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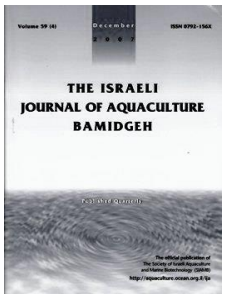

Effects of Dietary Nucleotide Yeast on Immune Responses and Antioxidant Enzyme Activities of Rainbow Trout Juveniles (*Oncorhynchus mykiss*)

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Key words: *Nucleotide yeast base protein, antioxidant enzymes, immune response, rainbow trout*

Abstract

This study aimed at demonstrating the effects of dietary supplementation of nucleotide yeast base protein (Nu-Pro®) (NP) on the antioxidant enzyme activities and immune response in liver and blood tissues of rainbow trout (*Oncorhynchus mykiss*). Fish with an average initial weight of 27.75±0.26 g were randomly assigned to four groups with three replicates. Throughout the 60 day grow-out period the control group was fed a fish meal based basal diet, and three other groups were fed diets in which 20 (NP 20), 40 (NP 40) and 60 % (NP 60) fish meal was substituted with nucleotide (Nu-Pro® (NP) yeast). There were no significant changes of superoxide dismutase (SOD) and catalase (CAT) activities in liver among the experimental groups. A significant decrease (P<0.05) in malondialdehyde (MDA) level of tissue was observed in all nucleotide supplemented groups when compared to the control group. Serum lysozyme (LYZ) and myeloperoxidase (MPO) activities and nitric oxide (NO) level of liver tissue were significantly (P<0.05) increased in fish fed with nucleotide yeast based protein diets. The results showed that the fish in all nucleotide supplemented groups showed significantly better antioxidant activity and immune responses.

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Introduction

The expansion in aquaculture has led to a growing number of disease outbreaks caused by an increasing range of pathogens. Conventional approaches such as use of disinfectants and antimicrobial drugs have had limited success in the prevention or cure of aquatic diseases. Increasing economic and social concern aimed to decrease the use of antibiotics and other therapeutic chemicals in aquaculture has encouraged more environmentally friendly approaches to improve growth and disease control (Hansen and Olafsen 1999). Research into the use of food additivities for aquatic animals is increasing as part of the demand for environmentally friendly sustainable aquaculture (Martinez-Cruz et al. 2012).

The use of micronutrients, such as vitamins, trace elements, probiotics, and immunostimulants as dietary supplements may improve animal health by strengthening their non-specific immune system. These also improve availability or utilization of nutrients in a number of ways. The benefits of such supplements include improved feed value, enzymatic contribution to digestion, growth-promoting factors, and increased immune response (Verschuere et al., 2000; Ganguly et al. 2010; Özlüer Hunt et al., 2014). Immunostimulants are natural or synthetic substances which activate non-specific and specific immune responses (Anderson 1992; Li et al., 2005).

Dietary nucleotides have been shown to benefit many mammalian physiological and nutritional functions (Uauy et al., 1990). There have been few reports on the effectiveness of dietary nucleotides in fish. Nucleotides are considered to significantly enhance feed intake in largemouth bass and increase the growth rates of rainbow trout (Adamek et al., 1996; Kubitza et al. 1997). Nucleotides have also improved the health status of salmonids by increasing resistance of fish to various bacterial, viral and rickettsial infections and reducing the severity of ectoparasitic infestation by elevating immune responses of Atlantic salmon (Burrells et al., 2001). However, physiological stressors such as vaccination, grading, net changing, salt water transfer, etc., are known to induce the release of cortisol resulting in immunosuppression. Supplementation of standard aquaculture diets with additional dietary nucleotides has been beneficial in such cases. Dietary nucleotides have several benefits including rapid intestinal repair, improved mucosal gut flora, and mucosal surfaces and elongation of the intestinal tract in aquatic animals (Li and Gatlin 2006; Li et al. 2007). Nucleotides have also enhanced the immune system and disease resistance of various fish and shellfish species (Lin et al., 2009; Burrells et al., 2001; Biswas et al., 2012; Tahmasebi-Kohyani et al., 2012).

