Effect of dietary supplementation with zinc enriched yeast (*Saccharomyces cerevisiae*) on immunity of rainbow trout (*Oncorhynchus mykiss*)

Gharekhani, A.[1]; Azari Takami, Gh.[2]; Tukmechi, A.[3*]; Afsharnasab, M.[4] and Agh, N.[5]

[1]Graduated from Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran; [2]Department of Aquatic Health and Disease, Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran; [3]Department of Pathobiology and Quality Control, Artemia and Aquatic Animals Research Institute, Urmia University, Urmia, Iran; [4]Department of Aquatic Health and Disease, Iranian Fisheries Research Institute, Tehran, Iran; [5]Department of Ecology and Nutrition, Artemia and Aquatic Animals Research Institute, Urmia University, Urmia, Iran

[*]***Correspondence:*** *A. Tukmechi, Department of Pathobiology and Quality Control, Artemia and Aquatic Animals Research Institute, Urmia University, Urmia, Iran. E-mail: a.tukmachi@urmia.ac.ir*

(Received 7 Feb 2015; revised version 26 May 2015; accepted 30 May 2015)

Summary

Zinc (Zn) is an essential trace element in all living organisms, and the first eukaryotic Zn uptake transporter was discovered in the yeast, *Saccharomyces cerevisiae*. Zinc-enriched yeast is a currently available Zn supplement. The purpose of the investigation was to compare and evaluate the effect of Zn enriched yeast in rainbow trout. The fish (mean body weight 10 ± 0.5 g) were fed a commercial diet supplemented with 0 (control), 1×10^6, 1×10^7 and 1×10^8 CFU/g of Zn-enriched yeast for 60-days. Results showed that significant increase in serum lysozyme activity, complement activity and total immunoglobulin were seen in all treatment groups during feeding trial when compared to the control group. On the basis of our findings, Zn-enriched improved rainbow trout growth, some immune parameters and disease resistance.

Key words: Disease resistance, Immunity, Rainbow trout, Zinc enriched yeast

Introduction

Zinc (Zn) is an essential trace element for all living organisms, its role in biology was first recognized by Raulin in 1869 (Prasad, 2009). It acts as a co-factor for a large number of proteins and enzymes (Schneider, 2013). Also, Zn affects many aspects of the immune system and it is essential for normal development and function of immunity such as phagocytosis, intracellular killing and cytokines production (Prasad, 2009). Zinc also functions as an antioxidant and anti inflammatory agent. Zinc requirements of fish are difficult to determine because fish may in part utilize trace elements that are present in solution. However, Zn is not taken from water in sufficient amounts to meet the needs of fish and must therefore be supplied by the diet to prevent deficiencies. In rainbow trout an adequate Zn content of the diet was estimated to be 15-30 mg per kg. This trace element is readily absorbed from the gastro-intestinal tract, gills, fins and skin of fish. Dietary Zn availability and absorption is reduced in the presence of phytates, and high dietary intakes of calcium, phopshorus and copper (Bury *et al*., 2006). Zinc could be supplemented in diets as inorganic mineral salts, typically as Zn oxide or Zn (Strnadov *et al*., 2011). Usually, the organic forms of trace minerals have higher bioavailability than inorganic forms. In addition, organic forms of them are less toxic and more environmentally friendly than inorganic forms (Yang *et al*., 2012).

Probiotics are live microbial feed supplements with beneficial effects on host by producing inhibitory compounds, competition or chemicals and adhesion sites, immune modulation and stimulation, and improving the microbial balance (Tukmechi *et al*., 2011). *Saccharomyces cerevisiae* contains various immuno-stimulating compounds such as β-glucan, nucleic acids, mannan oligosaccharides and chitin, and has been proved to enhance the immune responses and growth in fish (Abdel-Tawwab *et al*., 2008; Gopalakannan and Arul, 2010). Many studies of the processes involved in the uptake of trace elements by the *S. cerevisiae* have increased considerably in recent years. This yeast has become a model microorganism for studying metal transporters and their accumulation in the cells. *Saccharomyces cerevisiae* is known for its ability to accumulate metal ions from aqueous solutions by different physico-chemical interactions, e.g. by adsorption and absorption, or by a metabolism-dependent mechanism (Stehlik-Tomas *et al*., 2004). Production of yeast biomass rich in organically bound Zn is important to the animal industry because such forms of Zn are readily absorbed by the animal. In a study with growing heifers, it was found that those fed Zn methionine gained weight 8.1% faster and 7.3% more efficiently (Shet *et al*., 2011).

