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RESEARCH ARTICLE

Modulation of oxidative stress biomarkers and lysosomal functioning in gills and liver of rainbow trout (*Oncorhynchus mykiss* Walbaum) fed a diet supplemented with yeast β-glucans

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Abstract. The present study was performed to investigate the effects of dietary yeast β-1,3/1,6-D-glucans after 15-, 30-, and 45-day feeding periods on the development of oxidative stress, antioxidant defenses, and lysosomal functioning in gills and hepatic tissue of rainbow trout, *Oncorhynchus mykiss* (Walbaum). The fish were fed a control diet or an experimental diet containing the supplement Yestimun® at a dose of 1% in the basal feed (with 85% content of β-1,3/1,6-glucans). On days 15, 30, and 45 of the experiment, the following were analyzed in gills and hepatic tissue: oxidative stress biomarkers (2-thiobarbituric-acid-reacting substances [TBARS]); aldehydic and ketonic derivatives of oxidatively modified proteins; activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx); total antioxidant capacity (TAC); activities of lysosomal enzymes (alanyl aminopeptidase [AAP], leucyl aminopeptidase [LAP], acid phosphatase [AcP], β-N-acetylglucosaminidase [NAG]). The dietary β-glucans stimulated CAT, GPx, LAP, and AcP activities in gills and hepatic tissue compared to the control group; however, the opposite trend was observed in TAC levels in gills on days 30 and 45. The present study suggests that β-glucans can enhance immune response, antioxidant capacity, and lysosomal functioning effectively in rainbow trout.

Keywords: yeast β-glucans, oxidative stress, rainbow trout *Oncorhynchus mykiss*, gills, liver

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Introduction

Aquaculture and fish farming play an important role in food security and welfare; therefore, many fish species are now being cultured in response to increased demands for fish (Kumar 2014). Massive production losses in aquaculture are caused by many factors. Studies have demonstrated that almost fifty percent of production losses in developing countries are associated with diseases of different etiologies (Assefa and Abunna 2018). A variety of microbial agents (viruses, bacteria, fungi, parasites, etc.) have been shown to cause diseases in aquaculture (Ngugi et al. 2015), which is supported by high fish densities and favorable environmental conditions in intensive fish farming. Many manipulations in fish farming also cause stress reactions in fishes that strongly

impact their immune systems. Therefore, the risk of diseases and the vulnerability of fishes to various infections in fish farms is also increased by stress-induced weakening of the immune system (Nguyen et al. 2019).

In recent years, attention has been focused on finding novel drugs to develop alternative practices for disease management in aquaculture. Biological and chemical disease control strategies, such as the application of probiotics, prebiotics, and medicinal plants that help to increase resistance against infectious diseases by enhancing innate humoral and cellular defense mechanisms, are used widely (Magnadóttir 2006, 2010, Ganguly et al. 2010, Ringř et al. 2010, Meena et al. 2013, Song et al. 2014). Applying immunostimulants in aquaculture for disease control is a promising alternative to antibiotics (Assefa and Abunna 2018).

β-Glucans, i.e., naturally occurring polysaccharides with glucose as a structural component linked by β-glycosidic bonds, are natural feed additives used in fish diets to improve the immune system and are one of the most significant immunostimulant-based approaches to health care and growth performance (Novak and Vetvicka 2009, Meena et al. 2013). Various studies in a wide range of fish species have revealed the effect of β-glucans on growth, antibody production, immune-related gene expression, survival, resistance, and protection against pathogens, and their role as an adjuvant (Meena et al. 2013, Petit and Wiegertjes 2016, Petit et al. 2019, Song et al. 2020). β-Glucans are a heterogeneous group of glucose polymers found, inter alia, in the cell walls of plants, bacteria, fungi, and protozoa. β-Glucans have a basic chain, and in some cases, side chains, which comprise β(1,3), β(1,4), and/or β(1,6)-bound glucose groups, depending on the origin of the glucan (Douxfils et al. 2017, Felicioli et al. 2020). Depending on the source and mode of excretion, β-glucans have different degrees of branching and types of bonding in the main and side chains (Skov et al. 2012). The frequency and type of the bond in the side chain is largely consistent with the biological activity of the molecule. Glucans also differ significantly in their molecular weight and in the tendency of chains to agglomerate, both of which are essential to the efficiency profile of these molecules (Meena et al. 2013).

These large molecules do not undergo enzymatic fragmentation in the gastrointestinal tract. They are absorbed by cells in the intestinal mucosa and actively transferred to the submucosa layer, where they activate macrophages and, through them, lymphocytes responsible for protecting the endothelium, i.e., local immunity. Through a repopulation mechanism, activated lymphocytes disperse from the intestinal mucous membrane into the mucous membranes of various organs and thus protect against infections. In general, the mechanism of action of β-1,3/1,6-glucan can be explained by its marked selectivity to specific receptors on the surface of macrophages that bind only to an unbranched area of the β-glucan molecule (Medina-Córdova et al. 2018). This results in the activation of macrophages, which leads to the implementation of triggering mechanisms for several processes aimed at protecting the immune system (Selvaraj et al. 2006, Yar Ahmadi et al. 2014).

They both act on invading microorganisms and complement the activation and action of immune responses because of their ability to bind directly with macrophages and other white blood cells (neutrophils and natural killer cells) and to activate them (Sakai 1999, Gantner et al. 2003, Herre et al. 2004). The engagement of β-glucan receptors results in improvement of immune responses, i.e., phagocytosis, the release of certain cytokines – IL-1, IL-6, GM-CSF, interferons, and the processing of antigens (Meena et al. 2013). Activated phagocytic cells, i.e., B cells and T cells, in fish produce cytokines and antibodies, respectively, and enhance the efficacy of vaccines (Raa 2000). In addition to stimulating macrophages, β-glucans can also improve other immune responses (Vetvicka et al. 2013). β-Glucans contribute to a series of non-specific abilities to resist pathogens in fish. For example, they enhance serum lysozyme activity, rapid tissue growth, and the division of bone marrow cells (Meena et al. 2013).

The ability of fish to adapt to changing environmental conditions, with an increase in the probability of survival and self-reproduction at the cell and

tissue levels, is supported by various biochemical mechanisms (Valavanidis et al. 2006, Mendel et al. 2018). These mechanisms have been shown to rebuild metabolism through quantitative and qualitative changes in enzyme-related systems (Biller and Takahashi 2018, Kurhaluk 2019). An important role in maintaining complex integrative functions in the organism is played by lysosomes, i.e., specialized cellular organelles that directly participate in cell enzymatic hydrolysis reactions (Saftig and Klumperman 2009). Mammal and human lysosomes have been studied in detail, while there are substantially fewer studies on the participation of the lysosomal apparatus in maintaining homeostasis and adaptive reactions in response to changing environmental conditions and development of various pathologies in fishes and other hydrobionts (Misra et al. 2004).

Reactive oxygen species (ROS) are produced naturally via several cellular pathways of aerobic metabolism, including oxidative phosphorylation, electron transport chains in mitochondria and microsomes, the activity of oxidoreductase enzymes producing ROS as intermediate or final products, and even immunological reactions such as active phagocytosis (Halliwell and Gutteridge 2007, Regoli and Giuliani 2014). They differ greatly in terms of cellular reactivity and the potential to cause toxic insults to lipids, proteins, and DNA (Regoli and Winston 1999, Regoli and Giuliani 2014). In basal conditions, the adverse effects of oxyradicals are prevented by the antioxidant system consisting of a wide array of low molecular weight scavengers and antioxidant enzymes that interact in a sophisticated network with both direct and indirect effects (Regoli and Giuliani 2014). The balance between ROS production and removal by antioxidant systems is the "redox state." Oxidative stress is defined as the excess production of ROS relative to the levels of antioxidants. When the production of ROS exceeds the capacity of antioxidant defense, oxidative stress has a harmful effect on the functional and structural integrity of biological tissue (Tsutsui et al. 2011).

On the other hand, ROS activate various lysosomal enzymes and lysosomal signaling mechanisms, which allows cells to respond to oxidative bursts and adapt to oxidative stress (Zhang e al. 2016). The involvement of lysosomes in processes such as intracellular digestion, fertilization, cell division, differentiation and aging, metamorphosis, and immune responses has been shown (Moore et al. 2006a,b). Many hormone effects are realized through these organelles as is the regulation of metabolic pathways (DeMartino and Goldberg 1978). These lysosomal reactions are particularly sensitive to oxidation stress (Weng et al. 2013). Therefore, studying the relationship between immunological response, redox reactions, and the functioning of lysosomes in fishes is an important approach in the study of biochemical mechanisms underlying dietary treatment with β-glucans.

The relevance of this topic stems from the increasing demand for innovative solutions in aquaculture practice to improve the health and productivity of farmed fish. Research into the effects of β-glucans on fish is becoming increasingly important as alternative approaches are sought to enhance fish immunity and improve production efficiency (Rodrigues et al. 2020, Machuca et al. 2022). With the challenges of maintaining healthy fish populations and reducing the use of antibiotics in aquaculture, substances such as β-glucans offer valuable alternatives. Not only do they have a positive impact on fish health and welfare, they also contribute to sustainable aquaculture production (Vetvicka et al. 2013, Petit and Wiegertjes 2016). Therefore, the continued interest in this topic in aquaculture practice stems from the need to explore effective, environmentally friendly strategies to improve the quality and efficiency of fish farming. While some studies have investigated the general effects of β-glucans (Dalmo and Břgwald 2008, Goodridge et al. 2009, Meena et al. 2013), our focus is on the analysis of specific biochemical parameters that have been investigated to a lesser degree in this context (Tkachenko et al. 2022). Building on the findings of previous authors, we have extended our analysis to include markers of oxidative stress, antioxidant defenses, and lysosomal function to provide a more comprehensive understanding of the effects of β-glucan supplementation on fish health.

