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Effects of a Glucan from the Edible Mushroom (*Pleurotus florida*) as an Immunostimulant in Farmed Indian Major Carp (*Catla catla*)

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Abstract

The purpose of this study was to determine the effects of a dietary mushroom glucan on the non-specific immune responses and disease resistance of the Indian major carp, *Catla catla*. Glucan was extracted from farm-raised edible mushrooms (*Pleurotus florida*). The glucan was fed to test animals for two weeks at 0.5 g or 1 g/kg feed. Afterward, the fish received the control diet for another four weeks. Non-specific immune responses were measured at the end of the two-week experimental feeding (week 0), two weeks later (week 2), and four weeks later (week 4). Control and test fish were challenged by intraperitoneal injection of the fish pathogenic bacterium, *Aeromonas hydrophila*, seven days after the two-week experimental feeding period. Mortality was observed and the relative percent survival was calculated. In the 0.5 g treatment, significant enhancement ($p<0.05$) in superoxide anion production was observed at weeks 0 and 2. In the 1 g treatment, activity was enhanced only at week 2. Both concentrations significantly increased phagocytosis at weeks 0 and 2 and bactericidal activity at weeks 2 and 4 ($p<0.05$). Alternative complement pathway activity was unaltered by either treatment ($p>0.05$), but lysozyme activity was raised at weeks 2 and 4 in the 0.5 g treatment and at week 2 in the 1 g treatment. Both concentrations significantly enhanced relative percent survival.

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Introduction

Immunostimulants can enhance immunocompetence and disease resistance in cultured fish. Used as a dietary supplement, some immunostimulants can increase disease resistance in fish by improving the non-specific arm of the immune defense system (Raa, 1996; Sakai, 1999; Selvaraj et al., 2005ab).

The group of immunostimulants most studied in fish is β-glucans, polymers of glucose consisting of a linear backbone of β-1,3 linked D-glucopyranosyl residues with varying degrees of branching from the C_6 position (Bohn and Bemiller, 1995). β-glucans are major structural components of yeast, mushrooms, and fungal mycelia. Several have been reported to enhance non-specific cellular and humoral immunity and disease resistance in fish (Robertsen et al., 1994; Robertsen, 1999; Sakai, 1999). The mechanisms involved in the positive immunological effects of β-glucan in fish are yet unknown. In the mammalian system, molecular targets of β-glucans such as dectin-1 and toll-like receptors (TLR) have been demonstrated (Brown et al., 2003; Gantner et al., 2003). Though TLR gene sequences have been reported in zebrafish, pufferfish, and Japanese flounder (Hirono et al., 2004; Jault et al., 2004; Meijer et al., 2004), the cooperation of these receptor molecules with β-glucan in fish is yet to be demonstrated.

β-glucans induce immune responses in fish through enhancement of non-specific humoral defense factors such as complement and lysozyme activities (Engstad et al., 1992; Jorgensen et al., 1993; Thompson et al., 1995; Santarem et al., 1997; Bagni et al., 2005) as well as through enhancement of cellular mechanisms such as phagocytosis, respiratory burst, and bactericidal activity (Robertsen et al., 1990; Dalmo and Seljelid, 1995; Santarem et al., 1997; Castro et al., 1999; Selvaraj et al., 2005b). Administered in feed, they can enhance protection of fish against a variety of bacterial pathogens (Raa et al., 1992; Nikl et al., 1993; Siwicki et al., 1994; Noya et al., 1995), although some studies found no protection of fish through dietary treatment (Toranzo et al., 1995; Ogier de Baulny et al., 1996). Similarly, significant enhancement in humoral and cellular immune functions was not observed following treatment by dietary β-glucan (Ogier de Baulny et al., 1996; Verlhac et al., 1998; Selvaraj et al., 2005ab).

Yeast glucans are the most extensively studied in fish (Sakai, 1999). The effects of yeast glucans vary from species to species due to their variation in composition, molecular weight, and solubility (Robertsen, 1999). There are few reports regarding the use of mushroom glucan as an immunomodulator in aquaculture (Yano et al., 1989; Wang and Wang, 1997). Although the intraperitoneal route is more effective than the oral route of administration (Duncan and Klesius, 1996), application of the latter is more feasible. Immunomodulation strategies involving pulse feeding of the immunostimulant for a short period, generally 4-6 weeks, are useful in up-regulating immune responses (Bricknell and Dalmo, 2005).