There is no information on the effects of dietary Zn enriched yeast in aquaculture industry. Also, no information is available on the effects of Zn enriched *S. cerevisiae* in rainbow trout. Therefore, the objectives of this study were to determine the dietary Zn enriched

yeast on rainbow trout immunity.

Materials and Methods

Preparation of Zinc enriched yeast

The Zn nitrate used for the enrichment of *S. cerevisiae* (Persian type culture collection (PTCC) 5269) was purchased from Sigma, USA. The growth media used was yeast extract, peptone and glucose (YEPD) which was obtained from Merck, Germany. In present work, Wang *et al*. (2010) method was used for enrichment of yeast with Zn. Briefly, *S. cerevisiae* was cultured in 90 ml of YEPD medium at 27°C, pH = 5.8 and 160 rpm for 12 h on a shaker incubator (Biotech, South Korea). Then Zn nitrate was added into the medium at concentration of 10 mg ml^{-1} and yeast incubation was continued for 24 h at the same condition. The cells were harvested by centrifugation (3000 rpm for 15 min) and then washed with normal saline to remove the additional Zn nitrate. Finally, Zn-enriched cells were adjusted to the desired concentrations and used for feeding to fish. Atomic absorption spectrophotometer (AAS) (Shimadzu Scientific Instrument Inc., USA) was used to analyze Zn content in yeast cells and diet.

Experimental design

Rainbow trout (10 ± 0.5 g) were purchased from a commercial fish farm in Urmia, Iran. Acclimatization to the laboratory condition was performed for 10 days in 1000 L tanks using aerated free-flowing well water that had the following characteristics: temperature (15 ± 1°C); pH (7.5); dissolved oxygen (8 ± 0.2 mg/L); natural photoperiod (10 h light/14 h dark); flow rate (1.25 l/s). Fish were fed three times daily with commercial fish feed (40% protein), 3% of average initial body weight per day.

Diet preparation and feeding trial

Commercial basal diet (Faradaneh, Iran) was used in this study; three experimental diets were formulated to be supplemented at 1×10^6, 1×10^7 and 1×10^8 CFU per g of diet. After spraying the Zn-enriched yeast on commercial feed, pellets were dried at room temperature for 2 h and then the diets were stored at 4°C until use. Fish were divided into 4 groups (in triplicate) of 40 animals distributed per tank and were fed with Zn enriched yeast for a 60-day period. Study continued for 15 days and during this time all fish were fed with the control diet without Zn-enriched yeast supplementation. On days 0, 15, 30, 45, 60 and 75, a sample of three individuals per tank (nine per treatment) were taken to measure immunological parameters.

Immunological parameters

Fish were anesthetized with 200 mg/L clove oil, then blood was collected from cardinal vein using heparin coated syringe and transferred into sterile tubes. The blood was allowed to clot at room temperature for 1 h and stored in a refrigerator overnight. The clot was then centrifuged at 1500 × g for 5 min. Then the serum was collected and stored in sterile Eppendorf tubes at -20°C until use for immunological assays.

Serum lysozyme activity

The serum lysozyme activity was measured by the method of Tukmechi *et al*. (2014) based on the lysis of the lysozyme sensitive gram positive bacterium, *Micrococcus lysodiekticus* (Sigma, USA). The dilutions of hen egg white lysozyme (Sigma, USA) ranging from 0 to 20 mg/ml (in 0.1 Mphosphate-citrate buffer, pH = 5.8) were considered as the standard. This along with the undiluted serum sample (25 ml) was placed into wells of a 96-well plate in triplicate. 175 µl of *M. lysodiekticus* suspension (75 µg/ml) prepared in the same buffer was then added to each well. After rapid mixing, the change in turbidity was measured every 30 s for 5 min at 450 nm at approximately 20°C using a microplate reader. The equivalent unit of activity of the sample as compared to the standard were determined and expressed as mg/ml serum.

