FISHERIES & AQUATIC LIFE (2022) 30: 149 - 168
Archives of Polish Fisheries
DOI 10.2478/aopf-2022-0014

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RESEARCH ARTICLE

Effects of dietary yeast β-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.)

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Received – 11 March 2022/Accepted – 26 August 2022. Published online: 30 September 2022;
Citation: Tkachenko, H., Grudniewska, J., Kurhaluk N. (2022). Effects of dietary yeast β-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

Abstract. The present study was performed to investigate the effects of dietary yeast β-1,3/1,6-D-glucans supplemented over a 14-day feeding period on liver and cardiac function and the oxidative mechanism underlying these effects. We assessed relevant lipid peroxidation and protein oxidation biomarkers, antioxidant defense indices [superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), total antioxidant capacity (TAC)], and biochemical alterations [activities of aminotransferases (AT), lactate dehydrogenase (LDH), and succinate dehydrogenase (SDH), and levels of lactate (L) and pyruvate (P)] in rainbow trout, European whitefish, and grayling. The fish received a control diet and an experimental diet containing yeast β-1,3/1,6-D-glucans at a dose of 1% in the basal feed for 14 days. The results demonstrated enhancement in the phagocytic activity and phagocytic index in the groups fed β-glucans, compared to the control groups. In the groups fed β-glucans, the oxidative stress parameters such as lipid peroxidation, oxidatively modified proteins, and TAC did not vary significantly. The dietary β-glucans stimulated CAT and GPx activities in hepatic and cardiac tissues in comparison to the control group; however, a reverse trend was observed in the GR activity in these tissues.

Keywords: β-glucans, oxidative stress, metabolic alterations, *Thymallus thymallus*, *Oncorhynchus mykiss*, *Coregonus lavaretus*

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Introduction

β-glucans are linear polysaccharides with different molecular weights and degrees of branching (Dalmo and Bøgwald 2008). In nature, β-glucans are extracted from the cell walls of yeast, mushrooms, bacteria, algae, and plants (Dalmo and Bøgwald 2008, Abdelhamid et al. 2020). Given their prebiotic function, β-glucans are considered to be ideal immune-stimulating substances for aquaculture, and yeast β-glucans are the feed additive with the longest track record in aquaculture (Dalmo and Bøgwald 2008). They can stimulate growth performance and nonspecific immunity, i.e., they improve phagocytic activity, activate the complement cascade, and

increase the expression of selected cytokines in macrophages, neutrophils, and dendritic cells (Lin et al. 2011, Skov et al. 2012, Pionnier et al. 2014, Falco et al. 2014, Zhu and Wu 2018). Petit and Wiegertjes (2016) reported contrasting effects of β-glucans on the expression of immune-regulatory genes in the presence or absence of a microbial stimulus. Administering dietary β-glucans appears to down-regulate the expression of immune-regulatory genes (e.g., IL-1β and lysozyme) in the presence of a microbial stimulus (Djordjevic et al. 2009, Skov et al. 2012). On the other hand, they up-regulate the expression of genes such as IL-1β and cathelicidins (host defense peptides) in the absence of a microbial stimulus (Schmitt et al. 2015, Skov et al. 2012, Petit and Wiegertjes 2016).

As immunostimulants, β-glucans bind to macrophages and other white blood cells, improving the immunological responses of the host such as phagocytosis, lysozyme activity, respiratory burst, and cytokine release (Angulo et al. 2017). Ulvestad et al. (2018) reported that the head kidney macrophage-like cells of Atlantic salmon (*Salmo salar* L.) stimulated in vitro with bacterial lipopolysaccharide (LPS) and β-glucans showed increased production of reactive oxygen species (ROS) compared to unstimulated cells. Respiratory burst and associated ROS production also constitute important indicators of fish health status (Vera-Jimenez et al. 2013). Although β-glucans are not sources of ROS, they can stimulate ROS production via different mechanisms (Thitamadee et al. 2014, Ulvestad et al. 2018, Sánchez-Salgado et al. 2019). ROS can elicit adverse effects in aquatic organisms and induce oxidative damage to lipids, proteins, and nucleic acids (Livingstone 2001, Valavanidis et al. 2006).

Oxidative stress results from an imbalance between the production and removal of free radicals or oxidant agents (Ortiz-Ordoñez et al. 2011). Lipid peroxidation can be broadly defined as the process of the insertion of a hydroperoxy group into lipids present in a cell membrane, resulting in cell membrane instability with consequent cellular damage and death. Polyunsaturated fatty acids are often the targets of peroxidation, where the peroxyl groups are derived from an oxygen molecule or hydrogen peroxide (Anthonymuthu et al. 2016). The oxidative modification of proteins by free radicals proceeds through a large number of physiological and non-physiological reactions (Stadtman and Levine 2000). Oxidatively modified proteins are not repaired and must be removed through proteolytic degradation by proteasomes (Levine 2002). The accumulation of oxidatively modified proteins disrupts cellular function either by the loss of catalytic and structural integrity or by the interruption of regulatory pathways (Stadtman and Levine 2000). The accumulation of many lipid peroxidation products and protein damage is known to be toxic to cells (Levine 2002, Anthonymuthu et al. 2016). To decrease the negative effects of ROS, fish possess antioxidant defenses that include both enzymatic and nonenzymatic processes. The enzymatic system is composed of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase (Wilhelm Filho 1996, Janssens et al. 2000).

The tricarboxylic acid (TCA) cycle and oxidative phosphorylation have been studied extensively in immune cells. Both are fully functional in most T cell subsets. There is a shift towards glycolysis and away from the TCA cycle in effector T cells. In interleukin-4 (IL-4)-activated macrophages, the TCA cycle is intact and participates in oxidative phosphorylation, providing ATP for energy. The TCA cycle is broken after citrate and after succinate in M1-like macrophages activated by lipopolysaccharide (LPS) and interferon-γ (O'Neill et al. 2016). The alterations in the TCA cycle in M1 macrophages lead to the mitochondrial accumulation of metabolites that can promote their immune functions. These events can also be linked to nitric oxide production, which has been shown to inactivate the electron transport chain in macrophages (Clementi et al. 1998). A metabolic switch from oxidative phosphorylation to aerobic glycolysis has been observed in some processes of inflammation and LPS-induced sepsis (Bar-Or et al. 2018). Librán-Pérez et al. (2018) have hypothesized that β-glucans can induce metabolic changes used by hosts to fight pathogens. They evaluated changes in metabolic parameters in turbot that could affect their

survival after previous intraperitoneal treatment with β-glucans and the subsequent administration of Viral Hemorrhagic Septicemia Virus (VHSV) or bacteria (*Aeromonas salmonicida* subsp. *salmonicida*). The metabolic changes induced by β-glucans are beneficial for VHSV replication, but they are harmful to *A. salmonicida*, resulting in reduced mortality. β-glucans appear to have great therapeutic potential and can induce trained immunity against bacterial disease but not against viral disease, which seems to take advantage of β-glucan metabolic alterations (Librán-Pérez et al. 2018).

The effects of dietary β-glucans on the general health status of three fish species (rainbow trout, European whitefish, grayling) and oxidative stress biomarkers and biochemical alterations in different tissues specifically should be explored. This prompted us to investigate the effects of dietary yeast β-1,3/1,6-D-glucans supplemented for a 14-day feeding period on liver and cardiac function and the oxidative mechanisms underlying these effects. We assessed relevant lipid peroxidation and protein oxidation biomarkers, antioxidant defense indices (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, total antioxidant capacity), and biochemical alterations (activities of aminotransferases, lactate dehydrogenase, and succinate dehydrogenase, and lactate and pyruvate levels) in rainbow trout, European whitefish, and grayling after a 14-day period of supplementation with β-glucans.

Materials and Methods

Fish and experimental design

Thirty healthy grayling (*Thymallus thymallus*) weighing 34.9 ± 1.9 g, thirty healthy rainbow trout (*Oncorhynchus mykiss*) weighing 55.9 ± 2.1 g, and thirty healthy European whitefish (*Coregonus lavaretus*) weighing 43.3 ± 2.7 g were used in the experiments. The fish were kept in an indoor system with a supply of freshwater with adequate aeration and an internal power filter. The water quality parameters were as follows: temperature of 16 ± 2°C; 12 ± 0.5 ppm of dissolved oxygen; pH of 7.4–7.6. During the acclimation period (14 days), the fish were fed a commercial basal diet at a rate of 1.5% body weight (BW) four times daily. After acclimation, the fish were randomly divided into six groups and kept in aerated 250 L square tanks containing dechlorinated tap water (70 fish per tank). One tank comprised one group. Natural photoperiod conditions were maintained throughout the feeding trial. The experimental part of the study was conducted at the Department of Salmonid Research, Stanislaw Sakowicz Inland Fisheries Institute (Rutki, Poland).

