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## Immunostimulatory Effects of a Yeast (*Saccharomyces cerevisiae*) Cell Wall Feed Supplement on Rohu (*Labeo rohita*), an Indian Major Carp

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### Abstract

The immunostimulatory role of an orally administered yeast cell wall preparation from *Saccharomyces cerevisiae* (Nutrifer<sup>TM</sup>) was assessed in the Indian major carp, rohu (*Labeo rohita*). Fish were fed a diet containing the preparation for 15 days and then returned to the control diet for 20 days. Non-specific immunity was assessed at the end of the experimental feeding period (day 0) and on days 10 and 20. *In vitro* oxidative radical production, phagocytosis of leukocytes, nitrite production, and proliferation of lymphocytes were determined. All four parameters remained stable in control fish but, in fish fed the supplemented diet, reached a peak on day 10 and remained higher than in the control until day 20, indicating that ingestion of the diet containing the yeast cell wall preparation for 15 days had significant immunostimulatory effects.

### Introduction

Immunostimulants play a significant role in aquaculture health management strategies. Immunostimulants include biological and synthetic compounds that activate white blood cells (leukocytes) and enhance non-specific cellular and humoral defense mechanisms in animals. Such agents are used to rectify impaired immune function and stabilize the improved status.

Fish treated with immunostimulants usually have enhanced phagocytic cell activity, pathogen killing ability, and killing mechanisms involving reactive oxygen species and reactive nitrogen species in macrophages. Lymphocytes are also activated by immunostimulants.

The route of administration affects the function of immunostimulants. Oral adminis-

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tration is the most practical and preferred method for delivery of immunostimulants because it allows mass administration regardless of fish size and results in enhancement of leukocyte function and protection against infectious diseases. Injection, on the other hand, is labor intensive and time consuming.

The immunostimulatory effects of polysaccharides and  $\beta$ -glucans have been widely studied in fish. Yeast, which contains polysaccharides and  $\beta$ -glucans, is a good enhancer of the immune system. Intake of a yeast (*Saccharomyces cerevisiae*) supplement improved the cellular innate immune response in trout (Siwicki et al., 1994) and gilthead seabream (Ortuno et al., 2002) by increasing the oxidative radical release and phagocytic indices. However, reports on the immunostimulatory effects of yeast cell wall extracts on freshwater fishes of the Indian subcontinent are meager.

As Indian carps are the backbone of freshwater aquaculture in India, the objective of the present work was to study the effects of a dietary administered yeast cell wall extract on the cellular immune responses of the Indian major carp, rohu (*Labeo rohita*).

#### Materials and Methods

**Experimental fish.** Rohu (*Labeo rohita*) was used as a representative of the Indian major carps. Fish were purchased from a local market and maintained at the wet laboratory of the Department of Fishery Pathology and Microbiology at the West Bengal University of Animal and Fishery Sciences. Rohu (45-60 g) were maintained in circular 500-l fiberglass tanks with 24-h aeration. About 75% of the water was exchanged and waste feed and fecal materials were removed daily. Basic physico-chemical water parameters were measured every 15 days to maintain optimal levels of dissolved oxygen ( $5.65 \pm 0.72$  mg/l), pH ( $8.24 \pm 0.82$ ), nitrite ( $0.015 \pm 0.009$  mg/l), and ammonia ( $0.109 \pm 0.024$  mg/l). The water temperature was 25-30°C. The fish were acclimatized and fed a pelleted diet for three weeks prior to the experiment. The diet was prepared in our laboratory and fed at 1% of the body weight of the fish.

**Immunostimulant.** Nutriferm™ (AB Mauri) was used as the immunostimulant. It is composed of a purified cell wall fraction gained from a pure culture of the yeast, *Saccharomyces cerevisiae*. Soluble parts were removed by digestion and the remaining cell wall fraction was purified and dried.