Serum hemolytic complement activity (ACH50)

The hemolytic complement activity was assayed using rabbit red blood cells (RRBC) as targets (Amar *et al*., 2000; Tukmechi *et al*., 2011). Rabbit red blood cells (RRBC) were washed three times in ethylene glycol tetraacetic acid-magnesium-gelatin veronal buffer (0.01 M EGTA-MgeGVB, pH = 7) and the cell numbers were adjusted to 2×10^8 cells ml^{-1} in the same buffer. At first, the 100% lysis value was obtained by adding 100 ml of the above RaRBC to 3.4 ml distilled water. The hemolysate was centrifuged and the optical density (O.D.) of the supernatant was determined at 414 nm using a spectrophotometer (Awareness, USA). Following this, the test sera were diluted (100 times), different volumes ranging from 100 to 250 ml (total volume was adjusted to 250 ml with the buffer) was allowed to react with 100 ml of RaRBC in small test tubes. This mixture was incubated at 20°C for 90 min with intermittent mixing, following which 3.15 ml of 0.85% NaCl solution was added and the tubes were centrifuged at 1600 × g for 10 min at 4°C and the O.D. of the supernatant was measured as mentioned above. A lysis curve was obtained by plotting the percentage of hemolysis against the volume of serum added on a log-log graph. The volume yielding 50% hemolysis was used for determining the complement activity of the sample as follows:

ACH50 (units/ml) = K × (reciprocal of the serum dilution) × 0.5

where,
K: The amount of serum (ml) giving 50% lysis
0.5: The correction factor since the assay was performed on half scale of the original method

Serum total antibody level

Serum total immunoglobulin was determined following the method of Siwicki *et al*. (1994). After dilution of serum samples with 0.85% NaCl (100 times),

total protein content was determined by Bradford method (Kruger, 1996). Briefly, 100 ml of total serum samples were mixed with an equal volume of 12% solution of polyethyleneglycol (Sigma, USA) in wells of a 96-well microtiter plate. After 2 h of incubation at room temperature, plate was centrifuged at 5000 × g at 4°C. The supernatant was diluted 50 times with 0.85% of NaCl and the protein content was determined by Bradford method. This value was subtracted from the total protein level and the result was equal to the total immunoglobulin concentration of the serum that was expressed as mg/ml.

In situ *Y. ruckeri* challenge

After 60-day feeding trial, 30 fish from each dietary treatment (10 fish per each tank) were obtained, anaesthetized with clove powder (200 mg L^{-1}). Then, the fish were challenged by I.P. injection with 0.1 ml of a suspension of *Y. ruckeri* (BCCM/LMG 3279; Belgium Co-Ordinated Collection of Microorganisms) (1.1×10^7 CFU ml^{-1}). Dead and moribund fish were removed and examined microbiologically for up to 14 days. Moreover, agglutination test was performed on samples for confirmation of infection by *Yersinia*.

Statistical analysis

The results were subjected to analysis of variance (ANOVA) followed by Tukey's test to compare different treatments using the SPSS (ver. 19), correlation coefficients were significant at P<0.05.

Results

Before the experiment, no significant changes in all immunological parameters were observed between groups. Significant increase in lysozyme activity was shown in all treatment groups after 30-day feeding (Fig. 1). The lysozyme activity was significantly increased on day 60 in fish that received 1×10^7 and 1×10^8 CFU/g of Zn-enriched yeast in diet. The serum complement

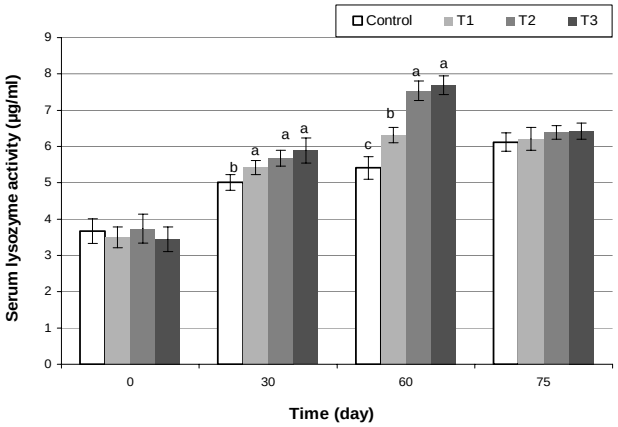

Fig. 1: The lysozyme activity of rainbow trout fed with zinc enriched *S. cerevisiae*. Each value (Mean ± SE) is the average performance of nine fish per treatment for a period of 75 days. Different letters represent significant differences between bars (P<0.05)