The groups were fed for 14 days as follows: the control groups of grayling (n = 15), rainbow trout (n = 15), and European whitefish (n = 15) received a control basal diet and the β-glucan groups were fed Yestimun® food supplement (with 85% β-1.3/1.6-glucans, Leiber GmbH, Bramsche, Germany). The basal feed was supplemented with 1% Yestimun® powder (dose: 1 kg per 99 kg, wt/wt). This insoluble, highly purified preparation contains natural polysaccharides, e.g., β-1,3/1,6-D-glucans derived from spent brewers' yeast (*Saccharomyces cerevisiae*). Yeast cell walls typically contain approximately 30% β-glucans (dry weight) (Stier et al. 2014).

The survival rate of fish in the different treatment groups was recorded during the feeding trial. An increase in fish weight was observed. At the end of the 14-day feeding period, the fish were decapitated, and the livers and hearts were dissected. Blood was sampled with plastic syringes from the caudal vein. A portion of the blood samples was transferred into tubes containing K_3EDTA for the phagocytic activity (PA) assay and for calculating the phagocytic index (PI). The fish were not anesthetized before tissue sampling. The experiments were performed in duplicate.

Reagents and solutions

Tris, EDTA, HEPES, KCl, K_2CO_3, KH_2PO_4, GSH, GSSG, $NADPH_2$, and 2-thiobarbituric acid were purchased from Sigma-Aldrich (Sigma-Aldrich Sp.

z o.o, Poznan, Poland). The reagents were prepared freshly. All other reagents were of analytical grade.

Phagocytic activity (PA) and phagocytic index (PI)

The PA and PI were determined with the microscopic counting method proposed by Siwicki and Anderson (1993). Briefly, a 100 μL blood sample was added to 100 μL of formalin-killed *Yersinia ruckeri* (1×10^7 cells). The suspension was thoroughly mixed and left in the well to incubate for 30 min. Next, the plate was mixed gently, and 0.05 mL of mixed suspension was placed on 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 viewed under a light microscope to count 100 cells per slide. The phagocytic activity and phagocytic index were determined as follows: phagocytic activity (%) = (number of phagocytic cells with engulfed bacteria/number of phagocytes) × 100; phagocytic index = number of engulfed bacteria/phagocytic cells.

Hepatic and cardiac tissue isolation

Tissue samples were collected from the fish after decapitation. One fish was used for each homogenate preparation. Briefly, the liver and heart were excised, weighed, and washed in ice-cold buffer. The minced tissue was rinsed clear of blood with ice-cold 100 mM Tris-HCl isolation buffer, homogenized in 10 vol. (v/w) in isolation buffer, and centrifuged at 3,000 × g at 4°C for 10 min. The resulting supernatant was stored at -22°C and used for analyses of enzyme activities and biomarkers of oxidative stress. The isolation buffer contained 100 mM Tris-HCl; the pH value was adjusted to 7.2 with HCl.

Biochemical assays

All enzymatic assays were carried out at 24 ± 0.5°C with a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany). The homogenate suspension was added to start the enzymatic reactions. The specific assay conditions are presented below. Each sample was analyzed in duplicate. The protein concentration in each sample was determined as in Bradford (1976) using bovine serum albumin as the standard (Bradford 1976).

2-Thiobarbituric acid reactive substances (TBARS).

Lipid peroxidation was determined in aliquots of 10% hepatic and cardiac tissue homogenates from the treated and control groups with the procedure developed by Kamyshnikov (2004). The absorbance of each aliquot was measured at 540 nm, and the lipid peroxidation level was expressed as nanomoles of TBARS formed per milligram of protein (nmol MDA·mg^{-1} protein) using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Content of carbonyl derivatives of oxidatively modified proteins (OMP)

The rate of protein oxidative destruction was estimated based on the reaction of carbonyl derivatives generated in the amino acid reaction with 2,4-Dinitrophenylhydrazine (DNPH) as described by Levine et al. (1990) and modified by Dubinina et al. (1995). The carbonyl content was calculated from the measurements of absorbance at 370 nm and 430 nm and an absorption coefficient of 22,000 M^{-1} cm^{-1}. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehydic derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}) and expressed in nmol per mg of tissue protein.

Superoxide dismutase activity

The activity of superoxide dismutase (SOD, E.C. 1.15.1.1) was determined by assessing its ability to dismutate superoxide produced during

auto-oxidation of quercetin in an alkaline medium (pH 10.0) as in Kostiuk et al. (1990). The reaction was initiated by adding 0.1 mL of quercetin (1.4 μM dissolved in dimethyl sulfoxide). Absorbance at 406 nm was measured immediately and after 20 min after the addition of the quercetin solution. The activity was expressed in units of SOD per mg of tissue protein.

Catalase activity

The activity of catalase (CAT, E.C. 1.11.1.6) was determined by measuring the decrease of H_2O_2 content in the reaction mixture with a spectrophotometer at a wavelength of 410 nm as proposed by Koroliuk et al. (1988). The reaction was initiated by the addition of 0.1 mL of the homogenate sample into the incubation medium (2 mL of 0.03% H_2O_2 solution) and to 1.0 mL of 4% ammonium molybdate dissolved in 12.5 mM H_2SO_4 solution (blank sample). The absorbance of the solution obtained was measured at 410 nm and compared with that of the blank. One unit of catalase activity was defined as the amount of enzyme required to decompose 1 μmol H_2O_2 per min per mg of tissue protein.

Glutathione reductase activity

The activity of glutathione reductase (GR, EC 1.6.4.2) in the tissues was measured with the method described by Glatzle et al. (1974). The enzymatic activity was assayed spectrophotometrically by measuring $NADPH_2$ consumption. In the presence of GSSG and $NADPH_2$, GR reduces GSSG and oxidizes $NADPH_2$ resulting in a decrease in absorbance at 340 nm. The enzyme assay mixture contained 2.4 mL of 67 mM sodium phosphate buffer (pH 6.6), 0.2 mL of 7.5 mM oxidized glutathione, and 0.1 mL of the homogenate sample. The rate of NADPH oxidation was determined spectrophotometrically at 340 nm. Quantification was performed based on a molar extinction coefficient of 6.22 mM^{-1} cm^{-1} of NADPH. The GR activity was expressed as nmol NADPH per min per mg of tissue protein.

Glutathione peroxidase activity

The activity of glutathione peroxidase (GPx, EC 1.11.1.9) was assayed 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) with the method developed by Moin (1986). The assay mixture contained 0.8 mL of 0.1 M Tris-HCl buffer with 6 mM EDTA and 12 mM sodium azide (pH 8.9), 0.1 mL of 4.8 mM GSH, 0.2 mL of homogenate sample, 1 mL of 20 mM t-butyl hydroperoxide, and 0.1 mL of 0.01 M 5,5-dithiobis-2-nitrobenzoic acid. The rate of GSH reduction was assessed spectrophotometrically at 412 nm. The GPx activity was expressed as μmol GSH per min per mg of tissue protein.

Total antioxidant capacity (TAC)

The TAC level was estimated by measuring the TBARS level after Tween 80 oxidation. It was determined spectrophotometrically at 532 nm by Galaktionova et al. (1998). The sample inhibited the Fe^{2+}/ascorbate-induced oxidation of Tween 80, which resulted in a decrease in the TBARS level. The absorbance of the solution obtained was measured at 532 nm. The absorbance of the blank was defined as 100%. The level of TAC in the sample (%) was determined from the absorbance of the blank.