Antioxidant enzymes are natural defenses that can be enhanced under stressful situations and used as indicators of oxidative stress (Livingstone 2001). In vitro and in vivo studies demonstrate the capacity of fish to generate reactive oxygen species (ROS) by xenobiotics, physical conditions, and/or diet. Defense systems that may inhibit ROS formation include antioxidant enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6). Measurements of antioxidative enzyme activity in fish are used to evaluate oxidative damage caused by environmental factors in aquatic ecosystems (Zhang et al., 2004). Malondialdehyde (MDA) is the main oxidation product of peroxidized polyunsaturated fatty acids and raised MDA level is an important index of lipid peroxidation (LPO) (Elia et al. 2002). If antioxidants in a body are unable to overwhelm the free radicals, free radical activity can lead to cell damage known as oxidative stress. Antioxidants can protect organisms from free radicals and ROS effects and slow down the progress of many chronic diseases (Kim et al. 2009). The antioxidant defense system is a multicomponent mechanism with enzymatic and non-enzymatic elements. Antioxidant defenses in fish are dependent on factors such as feeding behavior and nutrition (Martinez-Alvarez et al. 2005).

Lysozyme (LYZ; EC 3.2.1.17) is an important defense molecule of the immune system protecting against microbial invasion (Uribe et al. 2011).

Myeloperoxidase (MPO; EC 1.11.1.7) is an oxygen-dependent iron-containing heme non-protein which is located in macrophages and catalyzes oxidative reactions, serving as an indirect indicator of macrophage function (van der Veen et al. 2009). Nitric

oxide (NO) generated by NOS is a bactericidal molecule employed by aquatic organism immunocytes (Yeh et al. 2006).

The purpose of this research was to determine the effects of dietary nucleotide supplementation on antioxidant enzyme activity and immune response in liver tissue and blood serum of the juvenile rainbow trout. The activity of several antioxidant enzymes such as SOD, CAT, MDA and NO was measured in the liver tissues of rainbow trout. LYZ and MPO activity was also measured in the blood serum in order to understand the immune response of fish when fed diets supplemented with increasing amounts of Nu-Pro®.

Materials and Methods

Chemicals. Nu-Pro® was obtained from Alltech Inc., Nicholasville, KY, USA. All other chemicals were purchased from Sigma–Aldrich Chemical Corporation (USA).

Fish diets. Three balanced iso-nitrogenous and iso-energetic diets were formulated with supplemented commercial organic yeast (Nu-Pro® Alltech Inc., Nicholasville, KY, USA) and replaced fish meal at 20 (Nu-Pro®20), 40 (Nu-Pro®40) and 60 (Nu-Pro®60) % levels on a dry matter basis (Table 1), and a control (Nu-Pro® 0). Nu-Pro® is a functional protein from yeast containing highly concentrated levels of essential and functional nutrients. It is rich in nucleotides, inositol, glutamic acid, amino acids and peptides. After pelletizing, each diet was individually bagged and stored at -20 °C until use. The diets were analyzed in duplicate for proximate compositions according to the standard methods of AOAC (1996).

Table 1. Formulation (g/kg diet) and chemical composition of the experimental diets.

	Diets			
	Control	*NP20 (%)*	*NP40 (%)*	*NP60 (%)*
Fish meal[1]	605	501	432	355
Corn gluten meal	70	100	93	94
Nu-Pro®[2]	0	121	242	363
Fish Oil[3]	100	108	113	119
Dextrin[3]	140	91	55	10
Binder (CMC)	52	46	32	26
Mineral Mix.[4]	20	20	20	20
Vitamin Mix.[4]	13	13	13	13
Proximate Composition (g/kg)				
Dry Matter	896.5	904.4	909.1	908.6
Crude protein	441.7	436.1	448.6	439.3
Crude lipid	187.3	187.6	187.5	188.9
NFE[5]	159.2	168.2	162.8	179.4
Crude ash	108.4	112.5	110.2	101.0
Gross energy (MJ kg/DM)[6]	18.9	19.0	18.9	18.9
P:Eratio (g /MJ)	23.36	22.95	23.74	23.24
Calculated TAA Profile (g/kg)				
Arginine	16.46	16.01	15.99	15.87
Histidine	7.09	7.15	7.27	7.38
Isoloucine	13.94	14.08	14.35	14.59
Leucine	24.89	26.67	27.26	28.09
Lysine	20.86	20.28	20.62	20.75
Methionine	8.77	8.71	8.73	8.73
Cystine	2.96	3.26	3.41	3.59
Phenyalanine	13.00	13.58	13.88	14.23
Tyrosine	10.53	11.22	11.65	12.12
Thyreonine	12.32	12.89	13.63	14.33
Tryptophane	3.22	3.26	3.36	3.45
Valine	15.52	15.98	16.53	17.06