The present study aimed to explore the potential effects of dietary yeast β-1,3/1,6-D-glucans after 15-, 30-, and 45-day feeding periods on the development of oxidative stress, antioxidant defenses, and lysosomal functioning in gills and hepatic tissue of rainbow trout, *Oncorhynchus mykiss* (Walbaum). In this study, we attempted (i) to determine changes in levels of oxidative stress biomarkers, i.e., 2-thiobarbituric-acid-reacting substances (TBARS), aldehydic and ketonic derivatives of oxidatively modified proteins, the activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx), and the total antioxidant capacity (TAC) in gills and hepatic tissue of juvenile rainbow trout following β-glucan treatment; (ii) to evaluate the relationship between lysosomal activity and oxidative stress biomarkers after the three feeding periods; (iii) to characterize trends of the main effects (i.e., feeding periods, type of tissues, and impact of β-glucans) on levels of oxidative stress biomarkers and lysosomal functioning in gills and hepatic tissue; (iv) to evaluate important determinants in oxidative stress, antioxidant defenses, and lysosomal functioning in gills and hepatic tissue of rainbow trout following β-glucan treatment.

Materials and methods

Fish and experimental design

Ninety healthy *O. mykiss* weighing 54.9 ± 2.2 g were used in the current study. The fish were maintained in an indoor system supplied with freshwater with adequate aeration and an internal power filter. Water quality was maintained at a temperature of 16 ± 2°C, dissolved oxygen 12 ± 0.5 ppm, and pH 7.4–7.6. The fish were fed at 1.5% body weight (BW) four times daily with a commercial basal diet during the acclimation period (14 days). After two weeks, the fish were divided randomly into six groups and stocked into aerated 250-L square tanks supplied with dechlorinated tap water (70 fish per tank). One tank was assigned per group. The fish were kept in natural photoperiod conditions throughout the feeding trial. The experimental part of the dietary study was carried out in the Department of Salmonid Research, National Inland Fisheries Research Institute (Rutki, Poland).

The groups were fed for 15, 30, and 45 days as follows: the control groups of rainbow trout (n = 15) received a control basal diet; the β-glucan groups were fed with the supplement Yestimun® at a dose of 1% in the basal feed (with 85% content of β-1,3/1,6-glucans, Leiber GmbH, Bramsche, Germany). Yestimun® powder (at a 1% dose of 1 kg per 99 kg, wt/wt) was added to the basal feed. Yestimun® is an insoluble and highly purified preparation containing natural polysaccharides, including β-1,3/1,6-D-glucans derived from spent brewers' yeast (*Saccharomyces cerevisiae*). Yeast cell walls typically contain about 30% β-glucan by dry weight (Stier et al. 2014).

At the end of the 15-, 30-, and 45-day feeding periods, the fish were sacrificed by decapitation, and the liver and gills were dissected. Blood samples were taken from the caudal vein with plastic syringes. A part of the blood samples was transferred into tubes containing K_3-EDTA for the phagocytic activity (PA) and phagocytic index (PI) assays. The fish were not anesthetized before tissue sampling. The experiments were performed in duplicate. This study was conducted in the National Inland Fisheries Research Institute (Olsztyn, Poland). The research was in compliance with Polish animal welfare regulations and approved by the Local Ethics Committee for Animal Experimentation of the National Inland Fisheries Research Institute in Olsztyn, Poland.

Reagents and solutions

Tris, EDTA, HEPES, KCl, K_2CO_3, KH_2PO_4, GSH, GSSG, $NADPH_2$, and 2-thiobarbituric acid reagents were purchased from Sigma-Aldrich (Sigma-Aldrich Sp. z.o.o, Poznan, Poland). The reagents were prepared before use. All other reagents used were of analytical reagent grade.

Phagocytic activity (PA) and phagocytic index (PI)

PA and PI were measured using the microscopic counting method in Siwicki and Anderson (1993). Briefly, 100 μL of a blood sample was added to 100 μL of formalin-killed *Yersinia ruckeri* (1×10^7 cells). The suspension was thoroughly mixed and left to incubate for 30 min in a well. After incubation, the plate was mixed gently, and 0.05 mL of mixed suspension was transferred onto a glass slide and air-dried. The slides were fixed with ethanol (96%) for 5 min and stained with Giemsa solution for 10 min. Phagocytic cells and phagocytosed bacteria were counted. The slides were observed under a light microscope to count 100 cells per slide. The phagocytic activity and phagocytic index were calculated as follows:

Phagocytic activity (%) = (Number of phagocytic cells with engulfed bacteria/number of phagocytes) × 100; Phagocytic index = Number of engulfed bacteria/phagocytic cells.

Preparation of tissue homogenates

The gills and hepatic tissue samples were homogenized in an ice-cold buffer (100 mM Tris-HCl, pH 7.2). Blood was removed from the minced tissue with cold isolation buffer and the tissue was homogenized on ice in an H500 homogenizer with a motor-driven pestle. The homogenates were centrifuged at 3,000 g for 15 min at 4°C. After centrifugation, the supernatant was collected and frozen at -22°C for further analysis. Protein contents were determined with the Bradford method (1976) with bovine serum albumin as the standard. Absorbance was recorded at 595 nm. All enzymatic assays were carried out at 22 ± 0.5°C using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany) in duplicate. The enzymatic reactions were started by adding the tissue supernatant.

Biochemical assays

2-Thiobarbituric acid reactive substance (TBARS) assay

Lipid peroxidation was determined in aliquots of 10% homogenate of hepatic and gill tissues from the treated and control fish using the procedure proposed by Kamyshnikov (2004). The lipid peroxidation level was expressed as nanomoles of 2-thiobarbituric acid reactive substances generated per milligram of protein (nmol MDA·mg^{-1} protein) using a molar extinction coefficient of $1.56 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$.

Content of carbonyl derivatives of oxidatively modified proteins (OMP)

The estimation of the rate of protein oxidative destruction was based on the reaction of the resultant carbonyl derivatives of amino acid reaction with 2,4-Dinitrophenylhydrazine (DNPH). The assay is described in Levine et al. (1990) and modified by Dubinina et al. (1995). DNPH was used for determinations of the carbonyl content in soluble and insoluble proteins. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehydic derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}). The results were expressed in nmol per mg of tissue protein.

Superoxide dismutase activity assay

The activity of superoxide dismutase (SOD, E.C. 1.15.1.1) was assessed by its ability to dismutate superoxide generated in the process of quercetin auto-oxidation in an alkaline medium (pH 10.0) as proposed by Kostiuk et al. (1990). The activity was expressed in units of SOD per mg of tissue protein.

Catalase activity assay

The activity of catalase (CAT, E.C. 1.11.1.6) was determined by measuring the decrease in H_2O_2 in the reaction mixture using a spectrophotometer at a wavelength

of 410 nm and the method described by Koroliuk et al. (1988). One unit of catalase activity was defined as the amount of enzyme necessary to decompose 1 μmol H_2O_2 per min per mg of tissue protein.

Glutathione reductase activity assay

The activity of glutathione reductase (GR, EC 1.6.4.2) in the tissue was measured using the method described by Glatzle et al. (1974). GR activity was expressed as nmol NADPH per min per mg of tissue protein.

Glutathione peroxidase activity assay

The activity of glutathione peroxidase (GPx, EC 1.11.1.9) was determined by detecting the nonenzymatic utilization of GSH (reacting substrate) at an absorbance of 412 nm after incubation with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) as proposed by Moin (1986). GPx activity was expressed as μmol GSH per min per mg of tissue protein.

Total antioxidant capacity (TAC) assay

The TAC level in the sample was estimated by measuring the TBARS level following Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm by Galaktionova et al. (1998). The sample inhibited the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. The absorbance of the blank was defined as 100%. The level of TAC in the sample (%) was calculated based on the absorbance of the blank.

Tissue isolation for lysosomal enzyme assays

Tissue samples were excised, weighed, washed in ice-cold buffer, and minced. The minced tissues were rinsed with cold isolation buffer 0.15 M KCl to remove blood and homogenized on ice in an H500 homogenizer with a motor-driven pestle. The isolation buffer consisted of 0.25 M sucrose and 2 mM EDTA; the pH was adjusted to 7.0 with KOH. The homogenates (20%, w/v) were prepared for differential centrifugation according to the method described by DeMartino and Goldberg (1978). After centrifugation, the supernatant fractions were saved and used after resuspension in 50 mM acetic acid/sodium acetate buffer, pH 5.0. These isolated fractions were homogenized and subjected to two freeze-thaw cycles.