Alanine aminotransferase and aspartate aminotransferase activity

The activities of alanine aminotransferase (AlAT) and aspartate aminotransferase (AsAT) were analyzed spectrophotometrically with a standard enzymatic method described by Reitman and Frankel (1957). The substrates in the reaction included α-ketoglutaric acid (2 mM) plus L-aspartate (200 mM) for AsAT and α-ketoglutaric acid (2 mM) plus L-alanine (200 mM) (pH 7.4) for AlAT. The intensity of coloration was related to the enzymatic activity of the respective enzymes. Pyruvate was used as the standard for calibrating the graph for measuring both

AsAT and AlAT activities. One unit of AsAT or AlAT activity was defined as the liberation of 1 nmol of pyruvate per min at 37°C incubation per 1 mg protein. The AsAT to AlAT ratio (the De Ritis ratio) was also calculated (Botros and Sikaris 2013).

Lactate dehydrogenase (LDH) activity

The colorimetric method developed by Sevela and Tovarek (1959) was used to determine the activity of lactate dehydrogenase (EC 1.1.1.27). Pyruvate is formed through LDH action in the presence of a sample and NAD+. The reduction of the NAD+ level is coupled with the reduction of L-lactate. The addition of 2,4-dinitrophenyl hydrazine resulted in the formation of the hydrazone complex with ketoacid to generate their respective hydrazone derivatives, which were measured colorimetrically at 530 nm. The color red was produced upon the addition of 0.4M NaOH and was related to enzyme activity. Pyruvate was used as the standard for measuring LDH activity to calibrate graph composition. One unit of LDH was defined as the formation of 1 nmol of pyruvate per min at 37°C incubation per mg of protein.

Succinate dehydrogenase (SDH) activity

Succinate dehydrogenase (EC 1.3.99.1) activity was measured spectrophotometrically by following the reduction of potassium ferricyanide (K_3FeCN_6) at 420 nm with the method developed by Eschenko and Volski (1982) with some modifications. The assay mixture (pH 7.8) contained 0.1 M phosphate buffer, 25 mM EDTA, 0.1 M succinate acid, 150 mM sodium aside, and 25 mM K_3FeCN_6. The reaction was initiated by adding 0.5 mL of the homogenate. The enzyme activity was expressed as nmol succinate per min per mg protein.

Lactate and pyruvate concentrations

Lactate and pyruvate concentrations were measured according to the procedure described by Herasimov and Plaksina (2000). One mL of the sample was added to 6 mL of distilled water and 1 mL of 10% metaphosphoric acid. The mixture was centrifuged at 800g for 5 min to separate the supernatant. One mL of 25% copper (II) sulfate and 500 mg of calcium hydroxide were added to the supernatant, which was then mixed for 30 min. The mixture was centrifuged at 1,000 g for 10 min. For the lactate concentration assay, the resulting supernatant was resuspended in 3 mL of p-dimethylaminobenzaldehyde (0.5% solved in dimethyl sulfoxide) and 1 mL of 25% NaOH. The mixture was incubated at 37°C for 45 min and then centrifuged at 1,000 g for 10 min. Absorbance was measured at 420 nm. A mixture with 0.5% p-dimethylaminobenzaldehyde and 25% NaOH was used as the blank. For the pyruvate concentration assay, the resulting supernatant was resuspended in 0.1 mL of 10% copper (II) sulfate, 4 mL of concentrated H_2SO_4, and 0.1 mL of 20% hydroquinone dissolved in 96% ethanol. It was then heated in a water bath at 95°C for 15 min. The absorbance was measured at 430 nm. Calibration curves of lactate (0.1–50 μM) and pyruvate (0.1–50 μM) were used, and the results were expressed in nmol per mg of protein. The lactate-to-pyruvate ratio (L/P) was also calculated.

Statistical analysis

The basic statistical analysis was performed using Statistica 13.3 (TIBCO Software Inc.). The data were tested for homogeneity of variance using Levene's test of equality of error variances. Normality was checked with the Kolmogorov-Smirnov test.

The results were expressed as mean ± S.D. Significant differences among the means were measured with a multiple range test at a minimum of $P < 0.05$. Non-normally distributed data were log-transformed. The correlation of parametric values was based on Pearson's regressions analysis 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 substrate

concentrations and enzyme activities in liver and heart tissues were estimated using MANOVA. Multivariate significance tests of the main effects (species, tissue type, experimental conditions, and combined effects) facilitated determining statistically significant relationships for all three values. In the model approach, the following three-way classification model was adopted to combine the impact of three factors [tissues, grayling, rainbow trout, European whitefish, and type of experiment):

$X_{ijk} = \mu + \alpha_i + \beta_j + \gamma_l + (\alpha\beta)_{ij} + (\beta\gamma)_{jl} + (\alpha\gamma)_{il} + (\alpha\beta\gamma)_{ijl} + \varepsilon_{ijlk}$,

where X_{ijk} – value of the dependent variable, μ – mean, α_i – main effect of the tissue factor, β_j – main effect of the species factor; γ – main effect of the type of the experiment factor, $(\alpha\beta)_{ij}$ – effect of the interaction of tissue type and species factors; $(\beta\gamma)_{jl}$ – effect of the interaction of species and experiment factor; $(\alpha\gamma)_{il}$ – effect of the interaction of tissue type and experiment factor; ε_{ijk} – random experimental error.

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

RESULTS

Immune-related variables. Relative to the value for the control group, the phagocytic activity (Fig. 1A) was higher in the rainbow trout (by 27.3%, $P < 0.05$), European whitefish (by 19.3%, $P < 0.05$), and grayling (by 26.8%, $P < 0.05$) fed the diet supplemented with β-glucans on day 14. The phagocytic index was higher in the β-glucan treatment group, i.e., in the rainbow trout (by 84%, $P < 0.001$), European whitefish (by 92%, $P < 0.001$), and grayling (by 119.5%, $P < 0.001$) than in the control groups.

Pro/antioxidant balance. Oxidative stress was associated with immunocompetent processes in the cardiac and hepatic tissues of grayling (*T. thymallus*), rainbow trout (*O. mykiss*), and European whitefish (*C. lavaretus*). TBARS were used as the final products of lipid peroxidation and biomarkers of these changes in the first step of the present study (Figs. 2, 3). Data obtained in this analysis are shown in Fig. 2A. The statistical analysis of the oxidative stress parameters obtained for the three fish species showed significant differences between the species in the untreated controls for the following parameters obtained for cardiac tissue: SOD ($F_{2.27} = 3.36$, $P = 0.049$); CAT ($F_{2.27} = 77.31$, $P = 0.000$); GR ($F_{2.27} = 25.04$, $P = 0.000$); GPx ($F_{2.27} = 13.48$, $P = 0.000$). Similar data were obtained for cardiac tissue from

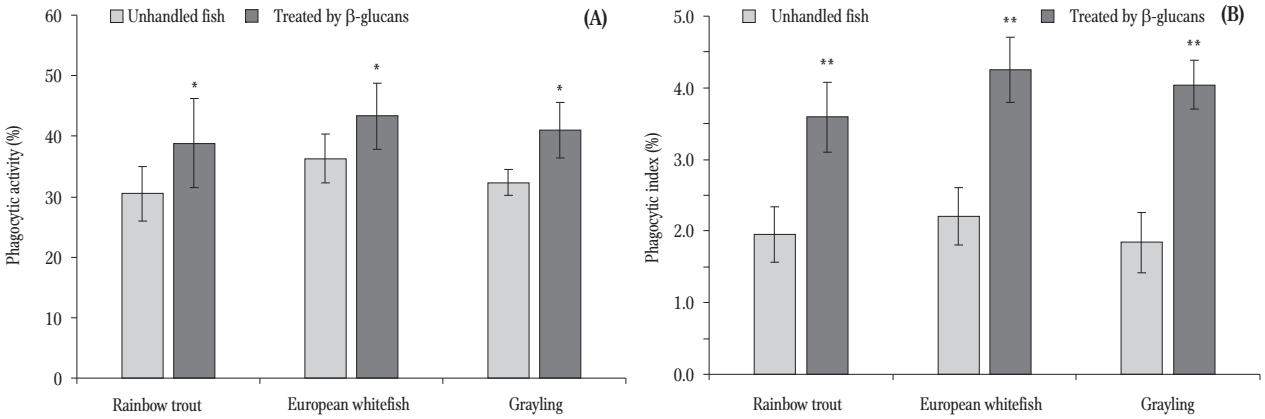

Figure 1. Phagocytic activity (A) and phagocytic index (B) in the groups of rrainbow trout (*O. mykiss*), European whitefish (*C. lavaretus*), and grayling (*T. thymallus*) fed the β-glucan-supplemented diet. The results are expressed as mean ± S.D. Differences between the control and experimental groups were analyzed with MANOVA and Bonferroni's post-hoc test. Differences were considered significant at $p < 0.05$. * $P < 0.05$; ** $P < 0.01$.

