immunity is serotype specific (Wang et al., 2002). Immunostimulation is one of the promising tools for aquaculture, since vaccination and/or treatment by injection is laborious and often expensive while repeated administration of chemotherapeutic agents can cause the emergence of drug resistant strains of pathogens. Immunostimulants activate non-specific defense mechanisms, thereby protecting fish against infectious pathogens (Siwicki et al., 1994).

Coriolus versicolor belongs to the common polypore mushroom of the Basidiomycetes family and has been highly prized as a Chinese medicine for thousands of years. It is marketed throughout the world for its energizing and healing properties. In China, Japan, and Korea, *C. versicolor* is used as an immunoadjuvant for anti-cancer activity *in vitro* (Liu et al., 1993) and *in vivo* (Li et al., 1990), and as a boosting agent for the immune system (Li et al., 1990). The present study investigates the effect of *C. versicolor*-supplementation diets on hematology, innate immune response, and disease resistance in olive flounder against *P. persalinus*.

Material and Methods

Fish. Healthy olive flounder (18.6±1.5 g) were purchased from a private fish farm in Jeju Island, South Korea, and transported to the laboratory. The fish were randomly distributed into 500-l flow-through tanks with a sea water flow rate of 6.8 l/min. Fish were acclimated for two weeks prior to the experiment and fed a basal diet *ad libitum* twice a day (09:00 and 15:00) at 5% of their body weight (Table 1). Tanks were provided continuous aeration using electric air compressors; 50% of the water was exchanged twice a week to remove unconsumed feed and feces. Ambient water conditions were maintained at 18±3.2°C, pH 7.97±1.24, salinity 33.6±1.8 mg/l, dissolved oxygen 6.78±1.56 mg/l, and photoperiod 14 h light:10 h dark.

Pseudocohnilembus persalinus. Ciliates were isolated from the brain, gill, or ulcerated skin of olive flounder collected from local fish farms in Jeju Island, South Korea, and cultured in minimum essential medium (MEM, Sigma) supplemented with 10% fetal calf serum or in filtered seawater supplemented with autoclaved yeast extracts at 20°C (Kim et al., 2004).

Table 1. Basal diet for olive flounder.

Ingredient	*%*
Mackerel meal	55
Wheat flour	18
Wheat gluten	8
Corn gluten	6
Fish oil	5
Dehulled soybean meal	2
α-potato starch	2
Mineral premix*	2
Vitamin premix*	1
Extract	1

* Provided by Suhyup Feed Co. Ltd. (South Korea).

Herbal extract. Mycelia of the mushroom *C. versicolor* were purchased locally and extracted using ethanol solvent extract fractionation. One hundred grams of dried mushroom mycelium were dissolved in 1000 ml of 70% ethanol in 2000-ml conical flasks. The flasks were tightly covered with aluminum foil, kept at room temperature for seven days, and agitated daily. Later, the extract was filtered using 3 M filter paper (0.45 mM) to remove debris and the filtrate was collected. The solvent was evaporated (freeze-dried) in a rotary vacuum evaporator (Buchi SMP, Switzerland) and stored at -4°C until use.

Preparation of enriched diets. Mackerel, dehulled soybean,

and corn gluten meals were used as the protein sources. Wheat flour, α-potato starch, wheat gluten, and fish oil were used as the carbohydrate and lipid sources. Ingredients were well mixed and extruded in a pellet extruder (EX 920, Matador, Denmark). Four experimental diets were prepared from the pellets with 0%, 0.01%, 0.1%, or 1.0% *C. versicolor* extracts slowly sprayed onto the basal diet. The diets were evenly mixed in a drum mixer, then air dried under sterile conditions for 12 h. The same volume of solvent without the extract was added to the control diet. The pellets were dried in an oven at 30ºC for 18 h, packed, and stored in a freezer at -20ºC until use. The proximate composition of the diet, quantified following AOAC methods, was 53.9% crude protein, 8.1% crude lipid, 7.6% crude ash, and 15.4% crude carbohydrate.

