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RESEARCH ARTICLE

Role of β-glucans in reducing oxidative stress and improving muscle tissue health in European whitefish (*Coregonus lavaretus* L.)

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Abstract. β-glucans are currently a notable topic in research on enhancing immunity in fishes and their significant role in improving fish health and productivity in aquaculture. Phagocytic activity (PA), phagocytic index (PI), analyses of oxidative stress biomarkers (2-thiobarbituric acid reactive substances [TBARS] as biomarkers of lipid peroxidation, aldehyde and ketone derivatives of oxidatively modified proteins [OMP]), total antioxidant status (TAS), and lysosomal enzyme activity (alanyl aminopeptidase [AAP], leucyl aminopeptidase [LAP], acid phosphatase [AcP], and β-N-acetylglucosaminidase [NAG]) were used to assess the effects of dietary β-glucans on the skeletal muscle and heart tissues of European whitefish (*Coregonus lavaretus* L.). Our study showed that dietary β-glucan supplementation improved the immune system of European whitefish. Fifteen, 30, and 45 days after supplementing diets with β-glucans, the fish showed increased blood PA and PI, indicating improved immune response. β-Glucans also reduced lipid peroxidation and oxidatively modified protein levels and effectively managed the overall antioxidant status. We observed an increase in β-N-acetylglucosaminidase (NAG) activity in the skeletal muscle tissue on days 15 and 30 after β-glucan dietary supplementation and in the cardiac tissue on days 15 and 45 after β-glucan dietary supplementation. This highlights the role of NAG in the metabolic changes in muscle caused by the action of β-glucans. Furthermore, NAG activity was significantly associated with the tissue type and the duration of β-glucan dietary supplementation, as shown by regression analysis. MANOVA analysis confirmed that the long-term effect of β-glucans was more pronounced. β-glucan supplementation is an effective strategy for improving immune function and managing oxidative stress in the skeletal and cardiac muscles of European whitefish.

Keywords: aquaculture, β-glucans, lysosomal enzymes, MANOVA analysis, oxidative stress

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Introduction

Aquaculture plays a key role in the global economy, supplying a significant proportion of the world's demand for fresh fish and seafood. Veterinary inspection plays a vital role in aquaculture to ensure the health and welfare of fish, to monitor and manage the health of aquaculture animals, and to support the development of appropriate husbandry practices that promote the sustainability of the industry (Porter et al. 2022). β-glucans continue to be a hot topic in fish immunity enhancement, driven by increasing global demand for

fish protein, the potential to reduce antibiotic use by enhancing fish immunity, and the need to strengthen natural defenses against climate change and water pollution, highlighting their important role in improving fish health and aquaculture productivity (Dietrich-Muszalska et al. 2011, Douxfils et al. 2017).

β-glucans are natural polysaccharides derived mainly from yeasts, fungi, and cereals that have strong immunomodulating properties, and the use of them in fish farming is very important for aquaculture (Liu et al. 2021, Machuca et al. 2022). Supplementing fish feed with β-glucans can strengthen fish immune systems, resulting in better resistance to bacterial, viral, and parasitic infections (Rodrigues et al. 2020), which can help to reduce the use of antibiotics in aquaculture, which is important from public health and sustainable aquaculture perspectives. Continued interest in β-glucans in aquaculture is thanks to their dual role in improving immune function and reducing oxidative stress. These properties are essential for improving fish health and productivity, making β-glucans a valuable component of fish diets in modern aquaculture (Dalmo and Bøgwald 2008).

The immunomodulatory potential of β-glucans has been highlighted by studies on the effects of β-glucans on oxidative stress in different animal species (Douxfils et al. 2017, Song et al. 2020, Tkachenko et al. 2022). β-glucans, mainly derived from yeast cell walls, have been shown to alleviate oxidative stress and improve immune responses in several fish species (Velazquez-Carriles et al. 2018). Research has shown that β-glucan supplementation significantly reduced oxidative damage by lowering the levels of lipid peroxidation markers (Dietrich-Muszalska et al. 2011, Kurhaluk et al. 2024). This indicates an enhanced antioxidant defense system in fish, which is crucial for maintaining health and resilience to environmental stressors. Furthermore, research has shown that the timing and dosage of β-glucan administration can influence the magnitude of these beneficial effects. For example, early administration of β-glucans during periods of stress can prime the immune system, making it more responsive to subsequent challenges (Douxfils et al. 2017, Machuca et al. 2022). This is particularly relevant for salmonid fishes, which can be exposed to various stressors in aquaculture environments, such as changes in water quality, handling, and infections (Engstad et al. 1992, Meena et al. 2013).

Research on oxidative stress is critical to aquaculture for several reasons. Oxidative stress can adversely affect fish health and immune function, potentially leading to increased susceptibility to disease. Understanding oxidative stress facilitates developing strategies to mitigate its effects, thereby improving fish welfare and productivity. Importantly, research into oxidative stress will help to identify environmental stressors and refine aquaculture practices to ensure sustainable production and the long-term health of farmed fish populations (Tkachenko et al. 2022, Kurhaluk et al. 2024).