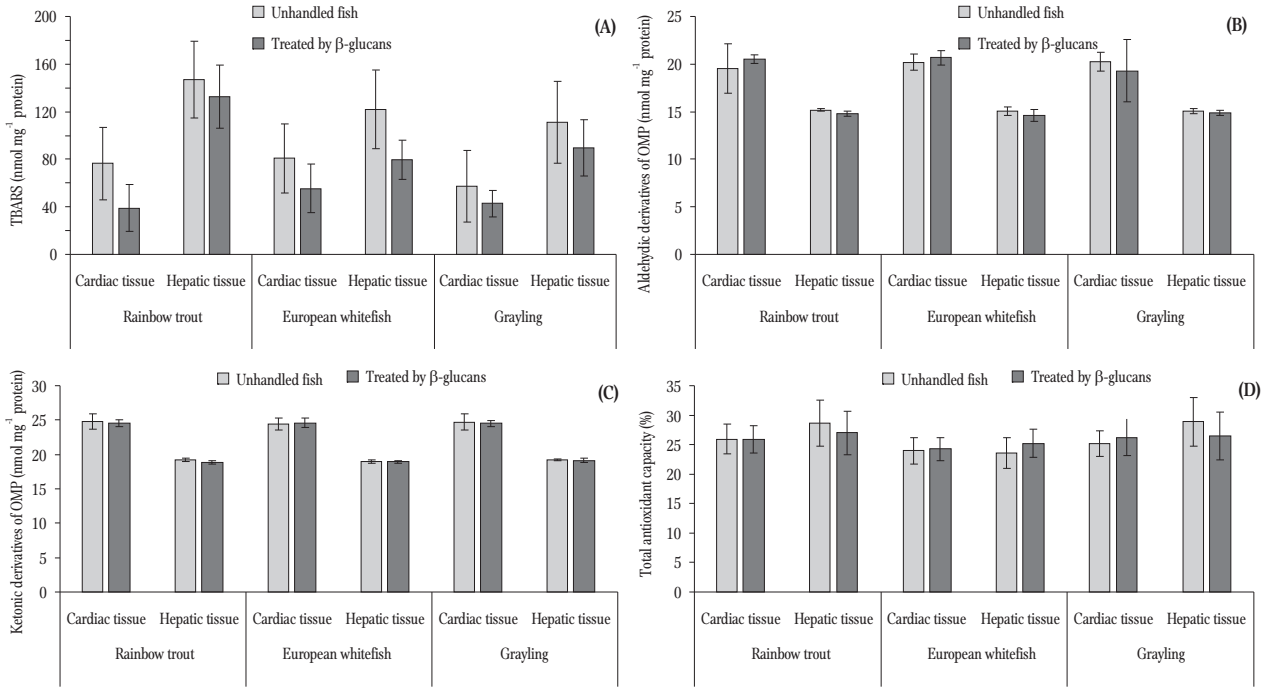

Figure 2. Levels of TBARS (A, nmol MDA·mg^{-1} protein), aldehydic derivatives (B, nmol·mg^{-1} protein) and ketonic derivatives (C, nmol·mg^{-1} protein) of oxidatively modified proteins (OMP), and total antioxidant capacity (%) in the cardiac and hepatic tissues of rainbow trout (*O. mykiss*), European whitefish (*C. lavaretus*), and grayling (*T. thymallus*) fed the β-glucan-supplemented diet. The results are expressed as mean ± S.D. Differences between the control and experimental groups were analyzed with MANOVA and Bonferroni's post-hoc test. Differences were considered significant at $P < 0.05$.

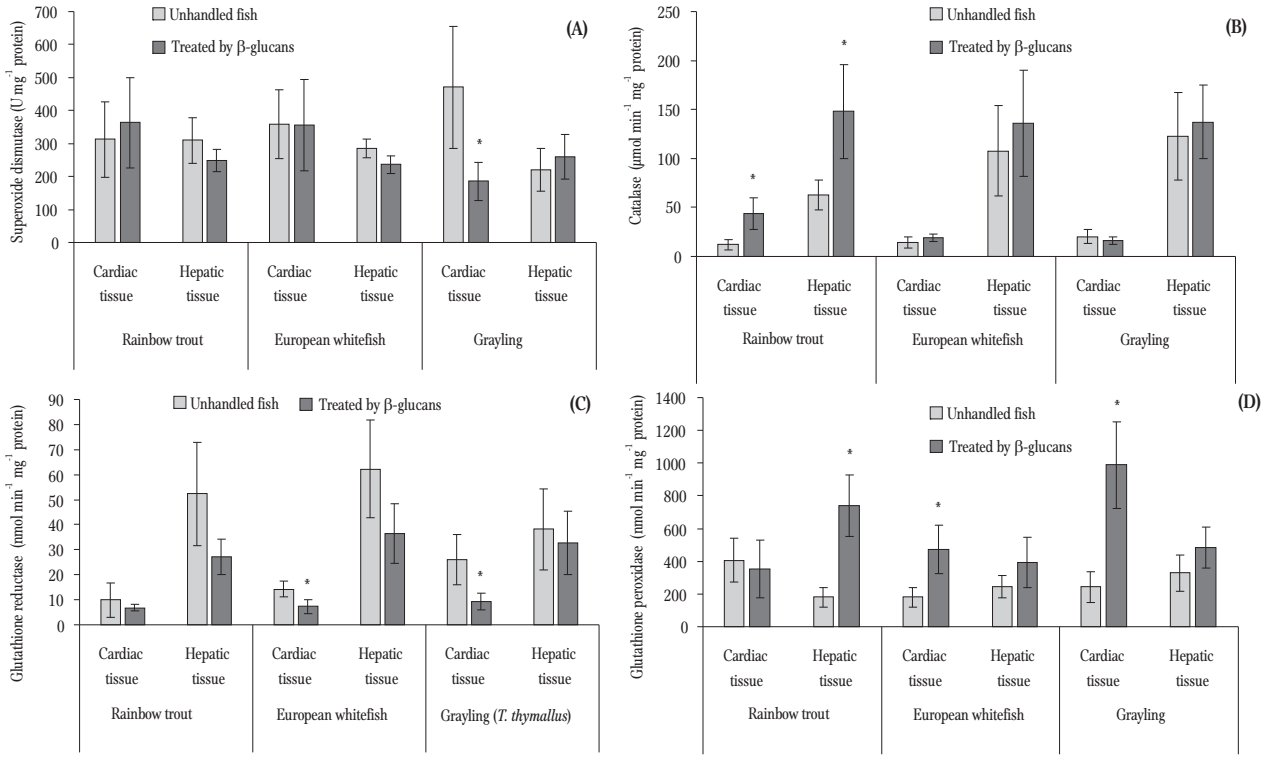

Figure 3. Activities of superoxide dismutase (A, SOD, U mg protein^{-1}), catalase (B, CAT, μmol min^{-1} mg protein^{-1}), glutathione reductase (C, GR, nmol min^{-1} mg protein^{-1}), and glutathione peroxidase (D, GPx, nmol min^{-1} mg protein^{-1}) in the cardiac and hepatic tissues of rainbow trout (*O. mykiss*), European whitefish (*C. lavaretus*), and grayling (*T. thymallus*) fed the β-glucan-supplemented diet.

the fish treated with β-glucans: SOD ($F_{2.27}$ = 7.32, P = 0.003); CAT ($F_{2.27}$ = 20.69, P = 0.000); GPx ($F_{2.27}$ = 27.59, P = 0.000). The statistical dependencies in the hepatic tissue of the untreated fish species were as follows: AD OMP ($F_{2.27}$ = 5.18, P = 0.012); SOD ($F_{2.27}$ = 6.61, P = 0.004); CAT ($F_{2.27}$ = 6.68, P = 0.004); GR ($F_{2.27}$ = 4.05, P = 0.029); GPx ($F_{2.27}$ = 8.024, P = 0.002). Significant differences among the species in TBARS ($F_{2.27}$ = 15.36, P = 0.000) and GPx ($F_{2.27}$ = 12.95, P = 0.000) were observed after treatment with β-glucans.