Experimental design and challenge experiment. Three hundred fish were divided into four treatment groups with 25 fish in each of three replicates. The groups were given diets supplemented with 0%, 0.01%, 0.1%, or 1.0% *C. versicolor* extract. On day 30 of feeding, all fish were injected intraperitoneally with 100 µl PBS containing *P. persalinus* at 2.6 x 10^7 ciliates/ml. Fish continued to receive their respective diets until the end of the experiment. One, two, and four weeks post-challenge, six fish were randomly collected from each tank and anesthetized with MS-222 ($NaHCO_3$ and tricaine methanesulphonate; Sigma Chemicals) 1:4000 in dechlorinated water for 2 min. Cumulative mortality was calculated by the formula of Amend (1981).

Collection of blood samples. Approximately 0.5 ml blood was collected by caudal venupuncture using a 1-ml syringe fitted with a 20 gauge needle. The blood was kept at 4°C in an aqueous solution using heparin as an anticoagulant. Half of the blood was used for hematological and immunological examination and half was stored at -4°C for biochemical analysis.

Hematology and biochemical indices. Red blood cell counts (10^6/mm^3) were determined in a 1:20 dilution of the blood sample in Hayem's solution and white blood cell counts (10^4/mm^3) in a 1:200 dilution of the blood sample in Turke's solution with a Neubauer hemocytometer. The average of triplicate microhematocrits (%) was used to determine the red blood cell volume at 10,000 × *g* for 5 min (Larsen and Snieszko, 1961). Hemoglobin (g/dl) was determined by the cyanhemoglobin method. A 20-µl blood sample was drawn from a heparinized capillary tube and mixed in 5 ml cyanhemoglobin reagent (Hycel). Hemoglobin concentrations were determined at 540 nm with a Beckman DU spectrophotometer (Larsen and Snieszko, 1961). After reading the hematocrit, the packed erythrocytes were discarded and the plasma was stored at -12°C. Plasma biochemical indices were subsequently determined with a Hitachi 704C instrument. These included total protein (g/dl), glucose (mg/dl), and cholesterol (mmol/l), determined spectrophotometrically in the UV area, and calcium (mmol/l), determined by flame emission photometry (Hawk et al., 1954).

Serum scuticocidal activity. Pooled sera were used to analyze the scuticocidal activity in 96-well flat-bottomed micro-titration plates according to Lee and Kim (2003). The sera were serially diluted ranging from 1:4 to 1:4096 in Hank's balanced salt solution. Ciliates were cultured in chinook salmon embryo, collected after one day by centrifugation at 200 × *g* for 5 min, washed three times in HBSS, and resuspended in the buffer. Ciliates were added to each well at a density of 2.5 × 10^2. Plates were incubated at 20°C and observed at 1 h intervals for 24 h to analyze scuticocidal activity in the sera. The titer of each serum up to the last dilution was observed under an inverted microscope at 40-100× magnification to quantify all lysed and non-motile ciliates. In all assays, control wells containing heat-inactivated pooled sera (50°C for 30 min; 1:4 dilution) were maintained for each experimental group.

Separation of leukocytes. Blood was drawn from the caudal vein of six fish from each group. Leukocytes were separated from the anterior kidney by the method of Chung and Secombes (1988). The anterior kidney was aseptically resected, homogenized in Leibovitz-15 (L-15) medium (Sigma, St. Louis, MO, USA) supplemented with 0.2% heparin, 1% penicillin-streptomycin solution (10 000 units/ml penicillin and 10 mg/ml streptomycin; Sigma), and 2% fetal calf serum (FCS; Gibco BRL, Grand Island, NY, USA), and filtered through a 100-mm nylon mesh. The resulting cell suspension was carefully

poured on a 34%/51% Percoll (Sigma) density gradient and centrifuged at 450 × *g* for 30 min at 4°C. The cells at the interface were collected and washed with L-15 containing 0.1% FCS by centrifugation at 450 × *g* for 10 min, and the concentration of viable cells was determined by trypan blue exclusion.