Lysosomal enzyme assays

The method developed by McDonald and Barrett (1986) using Fast Blue BB salt (4-benzoyloamino-2,5-diethoxybenzene-diazonium chloride) derivatives was applied for the activities of lysosomal enzymes, i.e., alanyl aminopeptidase (AAP, EC 3.4.11.2) and leucyl aminopeptidase (LAP, EC 3.4.11.1). L-alanyl-2-naphtylamine in 0.1M PBS buffer, pH 7.0, was used as a substrate for determinations of alanyl aminopeptidase activity. L-leucyl-2-naphtylamine in 0.1M PBS, pH 7.0, was used as the substrate for leucyl aminopeptidase activity. The activities of these lysosomal enzymes were determined spectrophotometrically at 540 nm. The reaction was initiated using 50 μL of the sample and 500 μL of substrate incubation media with DMF, pH 6.0 (Serva, Germany), in 60-min incubation at 37°C; next, 500 μL of stop buffer consisting of Fast Blue BB salt dissolved in 2% Tween 20 (Sigma, USA) were added.

The method proposed by Barrett and Heath (1977) was used for determinations of the activities of other lysosomal enzymes, i.e., acid phosphatase (AcP, EC 3.1.3.2) and β-N-acetylglucosaminidase (NAG, EC 3.2.1.30). The activities of these enzymes were determined spectrophotometrically as 4-nitrophenyl derivatives at 420 nm and expressed in nmol per h per mg of protein.

Statistical analysis

The results are expressed as mean ± S.D. The basic statistics were calculated using STATISTICA 13.3 (TIBCO Inc., USA). The data were tested for homogeneity of variance using Levene's test of the equality of error variances. Normality was checked with the Kolmogorov-Smirnov test. Significant differences between the means were measured using a multiple

range test at min. $P < 0.05$. Data that did not have normal distribution were log-transformed. The correlation of parametric values was based on the analysis of Pearson's regressions using the multiple regression module. The correlation and regression analysis comprised the correlation coefficient (r), regression equation, and the significance of these dependencies (P). The arithmetic means of the concentrations of substrates and the activities of enzymes in hepatic and gill tissues were estimated using MANOVA.

The coefficients of multiple correlation analysis (R) and the coefficient of determination (R^2) with its corrected form reduced by random errors (R^2 adjusted) were used in the data analysis for the description of the full model. We used the SS test to describe the share of all analyzed biomarkers of oxidative stress and biochemical parameters for the assessment of the antioxidant barrier with the F test of significance (Zar 1999).

The use of multivariate significance tests of the main effects (type of stages, type of tissue, experiment conditions, and combined effects) permitted determining statistically significant relationships for all three values. To combine the impact of three factors (tissues, three experimental stages, and type of experiment) in the model approach, we adopted a three-way classification model to analyze the value of the other parameters: the dependent variable, the mean, the main effect of the tissue factor, the main effect of the stage factor, the main effect of the experiment type factor, the effect of the interaction of the tissue type and stage factors, the effect of the interaction of the stage and experiment factor, the effect of the interaction of the tissue type and experiment factor, and random experimental error.

Results

Immune-related variables

Relative to the value obtained in the unhandled control groups, phagocytic activity (Fig. 1A) was 27.3%, 37.1%, and 64.3% higher ($P < 0.05$) in the rainbow trout fed the diet supplemented with β-glucans on days 15, 30, and 45, respectively. The phagocytic index (Fig. 1B) was higher in the β-glucan treatment group by 84.1% on day 15 ($P < 0.05$), 92% on day 30 ($P < 0.001$), and by 129.5% on day 45 ($P < 0.05$), in comparison to the unhandled control groups.

Pro/antioxidant balance

In the present study, the most widely used and accepted markers were employed to demonstrate the existence of oxidative stress in tissues (TBARS as a marker of lipid peroxidation, aldehydic and ketonic derivatives of OMP, antioxidant defenses, and total antioxidant capacity). Our results demonstrated that

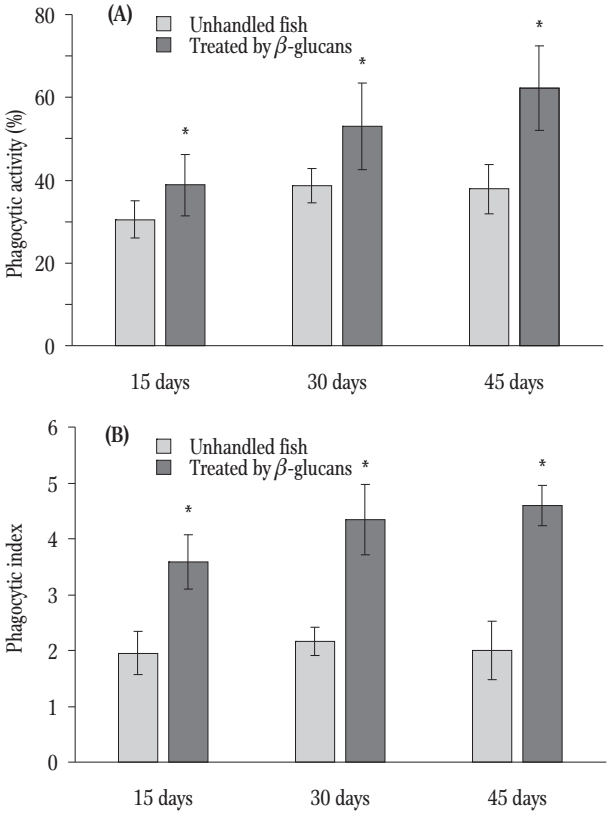

Table 1. Activities of superoxide dismutase (SOD, $U \cdot mg^{-1}$ protein), catalase (CAT, $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein), glutathione reductase (GR, $nmol \cdot min^{-1} \cdot mg^{-1}$ protein), and glutathione peroxidase (GPx, $nmol \cdot min^{-1} \cdot mg^{-1}$ protein) in gills and hepatic tissue of rainbow trout fed the diet supplemented with yeast β-glucans. Results are expressed as mean ± S.D. Differences between the control and experimental groups were analyzed with three-way ANOVA and Bonferroni's post-hoc test. Differences were considered significant at $P < 0.05$.

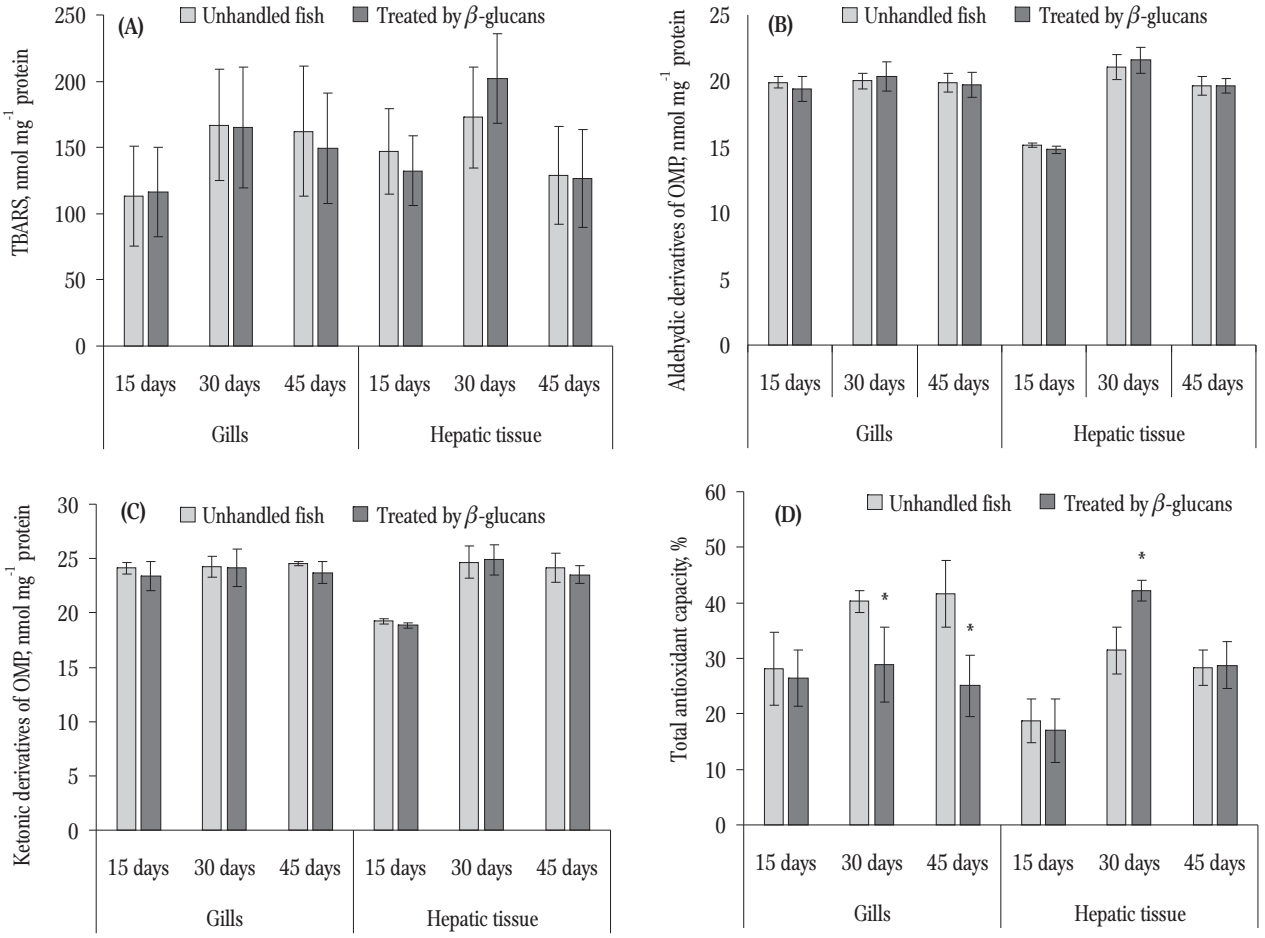

Figure 2. Levels of TBARS (A, nmol·mg^{-1} protein), aldehydic derivatives (AD) (B, nmol·mg^{-1} protein), and ketonic derivatives (KD) of oxidatively modified proteins (OMP) (C, nmol·mg^{-1} protein), and total antioxidant capacity (D, TAC, %) in gills and hepatic tissue of rainbow trout fed the diet supplemented with yeast β-glucans. Results are expressed as mean ± S.D. Differences between the groups were analyzed with three-way ANOVA and Bonferroni's post-hock test. Differences were considered significant at $P < 0.05$. Statistically significant differences between the groups of unhandled controls and the β-glucan-supplemented group are indicated. (* – $P < 0.05$).