**Feed preparation.** The control diet contained 40% fish meal, 35% ground nut oil cake, 12% wheat flour, 10% rice polish, 2% vitamins and minerals, and water. The experimental diet contained the above plus 5 g of yeast cell wall per kg feed. The feed was pelleted with a hand pelletizer in our laboratory then dried at room temperature and stored at 4°C.

**Experimental design.** Eighty fish were stocked in eight tanks (4 control and 4 experimental) at 10 fish per tank. Before the start of the experiment, four control fish (one from each tank) were randomly chosen and cellular immune parameters were measured as below. After acclimatization, the four experimental tanks received the yeast-supplemented diet once a day for 15 days and then the control diet for another 20 days; the control tanks received the control diet throughout. Cellular activity was measured on days 0 (the day after cessation of the experimental diet), 10, and 20 in four randomly chosen fish from each treatment (one from each tank).

**Oxidative radical production by leukocytes.** Oxidative radical production of leukocytes was assayed by the nitroblue tetrazolium (NBT) reduction test as described by Siwicki et al. (1985). Equal volumes (0.1 ml) of blood and filtered NBT solution (0.2% in phosphate buffered saline [PBS], pH 7.2 original stock) was mixed and incubated for 30 min at room temperature. Then 0.05 ml of the mixture was added to 1 ml N, N dimethylformamide. The suspension was centrifuged for 5 min at 3000 x g and the optical density of the supernatant was assessed at 540 nm in a spectrophotometer (Siwicki et al., 1998).

**Phagocytosis.** Phagocytosis was examined as described by Yoshida et al. (1993). The number of cells was adjusted to  $1 \times 10^7$  cells/ml in RPMI-1640 medium containing 10% fetal calf serum. One ml of cell suspen-

sion was allowed to adhere to a cover slip for 1 h at room temperature and cells that did not adhere were washed off with PBS. Cells from a young culture of *Staphylococcus* sp. ( $10^8$  cfu/ml PBS), obtained from the Department of Fishery Pathology and Microbiology, were added to the cover slip and the slips were incubated 2 h at 28°C. The cells on the cover slip were fixed with 95% methyl alcohol and stained with Giemsa. The number of phagocytic cells per 200 cells was counted and phagocytic activity (PA) was determined according to Findlay and Mundaym (2000) as  $PA = 100(\text{no. phagocytosing cells}/\text{total no. of phagocytes})$ .

**Isolation of head kidney leukocytes.** Fish were anesthetized by MS-222 and the head kidney was aseptically removed, cut into small pieces, minced to obtain cells, and pushed through a stainless steel mesh with PBS (pH 7.4) containing 100 µg/ml streptomycin, 200 µg/ml gentamycin, and 100 IU/ml penicillin. The cell suspension was layered onto Histopaque® 1077 (Sigma, USA) at a ratio of 1:3 and centrifuged at 4°C for 30 min at  $400 \times g$  (Chung and Secombes, 1988). Following centrifugation, the white blood cell interface layer was collected using a micropipette, transferred to a clean sterile test tube, and washed thrice with PBS. Viable cells were counted by the trypan blue exclusion method using a Neubauer counting chamber (Maji et al., 2006).

**In vitro production of reactive nitrogen.** The production of reactive nitrogen intermediates by leukocytes was assayed following the method described by Tafalla and Novoa (2000). This method is based on the Griess reaction that quantifies the nitrite content of macrophage supernatants, as nitric oxide is an unstable molecule and degrades to nitrite and nitrate (Green et al., 1982). The number of head kidney leukocytes was adjusted to  $2 \times 10^6$  viable cells/ml by diluting with RPMI-1640 growth medium containing 2 mM L-glutamine, 24 mM Hepes buffer, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% V/V fetal calf serum. Cell suspensions (100 µl/well) were dispensed in a 96-well flat bottom tissue culture plate (Nunc, Denmark). The final volume

of the wells was increased to 200 µl by adding LPS (Sigma, USA) at a concentration of 10 µg/ml and the plate was incubated at 28°C for 96 h in 5% CO<sub>2</sub> atmosphere.