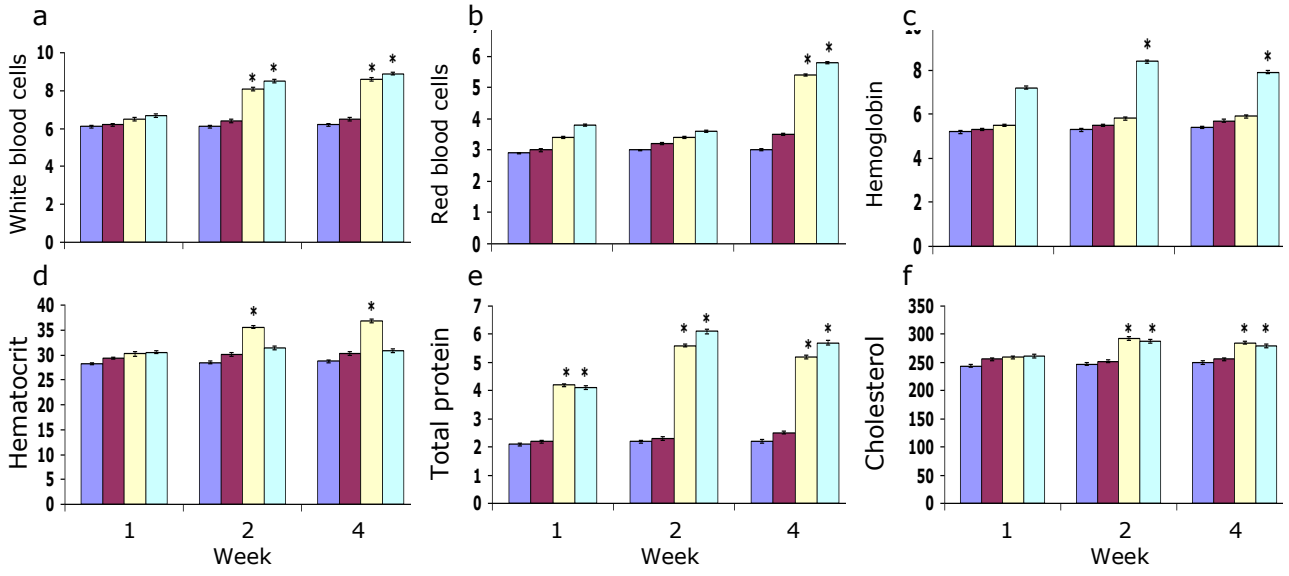
Nitroblue tetrazolium reduction analysis. Production of intracellular O_2^- was evaluated using nitroblue tetrazolium (NBT). Two experiments were carried out to evaluate the effect of glucans on the respiratory burst of fish phagocytes. In the first, leukocytes (2 x 10^6/ml) suspended in L-15 containing 0.1% FCS were transferred to 96-well plates. The plates were incubated at 18°C for 2 h. Non-adherent cells were removed by washing and cell monolayers were maintained in L-15 containing 5% FCS. The intracellular production of the superoxide anion was estimated, based on the formation of formazan crystals in the cells. A volume of 100 µl leukocyte solution (2 x 10^6/ml) was mixed with 100 µl/ml NBT (0.2% in phosphate buffered saline) containing zymosan (Sigma). After incubation at room temperature for 60 min with regular mixing, the plates were centrifuged at 500 × *g* for 3 min and the supernatants were discarded. The cells were washed twice with HBSS and fixed in 70% methanol. The formazan crystals were dissolved by adding 0.12 ml potassium hydroxide (KOH) and 0.14 ml dimethyl sulfoxide (DMSO). In the second, to study the effects of the soluble components of the glucan suspensions on the respiratory burst, cells were incubated for 1 or 2 h with the supernatants plus NBT. After turquoise-blue solutions were obtained, absorbance was measured at 620 nm using a multiscan spectrophotometer with KOH/DMSO as the blank.