[1] Anchovy fish meal and oil. SIBAL Black Sea Feed Inc., Sinop, Turkey
[2] Alltech Incorporated, Nicholasville, KY, USA.
[3] SUNAR Inc., Adana, Turkey.
[4] Vitamin and mineral premix added minimum to NRC recommendations, SIBAL Black Sea Feed Inc., Sinop, Turkey (NRC,1993)
[5] Nitrogen-Free Extract: Calculated as the remainder of crude protein + crude lipid + ash.
[6] Calculated based on the standard physiological fuel values: 19 kJ/g for protein, 36 kJ/g for lipid and 15 kJ/g for carbohydrate (Smith, 1989).

Fish and experimental conditions. Rainbow trout fingerlings were obtained from a commercial farm in Gözne-Mersin-Turkey. The fish were transported to the Mersin University Aquaculture Laboratory Unit and acclimatized to laboratory conditions. During the acclimation period, fish were fed a commercial diet (Crude protein: 48 %, crude lipid: 22 %, Çamli Yem-İzmir/Turkey) without dietary nucleotides for 2 weeks at a level of 3 % of their body weight. At the end of the acclimation period, fish (27.76 g ± 0.26, mean weight ± SEM) were randomly distributed into 12 tanks (12 fish/tank, total 144 fish). The tank system was installed in an environmentally-controlled laboratory where temperature was maintained between 15-16 °C, with a photoperiod of 12 h light and 12 h dark. Water depth in the 200 L tanks was kept at 50 cm throughout the experiment by adding fresh water continuously from a reservoir tank after daily siphoning of uneaten feed and feces. Fish were fed two equal portions of the experimental diets, twice daily (8:00-9:00am and 16:00-17:00pm) at 3 % body weight (BW) per day, throughout the 60-day growing period. Average water quality parameters were measured as; temperature 15.5±0.8 °C, dissolved oxygen 8.1±0.08 mg/L, pH 8.6±0.25, NO_3 0.5±0.1 mg/L, NO_2 0.03±05 mg/L, and NH_4 0.07±0.6 mg/L.

Tissue sampling for biochemical analysis. At the end of the study, 24 fish (6 fish per treatment) were randomly chosen from each dietary group, sacrificed, and used for analysis of liver tissue CAT, SOD activities and MDA and NO levels. The tissue samples were obtained from each individual fish, combined and prepared for analysis. Tissue samples were homogenized in 1/5 (w/v) ratio of physiological saline solution (0.8 % NaCl) with an homogenizer and then centrifuged at 13500 rpm for 10 min in a Sigma 2-16 K centrifuge at -4 ºC. The supernatants were then used for biochemical analyses.

Blood sampling and serum analysis. At the end of the trial, 36 fish (9 fish per treatment) were used for analyses of serum LYZ and MPO. 9 fish were randomly chosen from each dietary group and blood samples were collected from the caudal vein Serum was centrifuged at 3000 rpm for 10 min. Serum samples were transferred to Eppendorf tubes and stored at -70 °C until determination of LYZ and MPO activity.

Biochemical assays. CAT activity was determined according to the method of Aebi (1974). Enzymatic decomposition of H_2O_2 was directly followed by decrease in absorbance at 240 nm. The difference in absorbance per unit time was used as a measure of CAT activity. The enzyme activity was expressed as unit (U)/mg protein.

SOD activity was measured by inhibition of nitroblue tetrazolium (NBT) reduction due to O_2 generated by the xanthine/xanthine oxidase system (Sun et al. 1988). One unit of SOD activity was defined as the amount of protein causing 50% inhibition of NBT reduction rate. Reduction in NBT by superoxide anion to blue formazan was 560 nm. Enzyme activity was given in U/mg protein.

MDA levels were measured as an index of LPO, by the thiobarbituric acid reaction according to the methods of Yagi (1998 based on measurement of the absorbance of pink color produced by interaction of TBA with MDA at 530 nm. Values were expressed as nanomole (nmol) per mg protein.