Catla (*Catla catla*) is a commercially important farmed Indian major carp. Studies that focus on increasing the immunocompetence and disease resistance of this species are needed. There is a dearth of information regarding modulation in catla of the innate immune system by different substances. Therefore, in the present work we evaluated the short-term dietary influence of a β-glucan prepared from the farm raised edible mushroom, *Pleurotus florida*, on the innate immunity and disease resistance of catla.

Materials and Methods

Fish. Catla (60-80 g) were obtained from a local fish farm and 240 were randomly stocked into twelve 1000-l indoor circular tanks. The fish were acclimated in aerated tap water for two weeks and daily fed a pelleted diet containing 40% crude protein, prepared in our laboratory. The water temperature was 26-29°C. Water was exchanged daily to remove waste feed and fecal material.

Glucan. Glucan was isolated (Wasser, 2002) from the oyster mushroom, *Pleurotus florida*, by ethanol precipitation followed by cation and anion exchange chromatography.

Following the ion exchange chromatography, the neutral, concentrated polysaccharides were subjected to Sephadex G-100 gel permeation chromatography. The gel permeation produced three fractions. The second yielded the most polysaccharide, with a protein ratio of 24.6. It was frozen in a conical flask at -20°C, lyophilized, and stored in a desiccator at -20°C.

Experimental design. After acclimation, the twelve tanks were assigned to one of three dietary groups: two were fed a glucan-supplemented diet with 0.5 g or 1 g glucan per kg feed, one was fed a non-supplemented diet (control). The fish were fed the experimental diet for two weeks at a rate of 2% of their body weight. Seven days after the two-week experimental feeding, 15 fish from each tank were randomly selected and moved to twelve 1000-l indoor circular tanks in the challenge facility. The remaining five fish were fed the control diet for another four weeks and used to study innate immune responses.

Sampling, serum collection, and isolation of head kidney leukocytes. Immune parameters were measured in four fish from each dietary group at the end of second week of treatment (week 0) and two (week 2) and four (week 4) weeks later. Blood samples from anesthetized fish were collected by heart puncture, kept at 4°C for 4 h, and centrifuged at 300 x *g* for 10 min. The obtained sera were stored at -20°C for serum complement and lysozyme determination. Following blood collection, leukocytes were isolated from the head kidney as per Santarem et al. (1997) with some modifications. The head kidney was removed aseptically from the fish and a cell suspension was obtained by teasing the head kidney tissues with forceps in Roswell Park Memorial Institute medium (RPMI-1640) containing penicillin (100 IU/ml), streptomycin (100 µg/ml), heparin (10 IU/ml), and 10% fetal calf serum (FCS, Sigma). The cell suspension was washed by centrifugation for 10 min at 300 x *g* and the pellet, resuspended in RPMI growth medium, was carefully layered onto histopaque1077 (Sigma) and centrifuged at 450 x *g* for 30 min. Cells at the medium-histopaque interface were transferred to clean tubes and washed twice. Purified leukocytes were counted using a hemocytometer and cell viability was determined by the trypan blue exclusion method.

Production of superoxide anion (O_2^-). Production of intracellular O_2^- by the head kidney leukocytes was evaluated by using the nitroblue tetrazolium (NBT, Sigma) method (Rook et al., 1985). Leukocytes (1 x 10^6 cells/ml) were seeded into wells of a 96-well culture plate (Nunc, Denmark). NBT was dissolved in RPMI medium to a final concentration of 2 mg/ml. Medium containing NBT was filter sterilized and NBT (100 µl) was added to the leukocyte culture. After incubating the plate for 25 min at room temperature, the supernatant was removed from each well and the cells were fixed by adding 70% methanol (200 µl) for 1 min. Unreduced NBT was removed by washing cells several times with 70% methanol. Reduced NBT was dissolved by adding 2M KOH (120 µl) to the individual wells, then dimethyl sulfoxide (DMSO; 140 µl). Optical density was measured at 620 nm.

Phagocytosis assay. Phagocytosis was examined following the method described by Sakai et al. (1993). Head kidney leukocytes were prepared as described above and the number of cells was adjusted to10^7 cells/ml in RPMI. The cells were allowed to adhere to a clean glass slide for 1 h. Unbound cells were removed by washing with the medium. Overnight-grown *Streptococcus* sp. cells were suspended in RPMI at 10^8 CFU/ml, added to the glass slide, and incubated for 2 h at 25°C. The cells on the glass slide were fixed with methyl alcohol and stained with Giemsa. The number of phagocytic cells per 200 cells was counted microscopically. Phagocytic activity was determined as 100(no. phagocytozing cells/no. total cells).