Significant differences in the levels of lipid peroxidation and oxidatively modified proteins were observed between the fish species and their tissue specificity. However, we did not find statistically significant differences in the data between the untreated control and the β-glucan dietary treatment group. The influence of β-glucans was reflected in multidirectional changes in the activity of antioxidant enzymes depending on fish species and tissue specificity. In the groups of fish treated with dietary β-glucans, there was a significant decrease in SOD activity in the cardiac tissue of the grayling, in CAT activity in the cardiac and hepatic tissues of rainbow trout, and in GR activity in the cardiac tissue of European whitefish and grayling. We also observed an increase in GPx activity in the cardiac tissue of European whitefish and the hepatic tissue of rainbow trout.

Markers of hepato- and cardiotoxicity. The statistical analysis of the data on the markers of hepato- and cardiotoxicity obtained for the three fish species showed significant differences among the species in the untreated controls for the parameters of AlAT ($F_{2.27}$ = 104.28, P = 0.000) and AsAT ($F_{2.27}$ = 92.34, P = 0.000) of cardiac tissue. The results of this series of experiments are presented in Table 1. Our results showed that fish species had a significant effect on AlAT ($F_{2.27}$ = 4.98, P = 0.015) and AsAT activity in the cardiac tissue ($F_{2.27}$ = 6.27, P = 0.006) after β-glucan treatment. The statistical analysis of the data for the hepatic tissue of the untreated fish species showed significant differences in AlAT ($F_{2.27}$ = 5.49, P = 0.010) and AsAT activity ($F_{2.27}$ = 6.60, P = 0.005). The dietary treatment with β-glucans caused

Table 1

Effect of dietary β-glucan treatment on alanine aminotransferase and aspartate aminotransferase activities in the cardiac and hepatic tissues of three fish species

Groups of fish species	AlAT, nmol·min^{-1}·mg^{-1} protein	AsAT, nmol·min^{-1}·mg^{-1} protein	De Ritis ratio
Cardiac tissue			
Rainbow trout (*O. mykiss*)			
Control	9.33 ± 1.61	8.91 ± 1.68	0.952 ± 0.026
β-glucans	6.86 ± 0.81	6.99 ± 0.77	1.021 ± 0.055
European whitefish (*C. lavaretus*)			
Control	10.03 ± 1.19	10.25 ± 1.25	1.023 ± 0.057
β-glucans	6.18 ± 1.13*	6.39 ± 1.03*	1.039 ± 0.042
Grayling (*T. thymallus*)			
Control	43.86 ± 10.41	42.36 ± 10.59	0.964 ± 0.038
β-glucans	5.73 ± 0.19**	5.81 ± 0.16**	1.010 ± 0.031
Hepatic tissue			
Rainbow trout (*O. mykiss*)			
Control	4.48 ± 1.09	7.79 ± 2.23	1.738 ± 0.222
β-glucans	10.28 ± 3.12	17.12 ± 4.79**	1.689 ± 0.104
European whitefish (*C. lavaretus*)			
Control	6.82 ± 2.73	14.79 ± 5.51	2.200 ± 0.391
β-glucans	10.99 ± 2.85	18.30 ± 4.61	1.671 ± 0.058
Grayling (*T. thymallus*)			
Control	8.43 ± 3.58	14.15 ± 5.71	1.700 ± 0.121
β-glucans	9.68 ± 2.29	16.28 ± 4.19	1.677 ± 0.09

The results are expressed as mean ± S.D. Differences between the control and experimental groups were analyzed with MANOVA and Bonferroni's post-hoc test. Differences were considered significant at P < 0.05. * P < 0.05; ** P < 0.01.

multidirectional changes in the activities of alanine and aspartate aminotransferases depending on fish species and tissue specificity. There was a significant decrease in AlAT and AsAT activity in the cardiac tissue of European whitefish and grayling, while AsAT activity was statistically increased in the hepatic tissue of rainbow trout. These changes in aminotransferase activities were not associated with alterations in the De Ritis coefficient.

Markers of energy-related metabolism. The trends of enzyme activities and metabolites involved in metabolic pathways revealed that, under the influence of β-glucans, the main energy substrates in the cardiac and hepatic tissues were used. The activities of enzymes involved in metabolic oxygen-dependent processes, i.e., lactate dehydrogenase and succinate dehydrogenase, and the main metabolites involved in the processes analyzed are presented in Table 2.

The statistical analysis of the data obtained for the three fish species showed significant differences among the species in the untreated controls in the following parameters assessed in the cardiac tissue: LDH ($F_{2.27}$ = 96.00, P = 0.000); SDH ($F_{2.27}$ = 4.67, P = 0.018); lactate ($F_{2.27}$ = 45.54, P = 0.000); pyruvate ($F_{2.27}$ = 46.12, P = 0.000). Our results showed fish species had a significant effect on LDH activity ($F_{2.27}$ = 4.03, P = 0.029) and on the lactate level ($F_{2.27}$ = 5.11, P = 0.013) in the cardiac tissue after β-glucan treatment. The following statistical dependencies for the hepatic tissue of the untreated controls of the three fish species were determined: LDH activity ($F_{2.27}$ = 6.19, P = 0.006); lactate ($F_{2.27}$ = 5.32, P = 0.011); pyruvate levels ($F_{2.27}$ = 8.16, P = 0.002). The results showed the significant effect of β-glucans on lactate ($F_{2.27}$ = 38.40, P = 0.000) and pyruvate levels

Table 2

Activities of lactate dehydrogenase, succinate dehydrogenase, lactate, and pyruvate levels in the cardiac and hepatic tissues of rainbow trout (*O. mykiss*), European whitefish (*C. lavaretus*), and grayling (*T. thymallus*) treated with dietary β-glucans

	Enzyme activities, metabolite levels				
Fish species	LDH, nmol·min^{-1}·mg^{-1} protein	SDH, nmol·min^{-1}·mg^{-1} protein	Lactate, nmol·mg^{-1} protein	Pyruvate, nmol·mg^{-1} protein	Lactate to pyruvate ratio
Cardiac tissue					
Rainbow trout (*O. mykiss*)					
Control	27.15 ± 4.82	2.12 ± 1.56	631.701 ± 91.20	244.90 ± 79.49	2.71 ± 0.40
β-glucans	20.07 ± 2.43	3.47 ± 2.90	328.51±35.43**	218.53 ± 80.28	1.63 ± 0.45*
European whitefish (*C. lavaretus*)					
Control	30.07 ± 3.70	3.00 ± 2.45	763.08 ± 174.79	246.52 ± 86.04	3.29 ± 0.49
β-glucans	18.72 ± 3.37*	2.37 ± 1.19	437.31 ± 124.00*	247.22 ± 78.32	1.90 ± 0.77*
Grayling (*T. thymallus*)					
Control	124.26 ± 30.28	4.61 ± 1.36	2159.3 ± 636.4	1211.39 ± 234.13	1.89 ± 0.48
β-glucans	17.00 ± 0.60**	3.51 ± 2.46	355.66 ± 46.74**	192.29 ± 30.37**	1.91 ± 0.44
Hepatic tissue					
Rainbow trout (*O. mykiss*)					
Control	13.4 ± 02.82	2.37 ± 1.44	523.72 ± 164.29	407.09 ± 49.44	1.45 ± 0.39
β-glucans	29.39 ± 8.94**	1.46 ± 1.0	317.27 ± 101.29*	843.81 ± 46.15**	2.22 ± 0.22*
European whitefish (*C. lavaretus*)					
Control	20.01 ± 7.71	2.55 ± 1.03	676.24 ± 184.46	342.14 ± 121.11	1.84 ± 0.60
β-glucans	31.52 ± 8.69	1.45 ± 0.13*	685.90 ± 193.23	520.32 ± 96.25*	1.49 ± 0.70
Grayling (*T. thymallus*)					
Control	25.07 ± 9.93	2.43 ± 1.77	453.04 ± 110.70	219.29±55.12	2.12 ± 0.51
β-glucans	29.28 ± 6.95	1.92 ± 0.33*	213.27 ± 58.65**	830.11±145.28**	0.26 ± 0.08*

The results are expressed as mean ± S.D. Differences between the control and experimental groups were analyzed with MANOVA and Bonferroni's post-hoc test. Differences were considered significant at P < 0.05. * P < 0.05; ** P < 0.01.