the dietary β-glucan treatment did not alter rainbow trout gills or hepatic tissue. The statistical analysis based on parameter F showed significant statistical differences in the study groups depending on the tissue specificity (gills, liver), the three successive periods regarding the temporal effects of β-glucan treatment, and the differences between fish groups (unhandled control and β-glucan-treatment group). The final products of lipid peroxidation TBARS were used as biomarkers of these changes. Data obtained in this analysis are presented in Fig. 2.

The statistical analysis of the periods and type of study showed significant differences in the TBARS levels in gills ($F_{5.43} = 2.65$, $P = 0.036$) and hepatic tissue ($F_{5.43} = 5.12$, $P < 0.001$). Similar data were obtained in the case of aldehydic derivatives of OMP ($F_{5,43} = 178.33$, $p < 0.001$) and ketonic derivatives of OMP ($F_{5.43} = 68.42$, $P < 0.001$) in the hepatic tissue. The following statistical dependencies were noted for the gills: SOD ($F_{5.43} = 6.98$, $P < 0.001$), CAT ($F_{5.43} = 45.61$, $P < 0.001$), GR ($F_{5.43} = 24.47$, $P < 0.001$), and GPx ($F_{5.43} = 38.71$, $P < 0.001$). For the hepatic tissue, they were: SOD ($F_{5.43} = 63.17$, $P < 0.001$), CAT ($F_{5.43} = 50.69$, $p = 0.000$), GR ($F_{5.43} = 20.37$, $P < 0.001$), and GPx ($F_{5.43} = 51.90$, $P < 0.001$). Significant differences in the TAC levels were observed both in gills ($F_{5.43} = 10.89$, $P < 0.001$) and hepatic tissue ($F_{5.43} = 42.01$, $P < 0.001$).

Significant differences in the levels of lipid peroxidation and protein modifications were observed for the study periods and fish tissue specificity. However, we did not observe significant statistical differences in the relations between the unhandled controls and the β-glucan treatment group. There was a significant decrease in the TAC level in the gills of trout treated with β-glucans in the second and third periods of the current study. In contrast, an increased value of TAC level in the hepatic tissue in the second period of our study was noted. The dietary β-glucan treatment caused multidirectional changes in the activity of antioxidative enzymes, depending on the study periods and tissue specificity (gills, liver). The immunocompetence processes of the fish resulted in different tendencies of the activity of antioxidative enzymes. These changes had clear tissue specificity in comparison to the unhandled controls. For example, in the 15-day feeding period, the active formation of immunological status in the fish resulted in a statistically significant decrease in the activity of CAT, GR, and GPx in the gills, whereas an increase in the CAT and GPx activity was noted in the hepatic tissue (Table 1).

Lysosomal enzymes

Biological processes are largely determined not only by the composition and physiological status of membranes that might be modified by free radical action but also by membrane-bound, free (solubilized) and

Table 1

Activities of superoxide dismutase (SOD, U·mg^{-1} protein), catalase (CAT, μmol·min^{-1}·mg^{-1} protein), glutathione reductase (GR, nmol·min^{-1}·mg^{-1} protein), and glutathione peroxidase (GPx, nmol·min^{-1}·mg^{-1} protein) in gills and hepatic tissue of rainbow trout fed the diet supplemented with yeast β-glucans

	Days		
Parameters/Stages	15	30	45
Gills			
SOD, U·mg^{-1} protein			
Unhandled controls	541.31 ± 105.31	369.80 ± 69.70	441.17 ± 126.34
β-glucans	762.07 ± 164.55	343.63 ± 91.37	420.91 ± 164.15
CAT, μmol·min^{-1}·mg^{-1} protein			
Unhandled controls	7.90 ± 2.43	4.26 ± 2.11	2.52 ± 0.96
β-glucans	0.85 ± 0.31**	17.47 ± 5.37*	0.92 ± 0.13**
GR, nmol·min^{-1}·mg^{-1} protein			
Unhandled controls	14.18 ± 3.45	12.08 ± 3.36	7.82 ± 1.89
β-glucans	5.24 ± 1.72**	29.05 ± 7.18**	8.07 ± 2.19
GPx, nmol·min^{-1}·mg^{-1} protein			
Unhandled controls	419.03 ± 155.20	506.29 ± 105.56	215.02 ± 74.49
β-glucans	148.45 ± 52.81**	767.64 ± 168.49*	95.08 ± 18.28**
Hepatic tissue			
SOD, U·mg^{-1} protein			
Unhandled controls	309.16 ± 68.99	154.88 ± 46.53	571.04 ± 22.63
β-glucans	247.83 ± 33.93	271.32 ± 87.50*	558.48 ± 32.55
CAT, μmol·min^{-1}·mg^{-1} protein			
Unhandled controls	22.82 ± 5.03	12.75 ± 2.83	13.94 ± 1.81
β-glucans	48.08 ± 8.01**	12.70 ± 2.59	18.61 ± 2.18*
GR, nmol·min^{-1}·mg^{-1} protein			
Unhandled controls	52.31 ± 11.70	25.45 ± 3.49	7.85 ± 1.12
β-glucans	27.20 ± 7.10**	14.04 ± 3.50**	13.20 ± 2.50**
GPx, nmol·min^{-1}·mg^{-1} protein			
Unhandled controls	179.66 ± 60.53	158.65 ± 31.28	214.39 ± 53.07
β-glucans	738.44 ± 189.14**	152.96 ± 51.89	193.69 ± 45.92

Results are expressed as mean ± S.D. Differences between the groups were analyzed with three-way ANOVA and Bonferroni's post-hock test. Differences were considered significant at $P < 0.05$. Statistically significant differences between the groups of unhandled controls and the β-glucan-supplemented group are indicated. Other significant differences are presented in the text.
* – $P < 0.05$; ** – $P < 0.01$.

Table 2
Activities of alanyl aminopeptidase (AAP, nmol·h^{-1}·mg^{-1} protein), leucyl aminopeptidase (LAP, nmol·h^{-1}·mg^{-1} protein), acid phosphatase (AcP, nmol·h^{-1}·mg^{-1} protein), and β-N-acetylglucosaminidase (NAG, nmol·h^{-1}·mg^{-1} protein) in gills and hepatic tissue of rainbow trout fed the diet supplemented with yeast β-glucans

	Days		
Parameters/Stages	15	30 days	45 days
Gills			
AAP, nmol·h^{-1}·mg^{-1} protein			
Unhandled controls	121.31 ± 23.31	152.14 ± 44.25	136.11 ± 36.47
β-glucans	178.07 ± 33.12	185.14 ± 22.18	164.85 ± 52.11
LAP, nmol·h^{-1}·mg^{-1} protein			
Unhandled controls	76.22 ± 14.22	111.24 ± 42.16	105.84 ± 16.84
β-glucans	121.49 ± 9.55**	159.47 ± 33.04	198.35 ± 34.08**
AcP, nmol·h^{-1}·mg^{-1} protein			
Unhandled controls	114.17 ± 23.45	129.08 ± 27.36	89.82 ± 11.29
β-glucans	185.24 ± 30.72*	185.27 ± 33.18*	80.07 ± 23.19
NAG, nmol·h^{-1}·mg^{-1} protein			
Unhandled controls	119.03 ± 25.20	106.29 ± 35.36	115.02 ± 24.49
β-glucans	78.45 ± 12.81*	97.64 ± 38.49	125.08 ± 16.28
Hepatic tissue			
AAP, nmol·h^{-1}·mg^{-1} protein			
Unhandled controls	321.11 ± 48.09	271.32 ± 87.50	371.54 ± 72.63
β-glucans	207.03 ± 55.93	454.38 ± 56.54	538.41 ± 52.05
LAP, nmol·h^{-1}·mg^{-1} protein			
Unhandled controls	178.13 ± 47.03	257.74 ± 36.08	168.27 ± 52.81
β-glucans	205.47 ± 13.01*	97.48 ± 22.59**	309.45 ± 42.18**
AcP, nmol·h^{-1}·mg^{-1} protein			
Unhandled controls	152.41 ± 19.70	189.45 ± 37.49	117.85 ± 19.12
β-glucans	270.20 ± 47.10**	214.04 ± 33.50	132.20 ± 29.50
NAG, nmol·h^{-1}·mg^{-1} protein			
Unhandled controls	158.63 ± 20.13	122.15 ± 31.48	114.29 ± 13.07
β-glucans	238.44 ± 49.14**	172.66 ± 31.49	135.69 ± 15.92