Bactericidal assay. Bactericidal activity was examined using the method described by Graham et al. (1988) with some modifications. Head kidney leukocytes (1 x 10^7 cells/ml) were prepared in RPMI medium without antibiotics and seeded in some wells of a 96-well culture plate. RPMI medium (50 µl) was added to additional wells as blanks. Bacteria (*Aeromonas hydrophila*), cultured overnight in tryptic soy

broth (TSB; 1 x 10^6 cells/ml), were added to half the wells (20 µl/well) and 3% TSB (20 µl) was added to the other half (the blanks). The plate was shaken and incubated at 25°C for 5 h (T_5 wells). Bacterial solution (20 µl) was then added to the remaining uninfected half of the plate (T_0 wells). After shaking the plate, lysis buffer (5% Tween 20 in distilled water; 30 µl) was added to all the wells to lyse leukocytes in order to stop their bactericidal activity. The plate was incubated an additional 16 h at 25°C, then 3% TSB (100 µl) was added to each well, the plate was vigorously shaken, and 100 µl were transferred to another microtitre plate. A solution of 3-(4,5-dimethyl thiazol 2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma; 5 mg/ml; 10 µl) was added to all the wells to determine the amount of live bacteria present. The plate was read using a micro-plate reader at 595 nm after 30 min of incubation in the dark. The percentage of surviving bacteria was obtained by dividing the optical density of the T_5 wells by the optical density of the T_0 wells and multiplying the value by 100.

Alternative complement pathway activity. The alternative complement pathway (ACP) activity was measured according to Ortuño et al. (1998). Test serum, diluted in Hank's buffer (HBSS, Sigma), was added to sheep red blood cells (SRBC; 500 µl) to reach final concentrations of 5, 2.5, 1.25, 0.625, and 0.313%. After incubation at room temperature for 1 h, the samples were centrifuged at 800 x *g* for 10 min at 4°C to remove any intact erythrocytes. The relative hemoglobin content of the supernatants was assessed by measuring their optical density at 540 nm using a spectrophotometer. The maximum (100%) and spontaneous hemolysis values were obtained by adding 500 µl of distilled water and HBSS to 500 µl SRBC, respectively. The degree of percent hemolysis (Y) was calculated from 100% and spontaneous lysis values and a lysis curve for each sample was obtained by plotting Y/(1-Y) against the volume of serum added (ml) on a log-log scaled graph. The volume of serum producing 50% hemolysis (ACP_{50}) was determined and the number of ACP_{50} units/ml was calculated for each sample.

Lysozyme activity. The lysozyme activity of the serum was determined following a turbidimetric method (Ellis, 1990) modified to a microtitre plate assay. Serum samples (25 µl) were added to 175 µl of a suspension of the lysozyme sensitive gram-positive bacterium, *Micrococcus lysodeikticus* (0.2 mg/ml in 0.05 M sodium phosphate buffer, pH 6.2). The reaction was carried out with constant shaking and the absorbance was recorded at 450 nm after 1 and 5 min. One unit of lysozyme activity was defined as the amount of sample causing a reduction in absorbance of 0.001/min.

Challenge experiment. *Aeromonas hydrophila* (AH-4-05) was isolated from the ulcerated lesion of a diseased catla in the Department of Fishery Pathology and Microbiology of the West Bengal University of Animal and Fishery Sciences. The LD50 concentration of the pathogen was determined as 4 x 10^5 CFU/ml after a test challenge in catla prior to the experiment. After one week of acclimation in the challenge facility during which the fish were fed the control diet, each fish was injected intraperitoneally with 0.2 ml of the LD50 concentration of *A. hydrophila*. Before injection, the bacteria were grown in TSB for 24 h, harvested by centrifugation, and washed twice with 0.85% sterile NaCl. The pellet was resuspended and diluted to obtain the desired dose. Mortality was recorded up to ten days and the relative percent survival (RPS) was calculated as 100 - 100(test mortality/control mortality).

Statistics. Data were analyzed with SPSS 10.0 for Windows software and one-way analysis of variance (ANOVA). Means were compared by LSD Post Hoc test and a probability level of 0.05 was used.

Results

The 0.5 g treatment significantly increased the superoxide anion production at weeks 0 and 2, while the 1 g treatment increased the activity only at week 2 (Fig. 1a). Both glucan concentrations significantly increased the phagocytic activity at weeks 0 and 2 (Fig. 1b). Significantly more bacteria was killed by both glucan concentrations than by the control at weeks 2 and 4 (Fig. 1c). No statistically signif-

icant differences were observed in alternative complement pathway activity at any sampling time (Fig. 1d). The 0.5 g diet significantly increased lysozyme activity at weeks 2 and 4 while the 1 g diet produced a significant effect only at week 2 (Fig. 1e). Mortality and relative percent survival are given in Table 1.