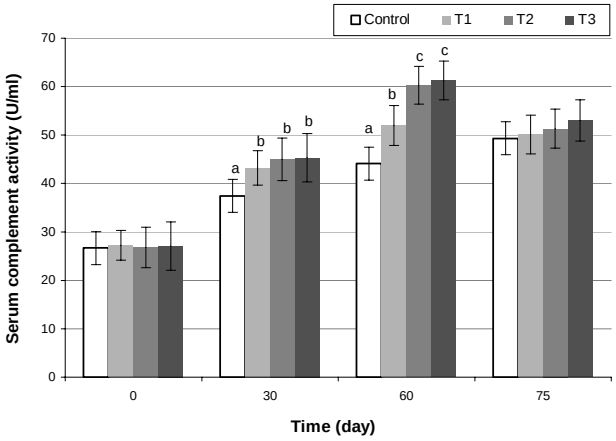

Fig. 2: The complement activity of rainbow trout fed with zinc enriched *Saccharomyces cerevisiae*. Each value (Mean ± SE) is the average performance of nine fish per treatment for a period of 75 days. Different letters represent significant differences between bars (P<0.05)

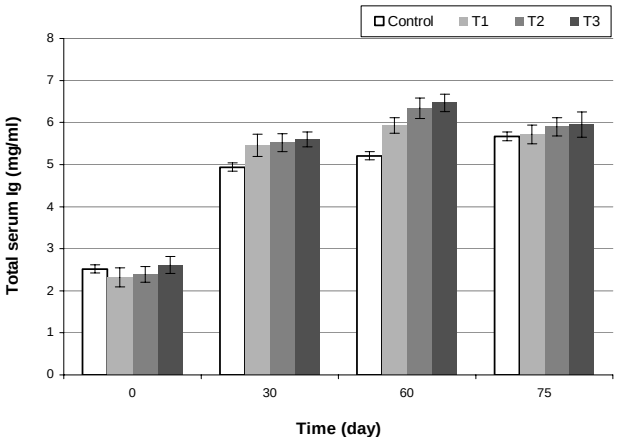

Fig. 3: The total antibody level of rainbow trout fed with zinc enriched *Saccharomyces cerevisiae*. Each value (Mean ± SE) is the average performance of nine fish per treatment for a period of 75 days. Different letters represent significant differences between bars (P<0.05)

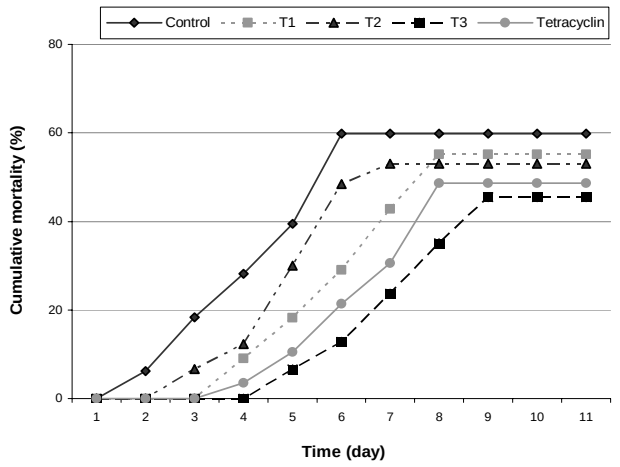

Fig 4: Cumulative initial mortality of rainbow trout fed diets containing Zn-enriched yeast after an i.p. bacterial challenge (*Yersinia ruckeri* BCCM/LMG 3279; Belgium, Coordinated Collection of Microorganism; 1.1×10^6 CFU/ml per fish). Values based on initial number of ten fish per replicate (three replicates) over a 10-day period

activity of fish exposed to Zn-enriched yeast was considerably higher than that of fish in control group (Fig. 2). Serum total immunoglobulin level showed a significant increase in fish fed with $\times 10^7$ and 1×10^8 CFU/g of Zn-enriched yeast compared to the control group (Fig. 3).