Results are expressed as mean ± S.D. Differences between the groups were analyzed with three-way ANOVA and Bonferroni's post-hock test. Differences were considered significant at $P < 0.05$. Statistically significant differences between the groups of unhandled controls and the β-glucan-supplemented group are indicated. Other significant differences are presented in the text. * – $P < 0.05$; ** – $P < 0.01$.

lysosomal enzymes. This study showed the role of lysosomal structures in both the internal injury system and in the maintenance of the stability of cell membranes and intracellular structures. Thus, we assessed these processes in gills and hepatic tissue of trout fed the diet supplemented with β-glucans. The influence of various factors resulted in lysosomal activities or an increase in the lysosomal enzyme content in the cytoplasm (in particular, because of progressive acidosis, which increased the yield of lysosomal enzymes and the subsequent activation thereof). Our results showed that the activities of aminopeptidase (AAP) ($F_{5.43} = 42.01$, $P < 0.001$), leucyl aminopeptidase (LAP) ($F_{5.43} = 16.09$, $P < 0.001$), acid phosphatase (AcP) ($F_{5.43} = 21.09$, $p = 0.000$), and β-N-acetylglucosaminidase (NAG) ($F_{5.43} = 18.86$, $p = 0.000$) changed statistically significantly in the gills of rainbow trout treated with β-glucans in the three periods studied. Differences in lysosomal enzyme activity in the hepatic tissue were also noted in the activities of AAP ($F_{5.43} = 12.45$, $P < 0.001$), LAP ($F_{5.43} = 12.45$, $P < 0.001$), AcP ($F_{5.43} = 63.45$, $P < 0.001$), and NAG ($F_{5.43} = 52.45$, $P < 0.001$). It is noteworthy that increased activity of lysosomal enzymes was observed in gills and hepatic tissue of rainbow trout fed the diet supplemented with β-glucans (Table 2).

The MANOVA test revealed statistically significant relationships in the analysis of the main factors.

Table 3
Multivariate significance tests and effective hypothesis decomposition for two types of tissue (gills, liver), three periods studied (15, 30, and 45 days) of β-glucan treatment, and effects on oxidative stress biomarkers and activities of lysosomal enzymes

Main Effects	Test value	F	P
Tissues	0.220	34.486	0.000
Studied periods	0.021	56.447	0.000
Experimental groups	0.490	10.130	0.000
Tissue and Studied periods	0.035	42.218	0.000
Tissues and Experimental groups	0.417	13.596	0.000
Studied periods and Experimental groups	0.345	6.840	0.000
Tissues, Studied periods and Experimental groups	0.180	13.178	0.000

Table 4
SS test of oxidative stress parameters for a full model data and SS for residues for two types of tissue (gills, liver), three periods studied (15, 30, and 45 days) of β-glucan treatment, and effects on oxidative stress biomarkers and activities of lysosomal enzymes

Parameters	Multiple R	Multiple R^2	Multiple adjusted R^2	F	P
TBARS	0.559	0.313	0.224	3.514	0.000
AD OMP	0.952	0.907	0.895	75.01	0.000
KD OMP	0.905	0.819	0.796	34.95	0.000
TAC	0.843	0.712	0.674	19.06	0.000
SOD	0.811	0.659	0.615	14.95	0.000
CAT	0.943	0.889	0.875	62.07	0.000
GR	0.870	0.758	0.727	24.19	0.000
GPx	0.918	0.843	0.823	41.56	0.000
AAP	0.404	0.163	0.079	1.93	0.043
LAP	0.892	0.795	0.775	38.55	0.000
AcP	0.942	0.888	0.877	78.79	0.000
NAG	0.917	0.839	0.823	51.80	0.000

The data are presented in Table 3. Multivariate significance tests and effective hypothesis decomposition for the two types of tissue (gills, liver) of rainbow trout fed the diet supplemented with β-glucans, three experimental periods for oxidative stress biomarkers, and lysosomal enzymes showed the significance of all the effects investigated and their interactions. These interactions permit concluding that the periods studied, type of tissue, and their interactions revealed the most important relationships.

The coefficient of multiple correlation analysis, the coefficient of determination, and its corrected form reduced by random errors for the data analysis are presented in Table 4. The SS test used to describe all data in the analyzed profiles of the oxidative stress biomarkers and lysosomal enzymes in gills and hepatic tissue with the significance F test allowed us to draw conclusions on the separate and combined effects of these factors. In the case of the oxidative stress biomarkers, these dependencies are as follows: aldehydic derivatives of OMP > CAT > GPx > ketonic derivatives of OMP > GR > TAC > SOD > TBARS. It is known that lysosomal enzymes play an important role in initiating redox metabolism. Thus, the statistical analysis revealed their role together with oxygen-dependent factors in cellular processes. The following dependencies were determined for metabolic enzymes and parameters: AcP > NAG > LAP > AAP.

Table 5
Correlations analysis of oxidative stress biomarkers and activities of lysosomal enzymes in gills and hepatic tissue of rainbow trout fed the diet supplemented with yeast β-glucans

	15 days			30 days			45 days		
Groups	relationships	r	p	relationships	r	p	relationships	r	p
Gills									
Unhandled controls				TBARS – AD OMP	0.712	0.047	KD OMP – CAT	-0.919	0.001
							KD OMP – TAC	0.815	0.048
β-glucans	TBARS – AD OMP	-0.757	0.001	AD OMP – CAT	0.722	0.043	TBARS – GPx	0.941	0.005
	TBARS – KD OMP	-0.764	0.001	AD OMP – TAC	-0.791	0.020	TBARS – LAP	-0.820	0.019
	KD OMP – LAP	-0.890	0.017	KD OMP – TAC	-0.796	0.018			
	TBARS – AcP	-0.786	0.018	TBARS – AcP	-0.776	0.015			
	TAC – AcP	-0.826	0.012						
Hepatic tissue									
Unhandled controls	AD OMP – KD OMP	0.754	0.012	AD OMP – KD OMP	0.979	0.000	CAT – GR	0.935	0.006
β-glucans	AD OMP – KD OMP	0.695	0.026	TBARS – AD OMP	0.720	0.029	TBARS – TAC	-0.890	0.017
	TBARS – AcP	-0.886	0.010	TBARS – KD OMP	0.695	0.038	KD OMP – CAT	0.813	0.049
	AD OMP – NAG	0.815	0.048	TAC – SOD	-0.768	0.016	TBARS – TAC	-0.890	0.017
				TAC – AcP	-0.826	0.012	TAC – LAP	-0.820	0.005

In the current study, we observed statistically significant dependencies between the biomarkers of lipid peroxidation, oxidatively modified proteins, activities of antioxidant enzymes, and lysosome-induced processes associated with β-glucan supplementation of the rainbow trout diet. Correlation and regression analyses are presented for two types of tissue and three periods of the study (Table 5).

Discussion

The current study aimed to perform time-dependent analysis of data indicating the relationship of immunomodulation effects induced by a diet supplemented with β-glucans and biomarkers of oxidative stress in gills and hepatic tissues of rainbow trout. These tissues differ in the intensity of metabolic functionality and immunological and oxidative stress parameters. We also selected different time points (15, 30, and 45 days) to assess alterations in the biomarkers of oxidative stress and the functioning of lysosomal enzymes in gills and hepatic tissues of rainbow trout fed β-glucans. These methodological approaches enabled us to evaluate both immediate (short-term) and long-term effects of the impact of β-glucans on the functioning of intracellular toxicity biomarkers, such as biomarkers of oxidative stress and lysosomal destruction. The activation of lysosomes destroys the cell membrane and intracellular structures, initiating processes of uncontrolled damage in pathological processes.

Although the effects of β-glucans on fishes have been investigated in previous studies by other researchers (Skov et al. 2012, Vetvicka et al. 2013, Stier et al. 2014, Song et al. 2020), our study contributes to existing literature by providing a comprehen-

sive analysis of specific biochemical parameters that have been studied less frequently in relation to β-glucan supplementation. Our study looks not only at well-established markers of oxidative stress and antioxidant defenses, but also at lysosomal function, which has received less attention in similar studies. By extending the scope of the analysis to include these parameters, we aimed to provide a more detailed understanding of the potential effects of β-glucans on fish health. In addition, our study included different feeding periods to assess both short- and long-term responses, further enhancing the scientific value of our results for aquaculture practice.