Discussion

The present work indicates that glucan supplementation in diets for catla can positively influence non-specific immune responses and resistance to bacterial infection. Glucans from a variety of sources were reported to enhance non-specific immune responses and disease resistance in teleosts (Robertsen et al., 1994). However, most studies examined yeast glucan (Sakai, 1999). The positive biological effects of the mushroom glucan tested in the present study further indicate that teleosts have developed the recognition mechanism for β-glucan through receptor binding, as suggested in macrophages of Atlantic salmon (Engstad and Robertsen, 1993) and in channel catfish (Ainsworth, 1994).

Our results suggest that administration of glucan stimulates the non-specific arm of the immune system. Both concentrations significantly enhanced the non-specific cellular factors (O_2^- production, phagocytosis, bactericidal activity). Likewise, enhancement of oxidative burst, phagocytic activity, myeloperoxidase, and potential killing activity was reported in rainbow trout fed yeast glucan for one week by Siwicki et al. (1994). However, no variation in oxidative burst and pinocytic activity of rainbow trout after two weeks of glucan feeding was reported by Verlhac et al. (1998). A significant increase in the oxidative burst activity of head kidney macrophages was observed in turbot fed yeast glucan for five-weeks but the enhancement was no longer observed two weeks after the fish returned to the control diet (Ogier de Baulny et al., 1996). African catfish fed yeast glucan for four weeks had more NBT-positive cells and greater bacterial clearance than the control (Yoshida et al., 1995).

Of the humoral factors, the serum complement level was not influenced in our experiment while lysozyme activity was significantly enhanced at weeks 2 and 4. Likewise, Verlhac et al. (1998) did not observe any significant enhancement in the complement activity after two weeks of glucan feeding in rainbow trout, although Bagni et al. (2005) observed a significant increase in complement level 15 days after the end of 15 days of feeding and significant enhancement of lysozyme activity 30 days after the end of feeding.

The inclusion of glucan in the diet was responsible for a significant reduction in mortality due to *A. hydrophila* infection. Yeast glucan enhanced the resistance of fish to bacterial infection when administered in the feed (Raa et al., 1992; Nikl et al., 1993; Siwicki et al., 1994; Ainsworth et al., 1994). Intraperitoneal injection of the mushroom glucan, lentinan, enhanced protection against *Edwardsiella tarda* in carp (Yano et al., 1991). However, glucan from the mushroom, *Pleurotus ostretus*, did not confer any protection against *A. hydrophila* in grass carp and tilapia (Wang and Wang, 1997). Mortality in the glucan group reached its plateau after four days, while mortality in the control group continued for seven days, suggesting increased resistance to infection via a more rapid immune response.

The different study results may be due to the use of different fish species, different experimental systems, or different mechanisms that might be induced during oral administration. The effect of glucan by oral administration should, therefore, be further investigated in relation to fish species, feeding period, and inclusion level.

The non-specific immune parameters tested in this study peaked two weeks after the fish returned to the control diet. The decreased immune response towards the end of the experiment suggests that immunostimulation may reach a resting level after which another dose should be given. Such a short-term pulse feeding strategy allows for flexibility in fish farming as the immunostimulant can be applied during periods of increased disease risk (Bricknell and Dalmo, 2005).