NO levels of liver tissue were measured as nitrites using the modified Griess reaction after converting nitrates to nitrites with vanadium chloride (Green et al. 1982; Miranda et al. 2001). The assay is based on reduction of nitrate by vanadium trichloride (VCl_3) combined with detection by the acidic Griess reaction. The diazotization of sulfanilic acid with nitrite at acidic pH and subsequent coupling with N-(10 naphthyl)-ethylenediamine produced an intensely colored product measured spectrophotometrically at 540 nm. Nitrite/nitrate concentration was calculated using a $NaNO_2$ standard curve and expressed as nmol/mg protein.

Tissue protein contents were determined according to the method developed by Lowry et al. (1951) using bovine serum albumin as standard. Absorbance of samples was measured at 750 nm wavelength by spectrophotometer.

Serum assays. The method used to determine the lysozyme activity in serum was based on the lysis of the LYZ-sensitive Gram positive bacterium *Micrococcus lysodeikticus* (Ellis 1990). A suspension of bacteria (0.2 mg/mL) in buffer was also prepared. Dilutions of the

standard and undiluted serum samples (2 µL) were used and *M. lysodeikticus* suspension was then added to each well. After mixing, the reaction was carried out at 25 °C, and absorbance was measured by spectrophotometer at 530 nm. LYZ activities were expressed as U/mL.

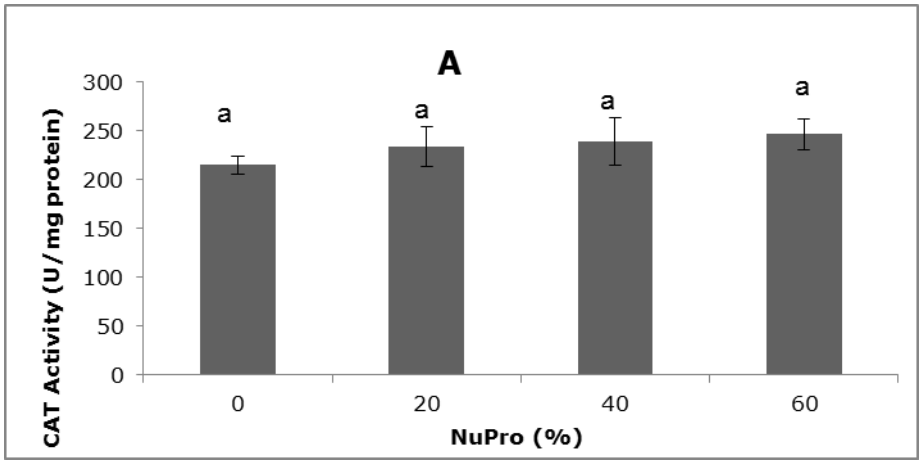
Serum MPO activities were measured spectrophotometrically using o-dianisidine and hydrogen peroxide (Krueger et al. 1990). In the presence of H_2O_2 as an oxidizing agent, MPO catalyses the oxidation of o-dianisidine yielding a brown colored product, oxidized o-dianisidine. Absorbance of samples was measured by spectrophotometry at the wavelength of 460 nm. MPO activities were expressed as U/ml.

Statistical analysis. Data were subjected to a one-way analysis of variance (ANOVA). After identification of differences among groups Duncan's new multiple range tests were used to make multiple comparisons between means. Differences were considered significant at $P<0.05$. Statistical analyses were performed using SPSS 22.0 for Windows.

Results

No mortalities were recorded during the 60-day feeding trial. There were no obvious effects of nucleotide supplementation on water quality.

CAT activity of tissue is shown in Figure 1A. Compared to the control, no significant changes ($P>0.05$) of CAT activity were found when amount of NP supplementation was increased. CAT activity was increased slightly in fish fed diets supplemented with increasing amount of NP (20 %; 233.54±20.53 U/mg), (40 %; 239.4±24.70 U/mg), (60 %; 246.62±15.51 U/mg) compared to the control (215.43±9.22 U/mg). SOD activity of tissue is shown in Figure 1B. Liver SOD activity was not significantly changed ($P>0.05$) in fish fed diets with increasing amounts of NP supplementation compared to that of fish in the control group. SOD activity increased slightly in fish fed the NP 60 % supplemented diet (3.64±0.15 U/mg) compared with control group (3.39.54±0.40 U/mg). MDA levels in liver tissue are shown in Figure 1C. MDA levels were significantly decreased ($P<0.05$) in all nucleotide supplemented groups compared with control group. A significant decrease in MDA levels were observed in groups receiving 20 %, 40 % and 60 % of dietary NP supplementation in comparison with control group (-35. 86 %; -39.36 % and -60.18 % respectively).