After incubation, 50 µl aliquots of supernatants from the wells were removed, added to 100 µl of Griess reagent containing 1% sulfanilamide, 0.1% N-1 naphthylethylene diamine dihydrochloride, and 2.5% phosphoric acid, and incubated at room temperature for 10 min. The optical density of the solution was measured with a microplate reader (ECIL, India) at 570 nm and quantified by comparison to NaNO<sub>2</sub> (Sigma, USA) as the standard (Joardar et al., 2003).

**Lymphocyte proliferation.** The number of head kidney leukocytes was adjusted to  $5 \times 10^5$  cells/ml in RPMI-1640 and 100 µl cell suspensions were seeded into a 96-well tissue culture plate (Nunc, Denmark). Each control ( $n = 4$ ) and treated ( $n = 4$ ) fish cell suspension was repeated in triplicate. The final volume of the wells was made up to 200 µl with Concanavalin A (Con-A) at a concentration of 10 µg/ml and the plate was incubated at 28°C for 48 h in a 5% CO<sub>2</sub> atmosphere.

The colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay described by Daly et al. (1995) was used to determine the proliferation of head kidney leukocytes. After 48 h culture, 20 µl of MTT (5 mg/ml PBS) were added to each well of the leukocyte culture and incubated at 28°C for 4 h. Formazan production was determined by the method of Plumb et al. (1989). The formazan crystals were dissolved by adding 150 µl of DMSO (Sigma, USA) to each well, followed by 25 µl glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5). The contents of the wells were mixed thoroughly with a micropipette and incubated at room temperature for 10 min. Formazan development was read at 595 nm using a microplate reader (ECIL, India) and a stimulation index (SI) was calculated as  $SI = (\text{mean optical density of leukocyte wells with test mitogen at time } x / \text{mean optical density of negative control wells at time } x) - 1$ .

**Statistical analysis.** Results are expressed as means  $\pm$  standard error (SE) and analyzed

by one-way analysis of variance (ANOVA) to test the significance of differences between the control and experimental groups (Neumann et al., 1995).

### Results

Oxidative radical production of leukocytes (Fig. 1), phagocytosis (Fig. 2), production of reactive nitrogen (Fig. 3), and lymphocyte proliferation (Fig. 4) were higher in treated fish on day 0 than in the control. In treated fish, all indicators peaked on day 10 and, except for nitrite production, remained elevated until day 20. Values in the control fish on days 0, 10, and 20 were similar to pre-experiment values and, therefore, are not given in the figures.

### Discussion

The *in vitro* production of reactive oxygen radicals increased from day 0, peaked on day 10, and gradually decreased to day 20, similar to some earlier reports on other species. Rainbow trout fed beta 1,3/1,6-yeast glucan for one week had a higher oxidative burst than control fish fed no glucan (Siwicki et al., 1994). The number of glass-adherent NBT-positive cells in catfish treated with oligosaccharide peaked at 12 days, decreased slightly by 30 days, dropped to the baseline level by 45

days, and was greater than but did not significantly differ from the control (Yoshida et al., 1995). In turbot fed beta 1,3/1,6-yeast glucan for five weeks, the oxidative burst of head-kidney macrophages was significantly enhanced but the enhancement was no longer observed two weeks after the fish returned to the control diet (Ogier de Baulny et al., 1996). Also in turbot, the influence of glucans on respiratory burst activity of leukocytes and production of  $O_2^-$  in all groups was higher on day 7 than on days 14 and 21 (Santarem et al., 1997). In gilt-head seabream, the respiratory burst activity of head kidney leukocytes, measured as the maximum slope of its kinetic, in fish fed a diet supplemented by 10 g yeast per kg diet was higher than that of fish fed any other diet after seven weeks of feeding (Ortuno et al., 2002). On the other hand, Verlhac et al. (1998) obtained no statistically significant differences in oxidative burst of head kidney phagocytes in rainbow trout fed glucan.