Lysozyme activity. Serum lysozyme activity was measured spectrophotometrically according to the method of Ellis (1990). A volume of 0.02% (w/v) suspension of *Micrococcus lysodeikticus* made up in 0.05 M phosphate buffer (pH 6.2) was used as the substrate. Lyophilized hen egg white lysozyme was used as the standard. A new standard curve was prepared for each assay. Standard solutions as well as samples were added to the substrate at 25°C. Results are expressed as µg/ml equivalent of hen egg white lysozyme activity.

Statistics. Experimental data are presented as means±SE and were analyzed by one-way ANOVA, followed by Tukey's test to compare means between treatments, in SPSS with a significance level of $p<0.05$.

Results

Hematological parameters, biochemical profile, serum scuticocidal activity, superoxide anion production, lysozyme assay, and mortality are given in Fig. 1.



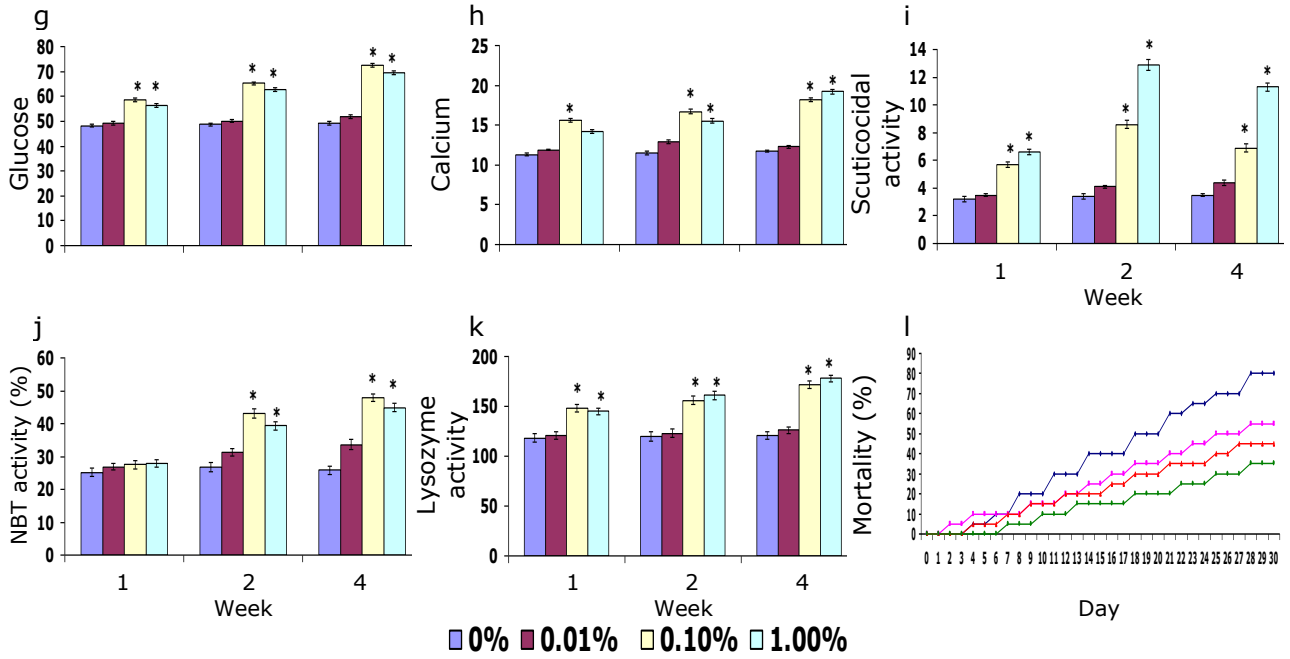


Fig. 1. Changes in (a) white blood cells, (b) red blood cells, (c) hemoglobin, (d) hematocrit, (e) total protein, (f) cholesterol, (g) glucose, (h) calcium, (i) serum scuticocidal activity, (j) serum superoxide anion production, (k) lysozyme activity, and (l) cumulative mortality in olive flounder (*Paralichthys olivaceus*) fed diets supplemented with *Coriolus versicolor* at 0%, 0.01%, 0.1%, or 1.0% and challenged with *Pseudocohnilembus persalinus* (means±SE; n = 6 per replicate). Statistical differences (p<0.05) between groups are indicated by asterisks.