Our results revealed that the β-glucans enhanced macrophage function evaluated by the phagocytic index and phagocytic activity in the three periods studied compared with the unhandled controls (Fig. 1). Recent studies have shown that β-glucans are components of pathogen-associated molecular patterns (PAMPs), as they are found on the surface of some pathogenic (micro)organisms, in particular fungi (Dalmo and Břgwald 2008). Higher organisms have developed a mechanism for recognizing these types of structures to detect and kill pathogens belonging to this class of organisms. Recognition of PAMPs by pathogen recognition receptors rapidly triggers an array of anti-microbial immune responses through the induction of various inflammatory cytokines, chemokines, and type I interferons (Kumar et al. 2011). In mammals, the cells of so-called innate immunity express specific receptors that recognize β-glucans, and Dectin-1 is one of the best-known receptors. Other receptors also participate in the recognition or signal cascade induced by β-glucans, including CD11b/CD18 (CR3), and Toll-like receptors 2 and 4 (TLR2 and TLR4) (Akramiene et al. 2007). This prototypical C-type lectin receptor (CLR) for β-glucan, Dectin-1, is limited to mammalian genomes (Sattler et al. 2012). Cells involved in the recognition of β-glucans are the typical phagocytes of innate immunity, i.e., monocytes, macrophages, dendritic cells, granulocytes, and natural killer cells (Goodridge et al. 2009). Several endothelial cells and cells specific to other tissues are capable of expressing β-glucan receptors (Vetvicka et al. 2013). Interestingly, β-glucans activate both local immunity protecting the organism against antigens and systemic immunity destroying the foreign genetic material that has already entered the body. They are also involved in the recovery of immune homeostasis. At the same time, it is necessary to emphasize the distinctive feature of the immunomodulatory component of the immune system. The enhanced immunomodulatory effect of β-glucans does not induce excessive stimulation leading to autoimmune diseases (Goodridge et al. 2009, Vetvicka et al. 2013).

An important step in the stimulation of biological responses in target cells is the initial binding to the receptor and the ability of β-glucans to mediate the cross-binding of sufficient receptors to trigger appropriate signaling pathways in cells. Soluble β-glucan products can induce a mass response by cross-binding to a large number of receptors and then phagocytizing them. Given the insoluble (or "crystal-like") structure of glucan, this can lead to lysosomal breaks within the cell that activate the NLRP inflammasome. Soluble β-glucans can also induce the formation of active oxygen forms, which also activates NLRP, leading to adverse inflammatory reactions (Kankkunen et al. 2010). Therefore, B-glucan products are being sought that can cause an inflammatory response activating some immune mechanisms without activating the inflammasome characteristics of some (aggregated and insoluble) β-glucan products.

The effects of β-glucans on the immune system of teleosts consists of inducing local intestinal inflammation resulting in subsequently increased resistance against pathogens (Dalmo and Břgwald 2008). Moreover, the oral administration of β-glucans might alter the composition of gut microbiota (Kühlwein et al. 2013). β-Glucans are demonstrated to be assimilated via phagocytosis. The opsonizing Fc-γ receptor (FcγR) and complement receptor 3 (CR3) are the best-characterized phagocytic receptors (Petit and Wiegertjes 2016). Toll-like receptors such as TLR2 can sense β-glucans and are expressed in several fish species (Pietretti and Wiegertjes 2014). β-Glucans can also induce and increase the robustness of

neutrophil extracellular traps (NETs) (Brogden et al. 2012, 2014).

Secondly, our study demonstrated the short-term and long-term effects of a diet supplemented with β-glucans on gills and hepatic tissues in rainbow trout. We tested the hypothesis of the positive, non-toxic effects of β-glucan treatment on biomarkers in two different tissues. Our results showed that the level of lipid peroxidation and aldehydic and ketonic derivatives of oxidatively modified proteins were not markedly influenced by the diet supplemented with β-glucans (Figs 2A, 2B and 2C). However, the unchanged level of lipid peroxidation and protein damage resulted in 28.4% and 39.9% ($P < 0.05$) decreases in the total antioxidant capacity of the gills, compared to the unhandled controls, on days 30 and 45, respectively (Fig. 2D). Moreover, the decreased TAC level might have caused increased aldehydic ($r = -0.791$, $P = 0.020$) and ketonic derivatives of oxidatively modified proteins ($r = -0.796$, $P = 0.018$) (Table 5).

The finding of the study is that β-glucans provide multilevel protection against oxidative stress and lysosomal destruction through several mechanisms, including unaltered lipid peroxidation estimated by TBARS levels and oxidatively modified proteins, coupled with improved activities of antioxidant enzymes and total antioxidant capacity in hepatic tissue. The results of the present investigation indicated that β-glucans significantly increased CAT and GPx activity (1.1- and 3.1-fold, respectively, $P < 0.05$) in the hepatic tissue on day 15, SOD activity (by 75.2%, $P < 0.05$) on day 30, and CAT and GR activity (by 33.5% and 68.2%, respectively, $p < 0.05$) on day 45 (Table 1). The alterations of the antioxidant activities might have been the result of the adaptation in compensatory mechanisms against oxidative stress. This was confirmed by our study that demonstrated the effects of the diet supplemented with β-glucans on gills and hepatic tissues, which were reflected by the low level of the biomarkers of lipid peroxidation and protein damage (Fig. 2). The CAT and GPx activities in the gills of trout treated with β-glucans were significantly decreased on day 15 (by 89.2% and 64.6%, respectively, $P < 0.05$) and on day 45 (by 63.5% and 44.2%, respectively, $P < 0.05$). The inhibitive response of the enzymes suggested the failure of the antioxidant system to maintain the antioxidant defense balance, which may have been related to the excessive ROS production after β-glucan exposure, resulting in the accumulation of oxidative substances in cells (Table 1). Correlation analysis revealed positive correlations between oxidative stress biomarkers, i.e., aldehydic derivatives of oxidatively modified proteins and CAT activity ($r = 0.7224$, $P = 0.043$), TBARS as a marker of lipid peroxidation, and GPx activity ($r = 0.941$, $P = 0.005$) in the gills of trout treated with β-glucans (Table 5).

Recent studies have shown that the intensity of synthesis and degradation of molecules, many metabolites, enzymes, and all biologically active substances are driven by homeostasis maintained by the regulatory systems of metabolic transformations that depends on oxygen consumption (Cheng and Ristow 2013). This evolutionary ability to control metabolic pathways consists of the efficient regulation of energy synthesis processes in the mitochondria and the economic utilization of energy. During these processes, "unwanted" free radicals appear in cells, at the same time activating antioxidant defense systems that counteract them (Naviaux 2014). When there is an imbalance between these events, oxidative stress appears, revealing how internal organism systems trigger their regulatory mechanisms. The antioxidant defense system contains various types of enzymes that are selectively represented in cells of various tissues, depending on their activity (Oyewole and Birch-Machin 2015).

Thirdly, the detailed comparative analysis demonstrated that the activation of some antioxidative and lysosome enzymes occurred in response to the β-glucan treatment. The β-glucan-induced immune responses resulted in the involvement of lysosome activation, which had clearly defined tissue specificity in our studies. For example, the activation of the lysosomal enzymes LAP and AcP in gill tissue was observed after the 15-day period of β-glucan supplementation (by 59.4% and 62.3%, respectively, $P < 0.05$) (Table 2). It should be noted that these changes occurred without the activation of oxidative stress biomarkers (Fig. 2). These data showed that no

destructive effect on the cell membranes was exerted by the activation of the CAT, GR, or GPx antioxidant enzymes (Table 1). Determinations of free and total activity of lysosomal enzymes and modifications of multiple molecular forms of some of them is recommended, as they are informative biomarkers in assessments of the physiological condition and stability of animal growth and diagnostics of toxicosis, parasitic, and other pathologies. They can also be used for ecological and biochemical monitoring of natural and other aquaculture environments (Moore 2008). This is also confirmed by our results of the correlation analysis showing the relationship between these processes both in the gills, i.e., ketonic derivatives of OMP and LAP activity ($r = -0.890$, $P = 0.017$), TBARS level and AcP activity ($r = -0.786$, $P = 0.018$), and TAC level and AcP activity ($r = -0.826$, $P = 0.012$), and in the hepatic tissue, i.e., TBARS level and AcP activity ($r = -0.886$, $P = 0.010$), ketonic derivatives of OMP, and NAG activity ($r = 0.815$, $P = 0.048$) after the 15-day period of β-glucan supplementation (Table 5).

Recent studies have shown that the evolution of lysosomes was associated with greater complexity of their structure and the emergence of a variety of their functions in multicellular organisms. Thus, the functions of the lysosomal apparatus have expanded. Alongside intracellular digestion (the main, and oldest, target of lysosomes), the role of these organelles in specialized physiological processes has increased (Moore 2008). This is confirmed by our results of the distribution of lysosomal enzyme activity in various fish tissues. The role of the acidic hydrolase complex not only in providing the necessary nutrients but also in protecting against foreign and pathogenic factors became apparent quite early (Moore et al. 2006a,b). It has been established that the highest activity of lysosomal enzymes in fish is characteristic of organs where the processes of hydrolytic cleavage, neutralization, and the removal of foreign and toxic substances from the body are active. Enzymatic heterogeneity has been detected in lysosomes from various organs (DeMartino and Goldberg 1978).

Fourthly, a detailed comparative analysis demonstrated that, in response to immune activation caused by β-glucans, some antioxidant and lysosomal enzymes in the tissues were highly specific to rainbow trout. The results of the comparative analysis of changes in gills and hepatic tissues in the 15-, 30-, and 45-day periods of β-glucan supplementation confirmed the hypothesis that the levels of lipid peroxidation and oxidatively modified proteins can be inhibited by the β-glucan treatment, although different changes were observed. The physiological changes initiated by the dietary β-glucan treatment were reflected by the elevation of antioxidant enzyme activity (CAT, GR, and GPx) after the 30-day feeding period in our study (Table 1).