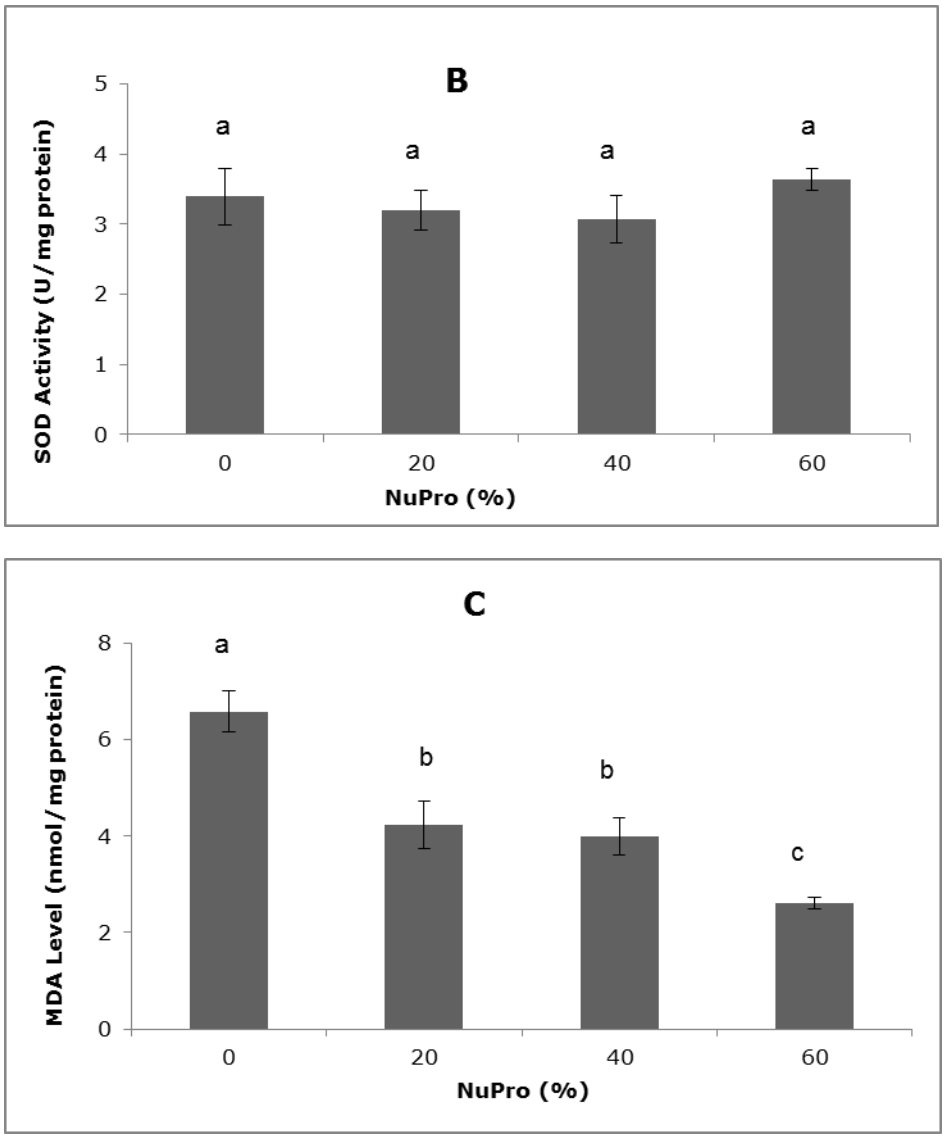


Fig.1 (A The activities of the antioxidant enzymes) catalase (CAT), (B) superoxide dismutase (SOD), and (C) malondialdehyde (MDA) content in liver tissue of rainbow trout fed different levels of nucleotide supplementation. Each value represents the mean±SEM. (n=4, 6 samples per treatment were used in the ANOVA). The different letters indicate significant (P<0.05) difference between dietary treatments.