The effects of dietary yeast β-glucans on lipid peroxidation in muscle and heart tissues of European whitefish were specifically investigated in one study. The objectives of our study were: i) to analyze the biochemical mechanisms by which β-glucans may alleviate oxidative stress in different muscle tissues (skeletal, heart) in European whitefish; ii) to evaluate the long-term effects of β-glucan consumption on the health of muscle tissues in European whitefish, focusing on oxidative functions (TBARS products, oxidatively modified protein level), metabolic health (total antioxidant capacity), and the functioning of lysosomal enzymes; iii) to compare the effects of β-glucans on oxidative stress in skeletal muscle and heart tissues in European whitefish under prolonged exposure and investigate differences in the response between these tissues in terms of oxidative stress and long-term health outcomes using MANOVA analysis.

Materials and Methods

Fish and characteristics of the study groups

Sixty-six European whitefish (*Coregonus lavaretus* L.) with an initial weight of 56.3 ± 1.9 g were used in the present study. The fish were kept in an indoor system supplied with fresh water with adequate

aeration and an internal power filter. The water temperature was maintained at 16 ± 2°C, dissolved oxygen at 12 ± 0.5 ppm, and pH at 7.4–7.6. The fish were fed a commercial basal diet at 1.5% body weight (BW) four times daily during the acclimation period (14 days). After two weeks, the fish were divided randomly into groups and transferred to square, aerated 250 l tanks supplied with dechlorinated tap water (70 fish per tank). Each group was stocked into a separate tank. The photoperiod throughout the feeding trial was natural. The experimental part of the dietary study was conducted at 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 European whitefish (n = 33) received a control basal diet; the β-glucan groups (n = 33) received Yestimun® supplement at a dose of 1% of the basal diet (containing 85% 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 diet. Yestimun® is an insoluble and highly purified preparation of natural polysaccharides, including β-1,3/1,6-D-glucans, derived from brewer's yeast (*Saccharomyces cerevisiae*). Yeast cell walls typically contain approximately 30% β-glucans based on 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 heart and muscle tissue (the sides near the middle of the back) were dissected. Blood samples were taken from the caudal vein using plastic syringes. Blood samples were transferred to tubes containing K_3-EDTA for phagocytic activity (PA) and phagocytic index (PI) assays. The fish were not anesthetized before decapitation. The experiments were performed in duplicate. The study was conducted at the National Inland Fisheries Research Institute (Olsztyn, Poland). The research complied with Polish animal welfare regulations and was approved by the Local Ethics Committee for Animal Experimentation of the National Inland Fisheries Research Institute, Olsztyn, Poland.

Preparation of tissue homogenates

Heart and muscle tissue samples were homogenized in ice-cold buffer (100 mM Tris-HCl, pH 7.2). Blood was removed from the 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 3,000g for 15 min at 4°C. After centrifugation, the supernatant was collected and frozen at -25°C for further analysis. Protein content was determined using the Bradford method (1976) with bovine serum albumin as the standard.

Phagocytic activity (PA) and phagocytic index (PI)

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

Analyses of oxidative stress biomarkers

Determination of 2-thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation in the homogenate was determined as 2-thiobarbituric acid reactive substances (TBARS) according to the method developed by

Buege and Aust (1978) with some modifications. Briefly, the homogenate was mixed with 2-thiobarbituric acid-trichloroacetic acid (TBA-TCA) reagent with thorough shaking and heated to 85°C for 20 min. The samples were then cooled to room temperature. The absorbance of the pink chromogen present in the clear supernatant after centrifugation at 1200 g for 10 min at room temperature was measured at 532 nm. Tetraethoxypropane was used as the standard. The values are expressed in nmole of TBARS per mg of protein.

Determination of carbonyl groups in oxidatively modified proteins (OMP)

The rate of oxidative protein degradation was assessed by measuring the reaction of carbonyl derivatives of amino acids with 2,4-dinitrophenylhydrazine (DNPH). This method, described by Reznick and Packer (1994), is based on using DNPH to determine the carbonyl content of both soluble and insoluble proteins. Carbonyl groups were quantified spectrophotometrically by measuring the difference in absorbance at 370 nm and 430 nm for aldehyde derivatives (OMP AD) and ketone derivatives (OMP KD). The results are expressed in nmole per mg of tissue protein.

Total antioxidant status (TAS) assay

Total antioxidant status (TAS) was approximated using a 2,2'-azino di[3-ethylbenzthiazoline sulphonate] assay based on the absorbance of the $ABTS^{+}$ radical cation according to Miller et al. (1993).