Figure 4 shows the survival rate of fish challenged with a virulent strain of *Y. ruckeri*. Over the next 9 days after the i.p. challenge, the lowest mortality was observed in fish that received 1×10^8 CFU/g of Zn-enriched yeast, but was not significant at the difference level of $P<0.05$.

A difference in other groups was not significant even though a minor decrease in mortality was observed in these treatment groups compared with the control group ($P>0.05$).

Discussion

In the current research the supplementation of Zn-enriched S. *cerevisiae* in all 3 concentrations improved the immunity in rainbow trout. This result agrees with that obtained with administration of whole cell yeast, Zn-enriched yeast and inorganic form of zinc (zinc sulfate) in fish and crustaceans (Wang *et al*., 2012). This study showed that Zn-enriched yeast treatment groups were effective in enhancing serum lysozyme, complement activity and total antibody of rainbow trout. Neutrophil is the main cell type for production of lysozyme in fish. As documented in this study the significant increase in neutrophil count was just observed in all treatment groups (the data have not been published yet) that are in parallel with increase in serum lysozyme activity. However, contrary results have been reported regarding the influence of *S. cerevisiae* whole cells and their constituents on serum lysozyme activity in (Jorgensen *et al*., 1995; Tukmechi and Bandboni, 2014).

The alternative pathway of complement activity is a powerful innate mechanism for protecting animal against potentially invasive pathogens (Chiu *et al*., 2010; Jalili *et al*., 2013). Our results showed that Zn-enriched *S. cerevisiae* groups exhibited significant increase in alternative complement pathway activity than the control. Contrary result was documented in grouper that supplementation with 10^5 and 10^7 CFU/kg of whole yeast cell exhibited significant increase in this immune response (Chiu *et al*., 2010). In fish that received 1×10^7 and 1×10^8 CFU/g of Zn-enriched yeast showed significant increase of these parameters on day 60 compared with control group. It can be concluded that availability of cellular constituent in whole cell yeast was not appropriate for stimulation the immune system. By the way, it was proved that Zn is an intracellular signaling molecule and it plays an important role in cell-mediated immune functions and oxidative stress. Zinc is also an anti-inflammatory agent.

The results of the current study demonstrated that in general, fish fed with Zn-enriched *S. cerevisiae* supplemented diet enhanced the survival of rainbow trout against *Y. ruckeri* compared with control group. Similarly, dietary Se-enriched *S. cerevisiae* enhanced resistance against *Y. ruckeri* in rainbow trout (Tukmechi and Shahraki, 2013) in comparison with untreated Zn-enriched yeast group. Even though yeast cells are rich in Zn the stimulation of immune system and consequently protection against disease agents will be reduced.

It is concluded that Zn enriched *S. cerevisiae* positively influenced non-specific immune responses of rainbow trout. In addition, the results of immune response assays demonstrated that Zn-enriched yeast could be administered for relatively long periods without causing immunosuppression.

References

Abdel-Tawwab, M; Abdel-Rahman, MA and Nahla-Ismael, EM (2008). Evaluation of commercial live bakers' yeast, *Saccharomyces cerevisiae* as a growth and immunity promoter for fry of Nile tilapia, *Oreochromis nileoticus* (L.) challenged in situ with *Aeromonas hydrophila*. Aquaculture. 280: 185-189.

Amar, EC; Kiron, V; Satoh, S; Okamoto, N and Watanabe, T (2000). Effects of dietary b-carotene on the immune response of rainbow trout (*Oncorhynchus mykiss*). Fisheries Sci., 66: 1068-1075.

Bury, NR; Walker, PA and Glover, CN (2006). Nutritive metal uptake in teleost fish. J. Exp. Biol., 206: 11-23.

Chiu, CH; Cheng, CH; Gua, WR; Guu, YK and Cheng, W (2010). Dietary administration of the probiotic, *Saccharomyces cerevisiae* P13, enhanced the growth, innate immune responses, and disease resistance of the grouper, *Epinephelus coioides*. Fish Shellfish Immunol., 29: 1053-1059.

Gopalakannan, A and Arul, V (2010). Enhancement of the innate immune system and disease-resistant activity in *Cyprinus carpio* by oral administration of b-glucan and whole cell yeast. Aquacult. Res., 41: 884-892.