In conclusion, the present study demonstrated that yeast β-1,3/1,6-D-glucans was a beneficial dietary supplement that improved the innate immune response and some biochemical parameters in rainbow trout tissues. The best results were obtained after the 30 day feeding period. While acknowledging the existing body of literature on the effects of β-glucans in fishes, particularly rainbow trout, we believe that our manuscript makes a significant contribution to the scientific discourse by building on previous research and addressing knowledge gaps. By synthesizing existing knowledge with our novel findings, our aim was to advance the field by providing new insights into the mechanisms underlying the beneficial effects of β-glucans on rainbow trout. In addition, our study design, which included different feeding periods, permitted making a nuanced assessment of both short-term and long-term responses, adding further depth to the scientific value of our research.

Conclusions

In conclusion, the results of this study demonstrated that dietary supplementation with yeast β-1,3/1,6-D-glucans administered to rainbow trout for 15, 30, and 45 days improved immune response and antioxidant capacity. The present findings revealed that (1) the dietary yeast β-glucans improved immunity by increasing the phagocytic activity and phagocytic index in the blood of the fish in the three

periods studied; (2) the dietary β-glucans did not exert cytotoxic effects on gills or hepatic tissue of the rainbow trout evaluated, which was reflected in the oxidative stress biomarkers (lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins) at the dose tested; (3) β-glucan supplementation resulted in a significant increase in the total antioxidant capacity in the hepatic tissue on day 30, while a decrease in the gill TAC was observed on days 30 and 45; (4) the β-glucan treatment resulted in increased antioxidant defense in the gills on day 30, while similar tendencies were observed in the hepatic tissue on days 15 and 45; (5) the formation of β-glucan-induced immune responses resulted in lysosome activation that had clearly defined tissue specificity, i.e., an increase in LAP and AcP activity in both gills and hepatic tissue; (6) multiple correlation analysis revealed the following dependencies of oxidative stress biomarkers: aldehydic derivatives of OMP > CAT > GPx > ketonic derivatives of OMP > GR > TAC > SOD > TBARS. In turn, these dependencies for metabolic enzymes and parameters were as follows: AcP > NAG > LAP > AAP. These results indicated that β-glucan is a beneficial dietary supplementation for rainbow trout in aquaculture. Further investigations on the use of different concentrations of β-glucans and evaluations of various biochemical parameters in different fish species are encouraged since fish responses seem to change at different doses and in different fish species.

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References

Akramiene, D., Kondrotas, A., Didziapetriene, J., Kevelaitis, E. (2007). Effects of beta-glucans on the immune system. Medicina (Kaunas), 43(8), 597-606.

Assefa, A., Abunna, F. (2018). Maintenance of fish health in aquaculture: review of epidemiological approaches for prevention and control of infectious disease of fish. Veterinary Medicine International, 2018, 5432497. doi: 10.1155/2018/5432497.

Barrett, A.J., Heath, M.F. (1977). Lysosomal enzymes. In: Lysosomes, a Laboratory Handbook (Ed.) J.T. Dingle, North Holland: Amsterdam: 19-146.

Biller, J.D., Takahashi, L.S. (2018). Oxidative stress and fish immune system: phagocytosis and leukocyte respiratory burst activity. Anais da Academia Brasileira de Cięncias, 90(4), 3403-3414.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248-254.

Brogden, G., Krimmling, T., Adamek, M., Naim, H.Y., Steinhagen, D., von Köckritz-Blickwede, M. (2014). The effect of β-glucan on formation and functionality of neutrophil extracellular traps in carp (*Cyprinus carpio* L.). Developmental & Comparative Immunology, 44(2), 280-285.

Brogden, G., von Köckritz-Blickwede, M., Adamek, M., Reuner, F., Jung-Schroers, V., Naim, H.Y., Steinhagen, D. (2012). β-Glucan protects neutrophil extracellular traps against degradation by *Aeromonas hydrophila* in carp (*Cyprinus carpio*). Fish and Shellfish Immunology, 33(4), 1060-1064.

Cheng, Z., Ristow, M. (2013). Mitochondria and metabolic homeostasis. Antioxidants & Redox Signaling, 19(3), 240-242.

Dalmo, R.A., Břgwald, J. (2008). Beta-glucans as conductors of immune symphonies. Fish and Shellfish Immunology, 25(4), 384-396.

DeMartino, G.N., Goldberg, A.L. (1978). Thyroid hormones control lysosomal enzyme activities in liver and skeletal muscle. Proceedings of the National Academy of Sciences of the United States of America, 75(3), 1369-1373.

Douxfils, J., Fierro-Castro, C., Mandiki, S.N., Emile, W., Tort, L., Kestemont, P. (2017). Dietary β-glucans differentially modulate immune and stress-related gene expression in lymphoid organs from healthy and *Aeromonas hydrophila*-infected rainbow trout (*Oncorhynchus mykiss*). Fish and Shellfish Immunology, 63, 285-296.

Dubinina, E.E., Burmistrov, S.O., Khodov, D.A., Porotov, I.G. (1995). Oxidative modification of human serum proteins. A method of determining it. Voprosy meditsinskoi khimii, 41(1), 24-26 (in Russian).

Felicioli, A., Forzan, M., Sagona, S., D'Agostino, P., Baido, D., Fronte, B., Mazzei, M. (2020). Effect of Oral Administration of 1,3-1,6 β-Glucans in DWV Naturally Infected Newly Emerged Bees (*Apis mellifera* L.). Veterinary Sciences, 7(2), 52. doi: 10.3390/vetsci7020052.

Galaktionova, L.P., Molchanov, A.V., El'chaninova, S.A., Varshavskii, Bia. (1998). Lipid peroxidation in patients with gastric and duodenal peptic ulcers. Klinicheskaia laboratornaia diagnostika, (6), 10-14 (in Russian).

Ganguly, S., Paul, I., Mukhopadhayay, S.K. (2010). Application and effectiveness of immunostimulants, probiotics, and prebiotics in aquaculture: a review. Israeli Journal of Aquaculture - Bamidgeh, 62, 130-138.

Gantner, B.N., Simmons, R.M., Canavera, S.J., Akira, S., Underhill, D.M. (2003). Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. Journal of Experimental Medicine, 197(9), 1107-1117.

Glatzle, D., Vuilleumier, J.P., Weber, F., Decker, K. (1974). Glutathione reductase test with whole blood, a convenient procedure for the assessment of the riboflavin status in humans. Experientia, 30(6), 665-667.

Goodridge, H.S., Wolf, A.J., Underhill, D.M. (2009). Beta-glucan recognition by the innate immune system. Immunological Reviews, 230(1), 38-50.

Halliwell, B., Gutteridge, J.M.C. (1989). Free Radicals in Biology and Medicine. (2nd edition), Clarendon Press, Oxford, UK.

Herre, J., Gordon, S., Brown, G.D. (2004). Dectin-1 and its role in the recognition of beta-glucans by macrophages. Molecular Immunology, 40(12), 869-876.

Kamyshnikov, V.S. (2004). Reference book on clinic and biochemical researches and laboratory diagnostics. MEDpress-inform, Moscow.

Kankkunen, P., Teirilä, L., Rintahaka, J., Alenius, H., Wolff, H., Matikainen, S. (2010). (1,3)-beta-glucans activate both dectin-1 and NLRP3 inflammasome in human macrophages. Journal of Immunology, 184(11), 6335-6342.

Koroliuk, M.A., Ivanova, L.I., Maiorova, I.G., Tokarev, V.E. (1988). A method of determining catalase activity. Laboratornoe Delo, (1), 16-19 (in Russian).

Kostiuk, V.A., Potapovich, A.I., Kovaleva, Zh.V. (1990). A simple and sensitive method of determination of superoxide dismutase activity based on the reaction of quercetin oxidation. Voprosy meditsinskoi khimii, 36(2), 88-91 (in Russian).

Kühlwein, H., Emery, M.J., Rawling, M.D., Harper, G.M., Merrifield, D.L., Davies, S.J. (2013). Effects of a dietary β-(1,3)(1,6)-D-glucan supplementation on intestinal microbial communities and intestinal ultrastructure of mirror carp (*Cyprinus carpio* L.). Journal of Applied Microbiology, 115(5), 1091-1106.

Kumar, H., Kawai, T., Akira, S. (2011). Pathogen recognition by the innate immune system. International Reviews of Immunology, 30(1), 16-34.

Kumar, M.S. (2014). Sustainable management of fisheries and aquaculture for food security and nutrition: policies requirements and actions. Agricultural Research, 3(2), 97-103. doi: 10.1007/s40003-014-0111-0.

Kurhaluk, N. (2019). Formation of an antioxidant profile in the sea trout (*Salmo trutta* m. *trutta* L.) from the Slupia River. Zoology (Jena), 133, 54-65.

Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S., Stadtman, E.R. (1990). Determination of carbonyl content in oxidatively modified proteins. Methods Enzymology, 186, 464-478.

Machuca, C., Méndez-Martínez, Y., Reyes-Becerril, M., Angulo, C. (2022). Yeast β-Glucans as Fish Immunomodulators: A Review. Animals, 12(16), 2154. doi: 10.3390/ani12162154.

Magnadóttir, B. (2006). Innate immunity of fish (overview). Fish and Shellfish Immunology, 20(2), 137-151.

Magnadóttir, B. (2010). Immunological control of fish diseases. Marine Biotechnology, 12(4), 361-379.

McDonald, J.K., Barrett, A.J. (1986). Exopeptidases. In: Mammalian Proteases: A glossary and Bibliography. Academic Press, London: 114-144.