The NO levels of liver tissue are shown in Figure 2. The NO levels were increased significantly *(P*<0.05) in fish fed diets with increasing amounts of nucleotide supplementation. 60.71%; 121.43%; and 107.14 % increase in NO levels were observed in fish fed diets supplemented with 20, 40, and 60% of dietary NP respectively compared to that of fish fed fish meal based diet.

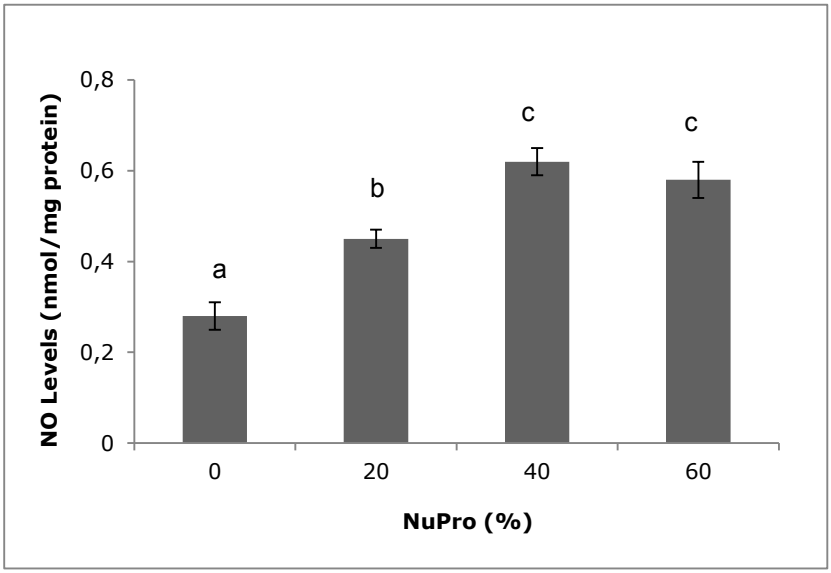
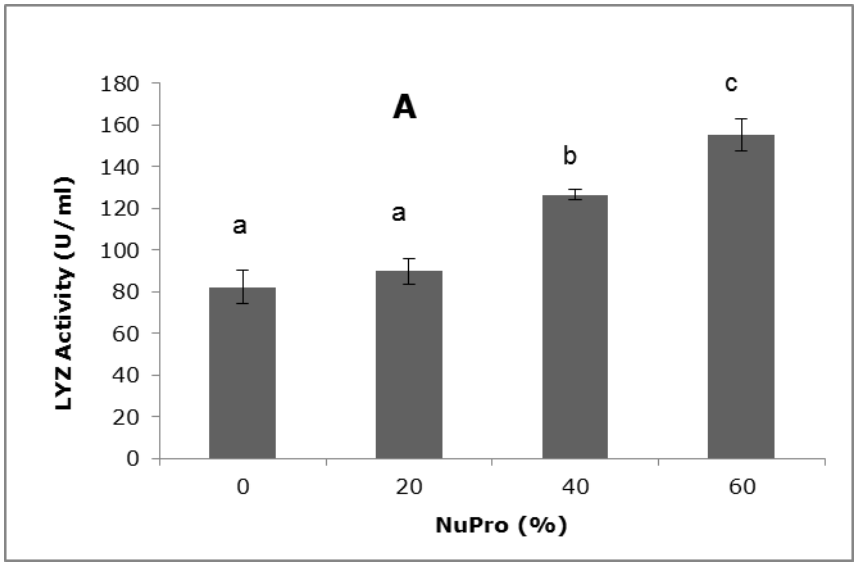


Fig. 2 The NO levels in liver tissue of rainbow trout fed different levels of nucleotide supplementation. Each value represents the mean ± SEM. (n=4, 6 samples per treatment were used in ANOVA). The different letters indicate significant (P<0.05) difference between diet groups

The serum LYZ and MPO activities are shown in Figure 3A and 3B. The dietary administration of nucleotide significantly enhanced (P<0.05) the serum LYZ activity at the end of the feeding period (Fig. 3A). NP 60 group showed the highest activity of LYZ (155.52±7.79 U/mL) at the end of the 60 days feeding period. The activities of MYO were also significantly increased (P<0.05) in fish fed diets supplemented with increasing amount of NP (20%; 47.50±2.02 U/mL), (40%; 52.53±1.59 U/mL), (60%; 67.15±1.94 U/mL) than the control (35.42±3.32 U/mL) (Fig. 3B).