The glucan concentrations differed in immune response. The higher concentration

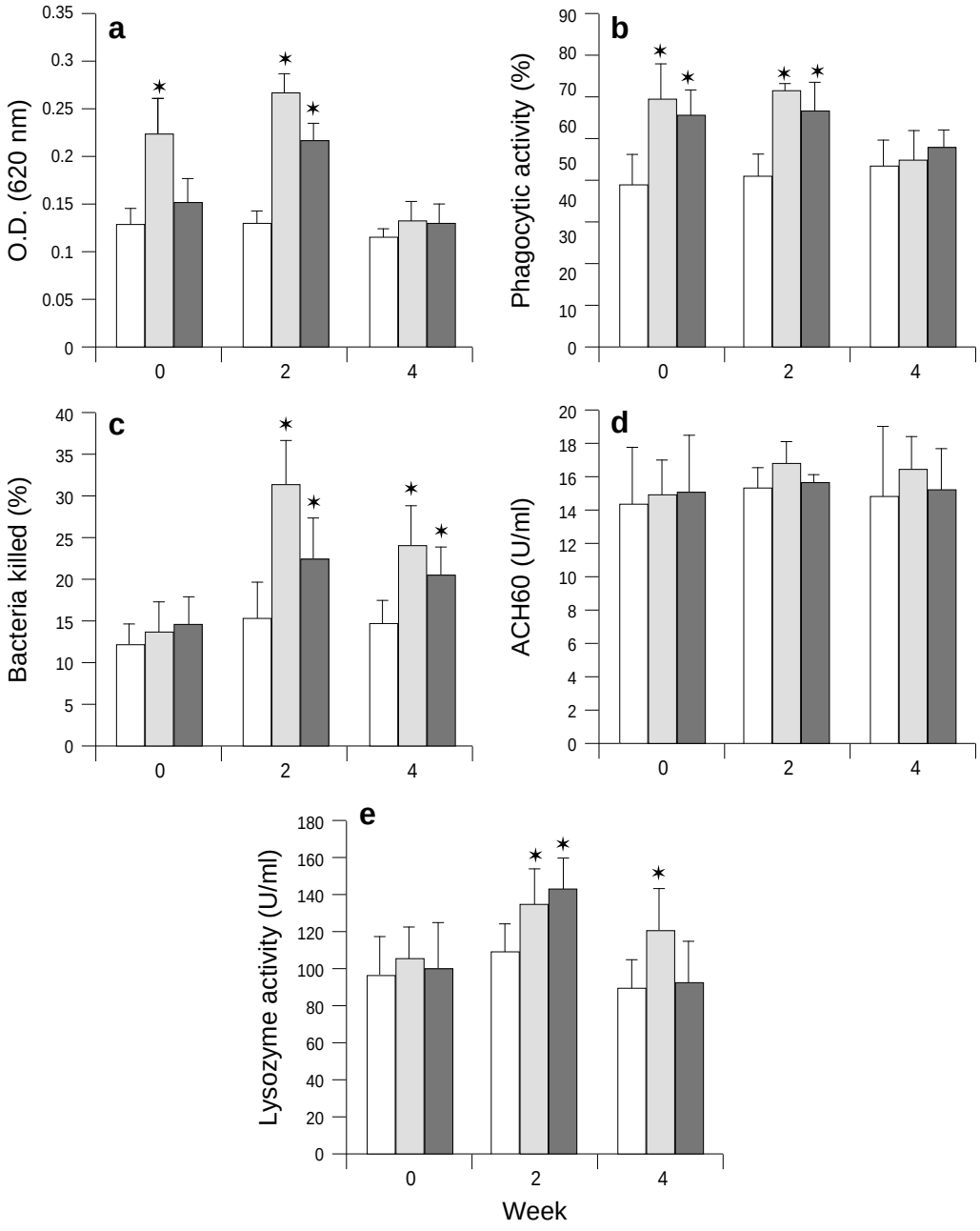

Fig. 1. Superoxide anion production (a), phagocytosis of *Streptococcus* sp. (b), bactericidal activity (c), alternative complement pathway (ACP) activity (d), and serum lysozyme activity (e) by head kidney leukocytes from *Catla catla* fed a diet supplemented with 0.5 g (■) or 1.0 g (▒) glucan per kg diet or no glucan (control □). Data presented as means±SD (n = 4). Asterisks indicate statistically significant difference (p<0.05) compared to the control at the same week.

Table 1. Effect of different concentrations of glucan on mortality and relative percent survival (RPS) of *Catla catla* following challenge with *Aeromonas hydrophila*.

Glucan (g/kg feed)	*Mortality (%)*	*RPS (%)*
0 (control)	56.67±13.88	0
0.5	26.67±10.89*	52.94*
1	31.67±14.78*	44.12*

* Significantly different from control (p<0.05).

(1 g/kg) was less effective than the lower concentration (0.5 g/kg), perhaps because glucan binding is specific and there might be competition for binding sites (Muller et al., 1996; Figueras et al., 1997). Indeed, less stimulation by higher doses of glucan was reported from *in vitro* studies (Robertsen et al., 1994; Castro et al., 1999). Hence, much research is needed in the area of immunostimulant dose, particularly during oral administration.

In conclusion, the present study indicates that inclusion of mushroom glucan in catla diets enhanced the catla non-specific immunity and resistance to a pathogenic bacterial challenge. However, such enhancement might be temporary and dependent on the feeding period and inclusion level.

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