Medina-Córdova, N., Reyes-Becerril, M., Ascencio, F., Castellanos, T., Campa-Córdova, A.I., Angulo, C. (2018). Immunostimulant effects and potential application of β-glucans derived from marine yeast *Debaryomyces hansenii* in goat peripheral blood leucocytes. International Journal of Biological mMcromolecules, 116, 599-606.

Meena, D.K., Das, P., Kumar, S., Mandal, S.C., Prusty, A.K., Singh, S.K., Akhtar, M.S., Behera, B.K., Kumar, K., Pal, A.K., Mukherjee, S.C. (2013). Beta-glucan: an ideal immunostimulant in aquaculture (a review). Fish Physiology and Biochemistry, 39(3), 431-457.

Mendel, J., Jánová, K., Palíková, M. (2018). Genetically influenced resistance to stress and disease in salmonids in

relation to present-day breeding practice – a short review. Acta Veterinaria Brno, 87, 35-45.

Misra, C.K., Das, B.K., Pradhan, J., Pattnaik, P., Sethi, S., Mukherjee, S.C. (2004). Changes in lysosomal enzyme activity and protection against Vibrio infection in *Macrobrachium rosenbergii* (De Man) post larvae after bath immunostimulation with beta-glucan. Fish and Shellfish Immunology, 17(4), 389-395.

Moin, V.M. (1986). A simple and specific method for determining glutathione peroxidase activity in erythrocytes. Laboratornoe Delo, (12), 724-727 (in Russian).

Moore, M.N. (2008). Autophagy as a second level protective process in conferring resistance to environmentally-induced oxidative stress. Autophagy, 4(2), 254-256.

Moore, M.N., Allen, J.I., McVeigh, A., Shaw, J. (2006a). Lysosomal and autophagic reactions as predictive indicators of environmental impact in aquatic animals. Autophagy, 2(3), 217-220. doi: 10.4161/auto.2663.

Moore, M.N., Allen, J.I., Somerfield, P.J. (2006b). Autophagy: role in surviving environmental stress. Marine Environmental Research, 62, Suppl., S420-425.

Naviaux, R.K. (2014). Metabolic features of the cell danger response. Mitochondrion, 16, 7-17. doi: 10.1016/j.mito.2013.08.006.

Ngugi, C.C., Oyoo-Okoth, E., Mugo-Bundi, J., Orina, P.S., Chemoiwa, E.J., Aloo, P.A. (2015). Effects of dietary administration of stinging nettle (*Urtica dioica*) on the growth performance, biochemical, hematological and immunological parameters in juvenile and adult Victoria Labeo (*Labeo victorianus*) challenged with *Aeromonas hydrophila*. Fish and Shellfish Immunology, 44(2), 533-541.

Nguyen, T.M., Mandiki, S.N.M., Tran, T.N.T., Larondelle, Y., Mellery, J., Mignolet, E., Cornet, V., Flamion, E., Kestemont, P. (2019). Growth performance and immune status in common carp *Cyprinus carpio* as affected by plant oil-based diets complemented with β-glucan. Fish and Shellfish Immunology, 92, 288-299.

Novak, M., Vetvicka, V. (2009). Glucans as biological response modifiers. Endocr. Metab. Immune Disord. Drug Targets, 9(1), 67-75.

Oyewole, A.O., Birch-Machin, M.A. (2015). Mitochondria-targeted antioxidants. FASEB Journal, 29(12), 4766-4771.

Petit, J., Embregts, C.W.E., Forlenza, M., Wiegertjes, G.F. (2019). Evidence of Trained Immunity in a Fish: Conserved Features in Carp Macrophages. Journal of Immunology, 203(1), 216-224.

Petit, J., Wiegertjes, G.F. (2016). Long-lived effects of administering β-glucans: Indications for trained immunity in fish. Developmental & Comparative Immunology, 64, 93-102.

Pietretti, D., Wiegertjes, G.F. (2014). Ligand specificities of Toll-like receptors in fish: indications from infection studies. Developmental & Comparative Immunology, 43(2), 205-222.

Raa, J. (2000). The use of immune-stimulants in fish and shellfish feeds. In: Advance en Nutricion Acuicola V. Memorias del V Simposium Internacional de Nutrcion Acouicola, vol. Memorias (Ed.) L.E. Cruz-Suarez, D. Ricque-Marie, M. Tapia-Salazar, M.A. Olvera-Novoa, R. Civera-Cerecedo Merida, Yucatan: 47-56.

Regoli, F., Giuliani, M.E. (2014). Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Marine Environmental Research, 93, 106-117.

Regoli, F., Winston, G.W. (1999). Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxyl radicals, and hydroxyl radicals. Toxicology and Applied Pharmacology, 156(2), 96-105.

Ringř, E., Olsen, R.E., Gonzales Vecino, J.L., Wadsworth, S., Song, S.K. (2012). Use of immunostimulants and nucleotides in aquaculture: a review. Journal of Marine Science: Research and Development, 2(1), 104. doi:10.4172/21-55-9910.1000104.

Rodrigues, M. V., Zanuzzo, F. S., Koch, J. F. A., de Oliveira, C. A. F., Sima, P., & Vetvicka, V. (2020). Development of Fish Immunity and the Role of β-Glucan in Immune Responses. Molecules (Basel, Switzerland), 25(22), 5378. doi: 10.3390/molecules25225378.

Saftig, P., Klumperman, J. (2009). Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. Nature Reviews Molecular Cell Biology, 10(9), 623-635.

Sakai, M. (1999). Current research status of fish immunostimulants. Aquaculture, 172, 63-92.

Sattler, S., Ghadially, H., Hofer, E. (2012). Evolution of the C-type lectin-like receptor genes of the DECTIN-1 cluster in the NK gene complex. ScientificWorldJournal, 2012, 931386. doi: 10.1100/2012/931386.

Selvaraj, V., Sampath, K., Sekar, V. (2006). Adjuvant and immunostimulatory effects of beta-glucan administration in combination with lipopolysaccharide enhances survival and some immune parameters in carp challenged with *Aeromonas hydrophila*. Veterinary Immunology and Immunopathology, 114(1-2), 15-24.

Siwicki, A.K., Anderson, D.P. (1993). Nonspecific defense mechanisms assay in fish. II. Potential killing activity of neutrophils and macrophages, lysozyme activity in serum and organs and total immunoglobulin level in serum. In: Fish Disease Diagnosis and Prevention Methods (Ed.) A.K. Siwicki, D.P. Anderson, J. Waluga, Wydawnictwo IRS, Olsztyn, Poland: 105-112.

Skov, J., Kania, P.W., Holten-Andersen, L., Fouz, B., Buchmann, K. (2012). Immunomodulatory effects of dietary β-1,3-glucan from *Euglena gracilis* in rainbow trout (*Oncorhynchus mykiss*) immersion vaccinated against *Yersinia ruckeri*. Fish and Shellfish Immunology, 33(1), 111-120.

Song, L., Zhou, Y., Ni, S., Wang, X., Yuan, J., Zhang, Y., Zhang, S. (2020). Dietary Intake of β-Glucans Can Prolong Lifespan and Exert an Antioxidant Action on Aged Fish *Nothobranchius guentheri*. Rejuvenation Research, 23(4), 293-301.

Song, S.K., Beck, B.R., Kim, D., Park, J., Kim, J., Kim, H.D., Ringř, E. (2014). Prebiotics as immunostimulants in aquaculture: a review. Fish and Shellfish Immunology, 40(1), 40-48.

Stier, H., Ebbeskotte, V., Gruenwald, J. (2014). Immune-modulatory effects of dietary Yeast Beta-1,3/1,6-D-glucan. Nutrition Journal, 13, 38. doi: 10.1186/1475-2891-13-38.

Tkachenko, H., Grudniewska, J., Kurhaluk, N. (2022). Effects of dietary yeast α-1.3/1.6-glucans on oxidative stress biomarkers in hearts and livers of rainbow trout (*Oncorhynchus mykiss* Walbaum), European whitefish (*Coregonus lavaretus* L.), and grayling (*Thymallus thymallus* L.). Fisheries & Aquatic Life, 30, 149-168.

Tsutsui, H., Kinugawa, S., Matsushima, S. (2011). Oxidative stress and heart failure. American Journal of Physiology – Heart and Circulatory Physiology, 301(6), H2181-2190.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M. (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicology and Environmental Safety, 64(2), 178-189.

Vetvicka, V., Vannucci, L., Sima, P. (2013). The Effects of β - Glucan on Fish Immunity. North American Journal of Medicine and Science, 5(10), 580-588.

Weng, Y.Z., Fang, Y.Q., Zhang, Y.S. (2013). (Application of lysosomal detection in marine pollution monitoring: research progress). Ying Yong Sheng Tai Xue Bao, 24(11), 3318-3324 (in Chinese).

Yar Ahmadi, P., Farahmand, H., Kolangi Miandare, H., Mirvaghefi, A., Hoseinifar, S.H. (2014). The effects of dietary Immunogen on innate immune response, immune related genes expression and disease resistance of rainbow trout (*Oncorhynchus mykiss*). Fish and Shellfish Immunology, 37(2), 209-214.

Zar, J.H. (1999). Biostatistical Analysis, 4th ed., Prentice Hall Inc., New Jersey.

Zhang, X., Yu, L., Xu, H. (2016). Lysosome calcium in ROS regulation of autophagy. Autophagy, 12(10), 1954-1955.