Phagocytosis is an important element of the defense of fish against invading microorganisms (MacArthur and Fletcher, 1985; Olivier et al., 1986). Destruction of ingested microorganisms might be due to degranulation and metabolic activation when toxic intermediates of oxygen are produced. In our

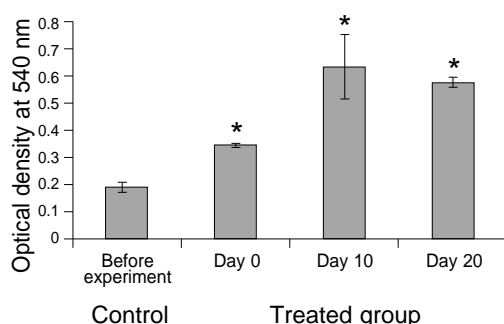


Fig. 1. Superoxide anion production by leukocytes of rohu (*Labeo rohita*) fed a yeast cell wall preparation, determined by nitroblue tetrazolium (NBT) reduction assay; means of four observations $\pm$ SE, asterisk indicates significant difference ( $p < 0.01$ ) from the control. Control did not vary throughout experiment.

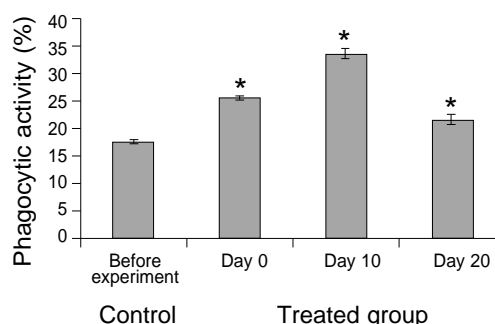


Fig. 2. *In vitro* phagocytic activity of head kidney leukocytes of rohu (*Labeo rohita*) fed a yeast cell wall preparation; means of four observations $\pm$ SE, asterisk indicates significant difference ( $p < 0.01$ ) from the control. Control did not vary throughout experiment.

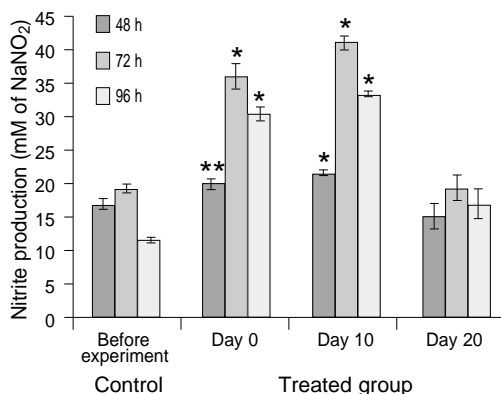


Fig. 3. *In vitro* nitrite (NO<sub>2</sub>) production by head kidney leukocytes of rohu (*Labeo rohita*) fed a yeast cell wall preparation after 48, 72, and 96 h of incubation; means of four observations ± SE, single asterisk indicates significant difference of  $p < 0.01$  from control, double asterisk indicates significant difference of  $p < 0.05$ . Control did not vary throughout experiment.

experiment, phagocytic activity increased from day 0, peaked on day 10, and gradually decreased to day 20. The phagocyte activation mechanism might be related to the *S. cerevisiae* cell wall supplementation of the feed, as was observed by earlier workers. However, the dose and time of administration, as with other animal models, varies from experiment to experiment. Yano et al. (1989), Chen and Ainsworth (1992), and Jorgensen et al. (1993) reported that glucan raises phagocytosis. In an earlier study, oral administration of 5 or 10 g yeast preparation from *S. cerevisiae* per kg feed enhanced the phagocytic function of gilthead seabream in week 4 although there was no significant difference between doses (Ortuno et al., 2002). No statistically significant differences in phagocytic parameters were observed in fish fed supplemented diets for 1 or 2 weeks compared to fish fed a control diet (Ortuno et al., 2002). In rainbow trout fed beta-1,3/1,6-glucan, phagocytic activity was higher than in control fish at 1 week (Siwicki et al., 1994). However, Verlhac et al. (1998) reported no effect of glucan on the tested macrophage activities.