Jalili, R; Tukmechi, A; Agh, N; Noori, F and Ghasemi, A (2013). Replacement of dietary fish meal with plant sources in rainbow trout (*Oncorhynchus mykiss*); effect on growth performance, immune responses, blood indices and disease resistance. Iranian J. Fisheries Sci., 12: 577-591.

Jorgensen, JB and Robertsen, B (1995). Yeast beta-glucan stimulates respiratory burst activity of Atlantic salmon (*Salmo salar* L.) macrophages. Dev. Comp. Immunol., 19: 43-57.

Kruger, NJ (1996). The Bradford method for protein quantitation. In: Walker, JM (Ed.), *The protein protocols handbook*. (1st Edn.), Totowa, NJ, USA, Humana Press. PP: 11-15.

Prasad, AS (2009). Zinc: role in immunity, oxidative stress and chronic inflammation. Curr. Opin. Clin. Nutr. Metab. Care., 12: 646-652.

Schneider, T; Persson, DP; Husted, S; Schellenberg, M; Gehrig, P; Lee, Y; Martinoia, E; Schjoerring, JK and Meyer, S (2013). A proteomics approach to investigate the process of Zn hyperaccumulation in Noccaea caerulescens. The Plant J., 73: 131-142.

Shet, AR; Patil, LR; Hombalimath, VS; Yaraguppi, DA and Udapudi, BB (2011). Enrichment of *Saccharomyces cerevisiae* with zinc and their impact on cell growth. Biotechnol. Bioinf. Bioeng., 1: 523-527.

Siwicki, AK; Anderson, DP and Rumsey, GL (1994). Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against

furunculosis. Vet. Immunol. Immunopathol., 41: 125-139.

Stehlik-Tomas, V; Gulan Zeti, V; Stanzer, D; Grba, S and Vahi, N (2004). Zinc, copper and manganese enrichment in yeast *Saccharomyces cerevisiae*. Food Technol. Biotechnol., 42: 115-120.

Strnadov, P; Svobodova, V; Pavlata, L; Misurova, L and Dvorak, R (2011). Effect of inorganic and organic zinc supplementation on coccidial infections in goat kids. Acta Vet. Brno., 80: 131-137.

Tukmechi, A and Bandboni, M (2014). Effects of *Saccharomyces cerevisiae* supplementation on immune response, hematological parameters, body composition and disease resistance in rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792). J. Appl. Ichthyol., 30: 55-61.

Tukmechi, A; Karimi Rad, F; Farrokhi, F; Agh, N and Jalili, R (2014). The effects of short- and long-term diet supplementation with Iranian propolis on the growth and immunity in rainbow trout (*Oncorhynchus mykiss*). Iranian J. Vet. Res., 15: 250-255.

Tukmechi, A; Rahmati Andani, HR; Manaffar, R and Sheikhzadeh, N (2011). Dietary administration of beta-mercapto-ethanol treated *Saccharomyces cerevisiae* enhanced the growth, innate immune response and disease resistance of the rainbow trout, *Oncorhynchus mykiss*. Fish Shellfish Immunol., 30: 923-928.

Tukmechi, A and Shahraki, R (2013). The effect of feeding with selenium enriched *Saccharomyces cerevisiae* on the growth and resistant of rainbow trout (*Oncorhynchus mykiss*) against environmental stresses and *Yersinia ruckeri*. J. Anim. Environ., 4: 49-58. (in Persian).

Wang, WX; Onsani, S and Dand, F (2012). Dietary bioavailability of cadmium, inorganic mercury, and zinc to a marine fish: effects of food composition and type. Aquaculture. 356: 98-104.

Wang, Z; Zhang, L and Tan, T (2010). High cell density fermentation of *Saccharomyces cerevisiae* GS2 for selenium-enriched yeast production. Korean J. Chem. Engin., 27: 1836-1840.

Yang, R; Meng, FC; Wang, P; Jiang, YB; Yin, QQ; Chang, J; Zuo, RY; Zheng, QH and Liu, JX (2012). Effect of organic and inorganic selenium supplementation on growth performance, meat quality and antioxidant property of broilers. African J. Biotechnol., 11: 3031-3036.