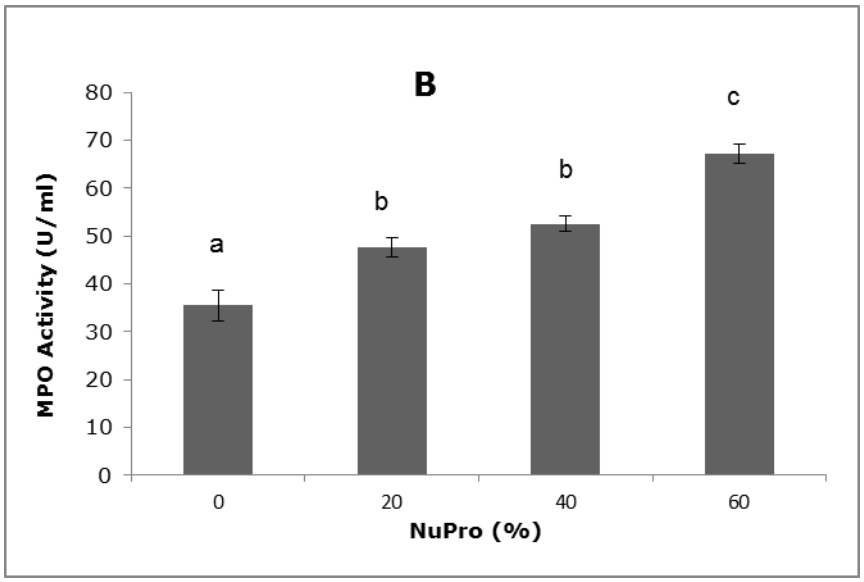


Fig. 3 Serum LYZ (A) and MPO (B) activity of rainbow trout fed different levels of nucleotide supplementation. Each value represents the mean±SEM. (n=4, 9 samples per treatment were used in ANOVA). The different letters indicate significant (P<0.05) difference between diet groups

Discussion

The present study was carried out to investigate the effects of increasing the amount of dietary nucleotide supplementation on the oxidative stress markers (CAT and SOD activity), determination of lipid peroxidation (MDA) and immune response in healthy rainbow trout. Almost no information is available regarding the effects of Nucleotide yeast base protein supplementation on growth parameters and wellbeing (specifically antioxidant defense mechanisms and immune parameters) of the rainbow trout, an example of an important farmed fish species including the rainbow trout. In a previous study we found that growth rates were positively affected when fish meal was replaced with nucleotides, and fish fed the NP 40 diet showed significantly higher growth rates (P<0.05) and higher weight gain than with other treatments (Özlüer-Hunt et al., 2014). Digestive tract enzyme activity measured in this study also revealed that there was increased gastric pepsin activity and intestinal trypsin and lipase activity in fish fed diets supplemented with nucleotides.

In this current study, CAT and SOD activity was not significantly altered in fish fed diets with increasing amount of NP. In addition, MDA level of liver tissue significantly decreased in relation to the increasing amount of nucleotide probably indicating a decrease in lipid peroxidation (LPO). The antioxidant enzymes play a crucial role in the inhibition of radical generation and prevention of oxidative damage in teleost (Rudneva 1997). CAT primarily occurs in peroxisomes where it detoxifies H_2O_2 to O_2 and water. SOD catalyzes the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide and is an important component of the antioxidant defense system of the organism. LPO is one of the main processes induced by oxidative stress and is the first step towards causing cellular damage. ROS can increase oxidation of polyunsaturated fatty acids and lead to peroxidation (Zhang et al. 2008). CAT activity was decreased and SOD activity was not affected by dietary supplementation with nucleotides in the serum of rainbow trout (Mohebbi et al. 2013). Moreover, they observed a significant decrease in MDA levels in the serum of rainbow trout after feeding dietary supplemented nucleotides. Similar trends were also in SOD activity and MDA levels. The result of CAT activity in the study by Mohebbi et al (2013) differed from those found in our study. This may be due to the amounts of nucleotide supplemented for fish meal in diets, the source of nucleotides, and the combination of both factors (Mohebbi et al., 2013). Cells may overecome the effects of oxidative stress either by repairing the damage or by directly reducing the pro-oxidative state via enzymatic and non-enzymatic