($F_{2.27}$ = 59.70, P = 0.000) in the hepatic tissue of the fish species analyzed.

The effects of the dietary β-glucans on metabolic changes in the cardiac tissue of rainbow trout were associated with a significant decrease in lactate levels, which might have caused the subsequent decrease in the lactate-to-pyruvate ratio. A significant decrease in both lactate and pyruvate levels, however, caused a significant increase in the lactate-to-pyruvate ratio. Thus, multidirectional changes were observed in the oxygen-dependent energy metabolic processes in the hepatic and cardiac tissues of rainbow trout treated with β-glucans.

The effects of β-glucans on the cardiac tissue of European whitefish were reflected in the significant decrease in LDH activity and lactate levels, which might have caused a subsequent decrease in the lactate-to-pyruvate ratio. A statistically significant decrease in SDH activity and increased pyruvate levels were observed in the hepatic tissue of this fish species.

The effects of β-glucans on the cardiac tissue of grayling were associated with a significant decrease in LDH activity and in lactate and pyruvate levels. Similar relationships were observed in the hepatic tissue of this fish species: the significant decrease in SDH activity and lactate levels and the increased pyruvate level, however, caused significant changes in the lactate-to-pyruvate ratio. Thus, multidirectional changes in the oxygen-dependent energy metabolic processes in the hepatic and cardiac tissues were observed in grayling treated with β-glucans.

The MANOVA test revealed statistically significant relationships between the effects of the main factors. The data are presented in Table 3. The multivariate significance tests and effective hypothesis decomposition for the two types of tissue (cardiac and hepatic) and three fish species (grayling, rainbow trout, European whitefish) treated with β-glucans vs. oxidative stress biomarkers showed the significance of the main effects and their interactions. These interactions allowed concluding that tissue type, fish species, and tissue and species interactions revealed the most important relationships.

The coefficient of multiple correlation analysis, the coefficient of determination, and its correction form reduced by random errors for the data analysis are shown in Table 4. The SS test used to describe all data in the profiles of the oxidative stress biomarkers, enzymes, and metabolites of energy metabolism in the cardiac and hepatic tissues analyzed with the significance of F test allowed drawing conclusions on the role of these data. The dependencies for the oxidative stress biomarkers were as follows: KD OMP > AD OMP > CAT > GPx > GR > TBARS > SOD > TAC. It is known that aminotransferases (alanine and aspartate aminotransferases) act as linking points between plastic and energy metabolism; thus, their role together with oxygen-dependent factors in cellular processes was determined by the statistical analysis. These dependencies for the metabolic enzymes and metabolites were as follows: AlAT > LDH > Lactate > AsAT > Pyruvate > SDH.

Table 3
Multivariate significance tests and effective hypothesis decomposition for two types of tissue (cardiac and hepatic) and three fish species (rainbow trout, European whitefish, grayling) treated with β-glucans vs. oxidative stress biomarkers, activities of energetic enzymes, and markers of hepato- and cardiotoxicity

Main effects	Test value	F	P
Tissues	0.0249	268.42	0.000
Species	0.225	23.59	0.000
Experimental groups	0.100	14.88	0.000
Tissues vs. Species	0.237	22.06	0.000
Tissues vs. Experimental groups	0.188	8.96	0.000
Species vs. Experimental groups	0.161	10.22	0.000
Tissue, Species, vs. Experimental groups	0.160	10.30	0.000

Table 4
SS test for a full model data and SS for residues for two types of tissues (cardiac and hepatic), three fish species (rainbow trout, European whitefish, grayling) treated with β-glucans vs. oxidative stress biomarkers, activities of energy-related enzymes, and markers of hepato- and cardiotoxicity

Parameters	Multiple R	Multiple R^2	Multiple adjusted R^2	F	P
TBARS	0.801	0.642	0.606	17.78	0.000
OMP AD	0.904	0.817	0.798	44.15	0.000
OMP KD	0.979	0.958	0.954	227.33	0.000
TAC	0.381	0.145	0.059	1.68	0.087
SOD	0.627	0.393	0.332	6.42	0.000
CAT	0.874	0.764	0.740	32.11	0.000
GR	0.824	0.679	0.647	21.00	0.000
GPx	0.856	0.732	0.706	27.19	0.000
AlAT	0.946	0.895	0.884	84.40	0.000
AsAT	0.911	0.829	0.812	48.10	0.000
De Ritis ratio	0.811	0.729	0.712	43.10	0.000
LDH	0.942	0.888	0.877	78.79	0.000
SDH	0.404	0.163	0.079	1.93	0.043
Lactate	0.917	0.839	0.823	51.80	0.000
Pyruvate	0.892	0.795	0.775	38.55	0.000

The evaluation of the effects of β-glucans on hepatic and cardiac tissues in the fish species investigated helped to determine the key points of interaction between oxidative stress biomarkers and metabolic pathways using correlation and regression analysis. This determined the observed multidirectional changes in the relationships and interconnected mechanisms of oxygen-dependent energy metabolic processes and biomarkers of redox reactions induced by the action of β-glucans. It should be noted that the specificity of certain relationships was related to tissue specificity and fish species. These parameters are presented in Table 5.

DISCUSSION

Natural dietary immunostimulants are considered to be good candidates for the prevention and control of fish diseases in aquaculture. Additionally, they have been reported to be an ideal alternative to antibiotics and a promising alternative to chemotherapy and vaccination (Devi et al. 2019). Many studies have described the positive results of the use of various natural immunostimulants and their *in vivo* effects on fish. β-glucans used as immunostimulants improve growth performance, hematology, cellular and humoral immune responses, and antioxidant capacity and can potentially be used as alternatives to antibiotics and chemicals (Do Huu et al. 2016, Pilarski et al. 2017). This study presents a comparative analysis of the effect of the oral administration of a diet supplemented with yeast β-glucans on the innate-adaptive immune response, biomarkers of oxidative stress and antioxidant defense, and markers of hepato- and cardiotoxicity in the three fish species of rainbow trout, European whitefish, and grayling.

Macrophages play the main role in non-specific cellular responses. Our results revealed that the β-glucans enhanced macrophage function in rainbow trout, European whitefish, and grayling (phagocytic index and phagocytic activity) compared to the untreated controls. β-glucans derived from fungi and yeast have immune-modulating properties, i.e., they increase leukocyte phagocytic, cytotoxic,

Table 5
Correlative intergroup interdependencies between the biomarkers of oxidative stress and metabolic parameters in cardiac and hepatic tissues of European whitefish (*C. lavaretus*) and grayling (*T. thymallus*) treated with dietary β-glucans