Discussion

In the present study, the number of white blood cells significantly increased in fish fed the 0.1% and 1.0% diets during weeks 2 and 4, in agreement with results in *A. hydrophila*-infected rainbow trout where the increase in white blood cells led to anemia attributed to destruction, loss, and suppression of red blood cells (Brenden and Huizinga, 1986). Similarly, chum salmon infected with erythrocytic necrosis virus showed abnormal, dense, and compact white blood cells that peaked at 72 h (Ellsaesser and Clem, 1986). Red blood cell abnormalities include viral inclusions, hemoglobin cysts, and hemoparasites (Martínez-Silvestre et al., 2001).

Hemoglobin was significantly higher in fish fed the 1.0% diet in weeks 2 and 4 while hematocrit was significantly higher in fish fed this diet in week 4. Decreased hemoglobin may result from swelling of red blood cells as well as poor mobilization of hemoglobin from the spleen and other hemopoeitic organs as in channel catfish (Scott and Rogers, 1981). This fact supports the present finding that the significant drops in red blood cells and hemoglobin may be due to hypochromic microcytic anemia caused by the *P. persalinus*. Decreased red blood cell counts, hematocrit, and hemoglobin indicate that red blood cells are being destroyed by leucocytosis activity in erythrocytic anemia (Scott and Rogers, 1981). However, hematocrit can rise as a result of oxygen deficiency (Holeton and Randall, 1967), indicating that manifestation is species specific.

Total protein, glucose, and calcium were significantly higher in fish fed the 0.1% and 1.0% diets in weeks 2 and 4. In rainbow trout, a high serum protein level was attributed to osmoregulatory dysfunction, damage to tissues surrounding blood vessels, and hemodilution (Hill, 1982). Increased protein indicates destruction of red blood cells and the consequent release of cell contents into the blood stream (Haney et al., 1992). However, protein levels in infected channel catfish did not significantly differ from the control (Scott and Rogers, 1981). In contrast, a rise in glucose may reflect a higher rate of metabolism that causes high glucose turnover due to the metabolic strain of parasitism, as suggested by the absence of glucose response in rainbow trout exposed to

hypoxic stress (Wright et al., 1989). Similarly, utilization of glucose increased in *A. crassus*-infected eels which were aggravated by stress imposed by netting and aerial exposure (Gollock et al., 2004).

Serum scuticocidal activity and superoxide anion production significantly increased in fish fed the 0.1% and 1.0% diets in weeks 2 and 4 while serum lysozyme activity significantly increased in weeks 1-4 in fish fed these diets. Lysozyme is an important parameter in the immune defense of invertebrates and vertebrates. Lysozyme is a bactericidal, hydrolyzing-β linked glycoside bond of bacterial cell wall peptidoglycans resulting in lysis (Fearon and Locksley, 1996). It is an opsonin that activates the complement system and phagocytes (Lie et al., 1989). This action primarily attacks gram-positive bacteria that can be lysed in conjunction with enhanced complement activity (Alexander and Ingram, 1992), increasing the protection of fish against infection.

Cumulative mortality was 80% in fish fed the control diet and challenged with *P. persalinus*. However, mortality was only 45% and 35% in fish fed the 0.1% and 1.0% diets. Incorporation of herbals or their compounds into fish feeds can control the incidence of diseases such as enteritis, gill rot, white head, and white mouth (Rath, 2000) and effectively activate the fish immune system (Jian and Wu, 2003). The present study indicates that diets containing 0.1% and 1.0% supplements of *C. versicolor* enhance the innate immune response and decrease mortality in fish infected by *P. persalinus*. Further studies are needed to assess the dosage of *C. versicolor* with reference to fish species and degree of pathogen virulence.

Acknowledgements

This work was supported by the National Foundation of Korea (NRF) grant funded by the Korean government (MEST) (No. 2011-0006155). The authors would like to thank Ms. Jayalakshmi Harikrishnanan, Department of Tourism Management, Jeju National University, South Korea for help in the statistical analysis.

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