antioxidants (Korkmaz et al. 2009). Nucleotides are not known to have antioxidant properties per se (Frankic et al. 2006). Nu-Pro® is a yeast extract derived from a select strain of yeast that provides an excellent source of protein, amino acids, nucleotides and vitamins. Nu-Pro® is a complex ingredient as it combines nutritional components (protein and vitamins) with functional components (nucleotides and free amino acids) (Fegan, 2006). Currently, specific mechanisms by which yeast and its components influence antioxidative status are unknown. However, one component of the yeast cell wall is β-D-glucan which has powerful antioxidant attributes with heightened free-radical scavenging activity and it might have improved the effects on oxidative status of juvenile rainbow trout in this study (Kim et al. 2009).

The present study clearly indicates that fish fed feed containing nucleotide yeast showed a significant increase of serum LYZ and MPO activities and level of NO in liver tissue. Our results support other findings which suggest that dietary nucleotides act as an immunostimulants. Several studies involving aquatic species have shown that dietary nucleotides can elevate cellular and humoral immune responses in Atlantic salmon (Burrells et al. 2001), hybrid striped bass (Li et al. 2004), catla (Jha et al. 2007), grouper (Lin et al. 2009), rainbow trout (Leonardi et al. 2003; Tahmasebi-Kohyani et al. 2012) and kuruma shrimp (Biswas et al. 2012). The immunostimulants mainly facilitate the function of phagocytic cells, increase their bactericidal activity, and stimulate the natural killer cells, complement system, LYZ activity, and antibody responses in fish and shellfish which results in enhanced protection from infectious diseases (Cheng et al. 2011). *Paralichthys olivaceus* fed diets containing 0.2 % and 0.4 % dietary nucleotide showed significantly higher serum MPO and LYZ activity than fish fed the basal diet (Song et al. 2012). The addition of yeast fermentation products to diets fed to hybrid tilapia, *O. niloticus X O. aureus*, grown in cages resulted in an increase in serum LYZ activity, macrophage phagocytic activity, and macrophage respiratory burst activity compared to fish fed a diet without the yeast product (He et al. 2009). Nucleotides from brewers' yeast RNA enhanced the phagocytic and oxidative activities of kidney phagocytic cells, LYZ in common carp (Sakai et al. 2001). The benefits of using brewer's yeast extract in feeds are both nutritional and as a functional nutrient (Barbu et al. 2008). Brewer's yeast (*Saccharomyces cerevisiae)* contains various immune stimulating compounds such as β-glucans, nucleic acids and chitin as well as mannan oligosaccharides, and it has been observed to be capable of enhancing immune responses as well as growth of various fish and shellfish species (Thanardkit et al. 2002; Lara-Flores et al. 2003; Wang et al. 2014; Meshram et al. 2015).

Studies with different fish have shown that dietary supplementation of nucleotides can alter gastrointestinal tract (GIT) morphology and potentially enhance digestive capacity. Nucleotide supplementation has been found to increase fold height in intestine and microvilli height in intestine of red drum and improved immune systems of fish (Cheng et al. 2011). The increase in fold height, microvilli height, and overall absorptive area might be another mechanism for improved feed utilization. Health of GIT is extremely important for livestock and aquatic animals because it is a major organ for nutrient absorption and immune responses. However, the relationship between improvement in GIT and stimulation mechanisms of the immune systems due to nucleotide supplementation has not yet been explained (Li et al. 2015).

Research with fish has shown that exogenous nucleotides can affect the immune system. However, there is limited information regarding the replacement of fish meal with nucleotide yeast based protein sources and how this affects immune responses and antioxidant enzyme activities in fish. Therefore, this experiment was conducted to examine the effect of dietary nucleotide yeast on immune response and antioxidant enzyme activity of rainbow trout in culture conditions.

In conclusion, the replacement of fish meal by nucleotide protein sources improved antioxidant capacity and immune response in juvenile trout. Taken together, results from the present study indicate that dietary supplementation with dietary nucleotides have a positive influence on immune responses and antioxidant capacity of rainbow trout. The use of nucleotides in aquaculture diets is an area to be investigated.

Further studies are needed to examine the mechanism of nucleotide supplementation in fish feed in increasing antioxidant activity and immune response in cultured fish species.

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