		Cardiac tissue			Hepatic tissue		
Fish species	Groups	relationships	r	P	relationships	r	P
Rainbow trout (*O. mykiss*)	Control	CAT – KD OMP	0.649	0.042	CAT – AlAT	0.954	0.000
		TAC – AlAT	0.641	0.046	CAT – AsAT	0.824	0.003
		TAC – AsAT	0.700	0.024	CAT – LDH	0.970	0.000
		TAC – LDH	0.725	0.018	AlAT – LDH	0.958	0.000
		AsAT – Lactate	0.719	0.019	AsAT – LDH	0.791	0.006
		AlAT – Lactate	0.740	0.014	GR – Pyruvate	-0.685	0.029
		LDH – Lactate	0.777	0.008			
		AlAT – Pyruvate	0.646	0.043			
		AsAT – Pyruvate	0.644	0.044			
	β-glucans	CAT – AD OMP	0.705	0.023	TBARS – Lactate	0.716	0.020
		SOD – KD OMP	0.744	0.014	CAT – AlAT	0.959	0.000
		AlAT – LDH	0.944	0.000	CAT – LDH	0.957	0.000
		AlAT – Lactate	0.063	0.036	AlAT – LDH	0.978	0.000
		AsAT – LDH	0.843	0.002	CAT – AsAT	0.971	0.000
		LDH – Lactate	0.801	0.005	AsAT – LDH	0.965	0.000
		TBARS – Lactate	0.711	0.021	Lactate – Pyruvate	0.900	0.000
		OMP KD – Lactate	-0.675	0.032			
		AsAT – Lactate	-0.810	0.004			
		TAC – Pyruvate	-0.697	0.025			
European whitefish (*C. lavaretus*)	Control	LDH – AlAT	0.979	0.000	CAT – GR	0.773	0.009
		LDH – AsAT	0.893	0.001	CAT – AlAT	0.903	0.000
		AsAT – Lactate	0.706	0.023	CAT – AsAT	0.938	0.000
		LDH – Lactate	0.729	0.017	CAT – LDH	0.878	0.001
					GR – CAT	0.773	0.009
					GR – AlAT	0.651	0.041
					GR – AsAT	0.714	0.020
					KD OMP – Pyruvate	0.797	0.016
					SDH – AD OMP	0.684	0.029
	β-glucans	CAT – Lactate	0.762	0.001	KD OMP – Lactate	-0.815	0.004
		LDH – AlAT	0.993	0.000	CAT – GPx	0.788	0.007
		AlAT – Lactate	0.785	0.007	CAT – AlAT	0.841	0.002
		LDH – AsAT	0.997	0.000	CAT – AsAT	0.851	0.002
		LDH – Lactate	0.786	0.007	CAT – LDH	0.843	0.002
		TBARS – SDH	0.678	0.031	AlAT – LDH	0.996	0.000
					AsAT – LDH	0.987	0.000
Grayling (*T. thymallus*)	Control	TBARS – SOD	0.742	0.014	CAT – AlAT	0.994	0.000
		GR – Lactate	0.635	0.048	CAT – AsAT	0.979	0.000
		TAC – AlAT	-0.812	0.004	CAT – LDH	0.990	0.000
		TAC – AsAT	-0.796	0.006	CAT – Pyruvate	0.655	0.040
		TAC – LDH	-0.818	0.004	AlAT – LDH	0.998	0.000
		TAC – Lactate	-0.919	0.000	AsAT – LDH	0.993	0.000
		SOD – SDH	0.634	0.049	AD OMP – GR	0.662	0.027
	β-glucans	KD OMP – AlAT	-0.736	0.015	CAT – AlAT	0.620	0.042
		AsAT – LDH	0.660	0.038	CAT – LDH	0.637	0.035
		LDH – Lactate	0.784	0.007	GR – KD OMP	0.684	0.020
		KD OMP – SDH	-0.722	0.018	GR – TAC	-0.636	0.035
					GR – AlAT	0.659	0.028
					GPx – Lactate	-0.633	0.037
					AsAT – LDH	0.982	0.000
					AlAT – SDH	-0.700	0.017
					SDH – LDH	-0.700	0.017

and antimicrobial activities and ROS production against various pathogens (Volman et al. 2008). Glucans are thought to mediate their effects via interactions with membrane receptors on macrophages. Atlantic salmon macrophages possess a receptor that can recognize even very short beta-1,3-linked glucosyl chains extending from yeast cell walls (Engstad and Robertsen 1994). A low dietary concentration of β-1,3 glucan (0.09%) enhanced the growth of and innate immunity in large yellow croaker following eight weeks of oral administration, but higher supplementation did not influence growth or further improve immunity in this species (Ai et al. 2007). The dietary administration of β-glucans potentiated innate immunity and disease resistance in Asian catfish (Kumari and Sahoo 2006). However, Bridle et al. (2005) revealed that dietary incorporation of β-glucans was unable to stimulate in vivo respiratory burst activity of head kidney macrophages or serum lysozyme production, and it did not increase resistance against amoebic gill disease in Atlantic salmon. The oral administration of β-glucans increased the phagocytic function of peritoneal macrophages in mice via induced phagocytic activity, oxidative bursts, and IL-1 production (Suzuki et al. 1990). Moreover, yeast β-glucans were also demonstrated to increase the phagocytic activity and oxidative metabolism of neutrophils and monocytes in rats (Wójcik 2010). The administration of yeast β-glucans was shown to increase lysozyme and ceruloplasmin activity as biomarkers of the acute phase of the humoral immune reaction in rats. Supplementation exerted positive effects on the oxidative metabolism of peripheral blood granulocytes and monocytes (Małaczewska et al. 2010). Moreover, orally administered β-glucans increased mucosal immunopotentiation by elevating the number of intraepithelial lymphocytes in mice intestines (Tsukada et al. 2003).

The current study highlighted the biochemical alterations in the cardiac and hepatic tissues of the three fish species treated with dietary β-glucans by evaluating the biomarkers of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, antioxidant enzyme activities, and total antioxidant capacity as well as the biomarkers of metabolic changes, i.e., aminotransferases, lactate dehydrogenase, succinate dehydrogenase, lactate, and pyruvate levels. In the current study, the most widely used and accepted markers were used to demonstrate the presence of oxidative stress in the tissues (TBARS as a marker of lipid peroxidation, aldehydic and ketonic derivatives of OMP, antioxidant defenses, total antioxidant capacity, markers of biochemical pathways). Lipid peroxidation measured by the amount of TBARS was not significantly decreased ($p > 0.05$) in the fish treated with β-glucans (Fig. 2A). The current study also revealed that the aldehydic and ketonic derivatives of OMP and the TAC levels did not change significantly following β-glucan treatment (Fig. 2B, C, D). All this resulted in an increase in catalase activity in both cardiac and hepatic tissues of rainbow trout (3.6- and 2.4-fold, $P < 0.05$, respectively) as well as GPx activity in the cardiac tissues of European whitefish (2.6-fold, $P < 0.05$) and grayling (4.1-fold, $P < 0.05$) and in the hepatic tissue of rainbow trout (4.1-fold, $P < 0.05$) after 14 days of the dietary β-glucan treatment (Fig. 3B, D). GR activity was significantly decreased in the cardiac tissue of European whitefish (by 48.2%, $P < 0.05$) and grayling (by 64.1%, $P < 0.05$). In turn, SOD activity was significantly decreased (by 60.6%, $P < 0.05$) in the cardiac tissue of grayling treated with β-glucans (Fig. 3A, C). SOD, CAT, and GPx are important parameters of the first line of enzymatic antioxidant defense. They protect cells from oxidative stress related to excessive generation of ROS. Thus, the measurement of these activities can indicate the antioxidant defense status in fish since they can also serve as biomarkers of oxidative stress (Zhang et al. 2013).

Our results demonstrated that the dietary β-glucan supplementation during the 14-day feeding period did not alter the cardiac or hepatic tissues of three fish species. In this study, the dietary treatment with β-glucans did not affect oxidative stress biomarkers at the dose tested, which indicated that the effect of β-glucans on cardiac and hepatic tissues did not cause cytotoxic effects in rainbow trout, European whitefish, or grayling, and they can be considered safe. Ideal natural immunostimulants should

activate the immune system without causing cytotoxicity to the host cells (Kim and Austin 2006). The results of the present study showed that β-glucan dietary supplementation in rainbow trout, European whitefish, and grayling resulted in a significant elevation of enzymatic antioxidants (CAT and GPx activities) with a non-significant decline in TBARS levels. The increase determined in this study could be explained by the possible enhancement of antioxidant capacity. The relatively low levels of TBARS and carbonyl derivatives of OMP observed in the tissues of the fish fed diets supplemented with β-glucans were further supported by the enhanced enzymatic antioxidant defense (CAT and GPx activities). The analysis of correlations among oxidative stress biomarkers confirmed our conclusions (Table 5). In the cardiac tissue of rainbow trout treated with β-glucans, catalase activity was positively correlated with the aldehydic derivatives of OMP ($r = 0.705$, $P = 0.023$), while SOD activity was positively correlated with the ketonic derivatives of OMP ($r = 0.744$, $P = 0.014$). In the hepatic tissue of grayling treated with β-glucans, GR activity was positively correlated with ketonic derivatives of OMP ($r = 0.684$, $P = 0.020$) and inversely correlated with TAC level ($r = - 0.636$, $P = 0.035$) (Table 5).

Previous investigations reported a relationship between β-glucans and the levels of oxidative stress biomarkers. Song et al. (2020) showed that β-1,3-glucans reduced the accumulation of lipofuscin in the gills of the annual fish *Nothobranchius guentheri* and the senescence-associated β-galactosidase in the caudal fins. Moreover, β-1,3-glucans were able to lower levels of protein oxidation, lipid peroxidation, and ROS in muscles. Finally, β-1,3-glucans were shown to promote the activities of antioxidant enzymes, including catalase, superoxide dismutase, and glutathione peroxidase in the fish, and to slow the increase in P66shc, i.e., a critical factor involved in the regulation of intracellular ROS contents (Song et al. 2020). Kim et al. (2009) estimated the effects of β-glucans on SOD and catalase activities in erythrocytes and Mx gene expression in grass carp challenged with grass carp hemorrhage virus. The results showed higher SOD and CAT activities in fish injected with β-glucans for 15 days than in the negative control group (Kim et al. 2009). It was shown that β-glucans had immunostimulatory activity and increased SOD activity in white marine shrimp, *Litopenaeus vannamei*. When juvenile shrimp were immersed in β-glucans and sulfated polysaccharide for 6 h, increased SOD activity was detected in hemocytes and muscle (Campa-Córdova et al. 2002).