Reactive nitrogen intermediates are important molecules in regulating immune functions and have a direct antimicrobial effect (Liew et al., 1990; Schoor and Plumb, 1994). Wang et al. (1994) first demonstrated that fish macrophages synthesize nitric oxide. A macrophage activating factor secreted by mitogen stimulated goldfish kidney leukocytes induced nitric oxide production in both primary cultures and cell lines of goldfish macrophages (Wang et al., 1995). In the present study, nitrite production of the head kidney leukocytes was assessed *in vitro*. In all treated fish, nitrite production increased and peaked on day 10. However, Novoa et al. (2003) found that nitric oxide production in turbot was not affected *in vitro* or *in vivo* by any nisin treatment and Sakai et al. (1999) found no evidence of activation of reactive nitrogen species in macrophages of fish treated with various immunostimulants.

Measurement of lymphoproliferation activity is required to evaluate stimulation of specific and non-specific mitogens. In the present study, yeast cell wall supplementation significantly enhanced the proliferative response of head kidney leukocytes induced by the mitogen Con A. Treated fish possessed *in vitro* lymphoproliferative ability up to day 20, with a peak on day 10. Verlhac et al. (1998) found a significant enhancing effect of dietary glucan on the response of peripheral blood lymphocytes of rainbow trout induced by Con A just after the experimental feeding (week 0) and four weeks later but no significant variations in the effect of dietary vitamin C on Con A-induced proliferation of peripheral blood lymphocytes and no response to LPS.

In conclusion, pelleted feed supplemented with the yeast cell wall preparation, Nutri-ferm™, enhanced *in vitro* production of reactive oxygen and nitrogen, phagocytic activity, and lymphocyte proliferation in the Indian major carp, rohu. Bricknell and Dalmo (2005) reported that administration of an immunostimulant may up-regulate the immune system to a heightened level until the immunostimulant is withdrawn, although continuous administration may cause adverse effects such as tolerance or immunosuppression. Conversely,

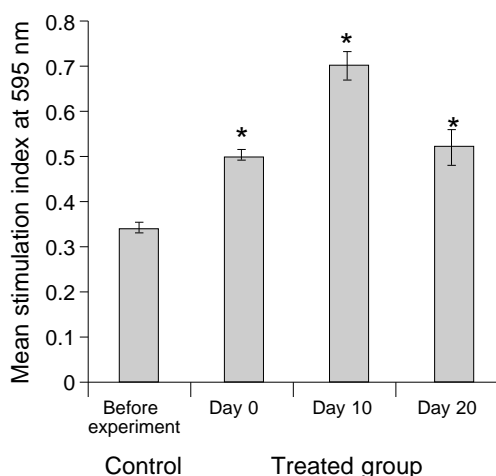


Fig. 4. *In vitro* lymphocyte proliferation assay of head kidney leukocytes of rohu (*Labeo rohita*) fed a yeast cell wall preparation; means of four observations  $\pm$  SE, asterisk indicates significant difference ( $p < 0.01$ ) from the control. Control did not vary throughout experiment.

pulse administration of immunostimulants may cause oscillations in the immune response from rest to enhanced and back to rest. As Nutriferm<sup>TM</sup> administered orally for 15 days resulted in a considerable immunomodulatory effect on the rohu, this preparation together with a pulse feeding strategy might be useful as a disease control strategy for Indian carps. A pulse-feeding strategy should be tested with 20-30 day intervals.

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