In the present study, we also assessed whether dietary treatment with β-glucans was able to alter fish metabolism. Fish have to mobilize energy substrates to cope metabolically with oxidative stress (Iwama 1998). As reported by Muthappa et al. (2014), the production of glucose during stress assists the animal by providing energy substrates to tissues such as the brain, gills, and muscles to cope with increased energy demand. The extent, magnitude, and direction of biochemical/metabolic changes to compensate for increased energetic demand (glucose production) in response to low-level stress determines how animals perform or perish in hostile environments (Muthappa et al. 2014). Fish exhibit unique biochemical plasticity in terms of changes in the activity of enzymes involved in the anaerobic metabolism of carbohydrates (LDH), transaminases in amino acid metabolism providing gluconeogenic substrates (AlAT and AsAT), and lactate and pyruvate levels. As shown by our results, protein catabolism after β-glucan treatment increased in the hepatic tissue to supply more glucose (energy) via the up-regulation of AsAT and AlAT, which plays a role in gluconeogenesis via the transamination reaction. Thus, we used these as markers along with several others. On the other hand, AlAT and AsAT activity in cardiac tissue decreased, while a statistically significant decrease was observed in European whitefish (by 38.4% and 37.7%, $P < 0.05$, respectively) and in grayling (by 38.4% and 37.7%, $P < 0.05$, respectively) treated with β-glucans (Table 1). In our study, the activities of aminotransferases were significantly determined by alterations in oxidative stress biomarkers. In the hepatic tissue of rainbow trout and European whitefish treated with β-glucans, catalase activity

correlated positively with AlAT (r = 0.959, P = 0.000 and r = 0.841, P = 0.002, respectively) and AsAT activity (r = 0.971, P = 0.000 and r = 0.851, P = 0.002, respectively), while AlAT activity in the hepatic tissue of grayling treated with β-glucans was correlated positively with catalase (r = 0.620, P = 0.042) and GR activity (r = 0.659, P = 0.028) (Table 5).

Chronic exposure to stressors affects fish energy requirements by changing anaerobic oxidation (Muthappa et al. 2014). In this study, the dietary β-glucans supplemented during the 14-day feeding period had significant effects on the aerobic and anaerobic metabolic pathways in cardiac and hepatic tissues. These changes differed. The significant decreases in lactate concentration and LDH activity in European whitefish and grayling cardiac tissues indicated dynamic alterations in anaerobic and aerobic metabolism and the total energy supply. The significant decrease in the lactate and pyruvate levels in grayling cardiac tissue with a simultaneous decrease in LDH activity indicated the inhibition of cardiac metabolic activity induced by the β-glucan treatment. On the other hand, the activation of aerobic metabolism was observed in hepatic tissues of fish treated with β-glucans. The significant increase in the pyruvate level with a simultaneous decrease in LDH activity and lactate levels indicated the dominance of an aerobic component in the hepatic energy supply in rainbow trout. A similar tendency was observed in the hepatic tissue of European whitefish and grayling. The correlation analysis revealed the role of the lactate level in the maintenance of oxidative stress biomarkers (TBARS and catalase) in the cardiac tissues of rainbow trout (r = 0.711, P = 0.021) and European whitefish (r = 0.762, P = 0.001) (Table 4). Catalase activity was positively correlated with AlAT activity in rainbow trout (r = 0.959, P = 0.000), European whitefish (r = 0.841, P = 0.002), and grayling (r = 0.620, P = 0.042), with AsAT activity in rainbow trout (r = 0.971, P = 0.000) and European whitefish (r = 0.851, P = 0.002), and with LDH activity in rainbow trout (r = 0.978, P = 0.000), European whitefish (r = 0.843, P = 0.002), and grayling (r = 0.637, P = 0.035) (Table 5).

Succinate dehydrogenase (SDH; mitochondrial complex II) plays a central role in mitochondrial metabolism, catalyzing the oxidation of succinate to fumarate and the reduction of ubiquinone to ubiquinol, thereby linking the tricarboxylic acid cycle and the electron transport system (Jardim-Messeder et al. 2015). SDH was suggested to be an essential contributor to physiological and pathological ROS production (Quinlan et al. 2012). ROS produced at the ubiquinol oxidation center of complex III seem to have additional physiological functions as signaling molecules during such cellular processes as adaptation to hypoxia (Bleier and Dröse 2013). SDH has been recognized as an indirect modulator of superoxide production by complexes I and III. Direct modulation of mitochondrial ROS generation during cardioprotection via complex II inhibition was reported (Dröse et al. 2011). In the current study, there was a decrease in the oxidative stress biomarker levels with a simultaneous decrease in SDH activity in the hepatic tissue of both European whitefish and grayling treated with β-glucans, which confirms the high adaptive capacity of the liver in compensation for metabolic alterations occurring as a result of β-glucan treatment. The correlations between the levels of oxidative stress markers and metabolites in the cardiac and hepatic tissues of rainbow trout, European whitefish, and grayling treated with β-glucans confirmed the significant role of metabolites and enzymes of energy transformation in the liver and heart as responses to the maintenance of balance between oxidative stress and antioxidant defenses.

CONCLUSIONS

The current study investigated the effects of dietary supplementation of yeast β-1,3/1,6-D-glucans for a 14-day feeding period on liver and cardiac function and the oxidative mechanism underlying these effects. The findings of our study demonstrated that (1) dietary β-glucans improved immunity by increasing the phagocytic activity and phagocytic index in the blood of fishes; (2) the dietary β-glucans did not

affect oxidative stress biomarkers (lipid peroxidation, oxidatively modified proteins, total antioxidant capacity) at the dose tested, indicating that the β-glucans did not cause cytotoxic effects on cardiac or hepatic tissues in rainbow trout, European whitefish, or grayling, and they can be considered safe; (3) the β-glucan dietary supplementation of rainbow trout, European whitefish, and grayling resulted in a significant elevation of enzymatic antioxidants (CAT and GPx activities) with a non-significant decline in the levels of oxidative stress biomarkers; (4) the β-glucan treatment resulted in increased protein catabolism in the hepatic tissue to supply more energy via the up-regulation of AsAT and AlAT, which play roles in gluconeogenesis via a transamination reaction, while the activity of both AlAT and AsAT decreased in cardiac tissue; (5) the significant decrease in the lactate concentration and LDH activity in the cardiac tissues of European whitefish and grayling reflected the dynamic alterations in anaerobic and aerobic metabolism and in the total energy supply. A significant decrease in the lactate and pyruvate levels in grayling cardiac tissue with a simultaneous decrease in LDH activity indicated the inhibition of the cardiac metabolic activity induced by the β-glucan treatment; (6) the activation of aerobic metabolism was observed in the hepatic tissue of the β-glucan-treated fish. The significant increase in pyruvate level with the simultaneous decrease in the LDH activity and lactate level indicated the dominance of the aerobic component in the hepatic energy supply of rainbow trout; (7) the decrease in SDH activity in the hepatic tissue in European whitefish and grayling treated with β-glucan confirmed the high adaptive capacity of the liver to compensate for metabolic alterations occurring from β-glucan treatment. However, this study is a single experimental model, and further investigations on the use of different concentrations of β-glucans in different fish species are encouraged to evaluate various biochemical parameters since it is likely that the responses of fishes seem to depend on doses and fish species.

Author contributions. Conceptualization: H.T., N.K., J.G.; Fieldwork: J.G.; Data analysis and biochemical study: H.T., N.K.; Manuscript draft: H.T., N.K.; review: H.T., N.K., J.G. All authors read, reviewed, and approved the final manuscript.

Funding. This research was funded by the Pomeranian University in Słupsk.

Conflicts of interest. The authors declare that they have no conflicts of interest.

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