Lysosomal enzyme assays

Homogenates (20%, w/v) were prepared for differential centrifugation using the method described by DeMartino and Goldberg (1978). The activity of alanyl aminopeptidase (AAP) and leucyl aminopeptidase (LAP) was determined spectrophotometrically according to DeMartino and Goldberg (1978). The reaction was initiated by incubating 50 µL of sample and 500 µL of substrate incubation medium with DMF (Serva, Germany) for 60 min at 37°C, pH 6.0, followed by the addition of 500 µL of stop buffer containing Fast Blue BB salt dissolved in 2% Tween 20 (Sigma, USA). Absorbance was measured at 540 nm. L-alanyl-2-naphtylamine in 0.1 M PBS buffer was used as a substrate to determine alanyl aminopeptidase activity. L-leucyl-2-naphtylamine in 0.1 M PBS pH 7.0 buffer was used as the substrate to determine leucyl aminopeptidase activity. Acid phosphatase (AcP) and β-N-acetylglucosaminidase (NAG) activities were determined spectrophotometrically at 420 nm with 4-nitrophenyl derivatives as substrates as in Barrett and Heath (1977). Enzyme activities were expressed in nmol per hour per mg protein.

Statistical analysis

The results were expressed in means ± S.D. Data from each individual were processed separately in Statistica 13.3 (TIBCO Software Inc., USA). Differences were considered significant at $P < 0.05$. Homogeneity of variance in the data was assessed using Levene's test for equality of error variances, while normality was assessed using the Kolmogorov-Smirnov test. Significant differences among means were determined using a multiple range test with a significance level of $P < 0.05$. Non-normally distributed data were log-transformed. Parametric value correlations were analyzed using Pearson's regression within the multiple regression module. This analysis included the correlation coefficient (r), the regression equation, and the significance of these relationships (P). Arithmetic means were calculated using multivariate analysis of variance (MANOVA). MANOVA was used to assess the main effects and interactions of three factors: study period (time points at 15, 30, and 45 days), tissue type (e.g. muscle, liver, etc.), and the effect of β-glucan supplementation. Interactions among these factors were also tested to determine their combined influence on the outcome variables. The significance of the main effects and interactions was determined with a significance threshold of $P < 0.05$. We also tested for potential interaction

effects among these variables to better understand their combined effects on oxidative stress markers and lysosomal activity.

For regression analysis, we used multiple regression models to explore the relationships between various biomarkers of oxidative stress and lysosomal activity in response to β-glucan supplementation. The model included independent variables such as β-glucan supplementation, study period, and tissue type. Coefficients (β) were calculated for each independent variable to assess their contributions to the overall model. We also calculated R-squared (R^2) and adjusted R-squared (adjusted R^2) values to assess the goodness of fit of the model and the proportion of variance explained by the predictors. All relationships were tested for statistical significance at $P < 0.05$. The regression equation and correlation coefficients (r) were included to quantify the strength and direction of the relationships. The SS test was used to assess the contribution of all oxidative stress biomarkers and lysosomal activity parameters, and the F test was used for significance testing. Multivariate significance tests for the main effects (study period, tissue type, and effect of β-glucans) were used to identify statistically significant relationships among all three variables.

Results

Phagocytic activity and phagocytic index

Enhancing fish immune systems with β-glucans is an important issue since it can improve fish health and production efficiency in aquaculture. Therefore, the first step of our study was to analyze the phagocytic activity and phagocytic index in the blood of European whitefish fed the diet supplemented with β-glucans after 15, 30, and 45 days. Phagocytic activity was 24.7, 31.6, and 55.3% ($P < 0.05$) higher in the muscle tissue of European whitefish fed the diet supplemented with β-glucans on days 15, 30, and 45, respectively. The phagocytic index was statistically higher in the β-glucan treatment group by 62.3% on day 15, 88.6% on day 30, and 119.7% on day 45 compared to the control group.

Thus, strengthening the immune system of fish with β-glucans contributed to improved fish health as was demonstrated by the analysis of PA and PI in the blood of European whitefish fed a diet supplemented with β-glucans for 15, 30, and 45 days. The increase in PA in the β-glucan-fed group, compared to the control. In addition, PI was significantly higher in the group that received β-glucan dietary supplementation throughout the study period, indicating that β-glucan supplementation effectively enhanced immune responses in this fish species and supports its use in aquaculture.

Oxidative stress analysis

The levels of lipid peroxidation and oxidative protein modification in fish are important indicators for research because the levels of these processes provide insight into the extent of oxidative damage to cell membranes, which may affect cell integrity and function. Therefore, in our study, we analyzed these processes by estimating TBARS levels ($F_{11.82} = 6.69$, $P < 0.001$) in muscle and heart tissues (Fig. 1A). Throughout our study, which consisted of three periods of sampling two types of fish tissues, we observed a statistically significant decrease in TBARS levels only in skeletal muscle tissues in the first period of the experiment (15 days of dietary supplementation) and no changes in the other periods compared to the untreated control group. Thus, the effects of β-glucans in our study were not associated with damage to cell membranes or the initiation of the lipid peroxidation process.

Since it is known that oxidative modifications of proteins can affect cell structure and function and disrupt important biological processes, we measured these markers using aldehyde (OMP AD, $F_{11.82} = 7.64$, $P < 0.001$) and ketone (OMP KD, $F_{11.82} = 7.90$, $P < 0.001$) derivatives of oxidative damage to proteins as important markers of oxidative stress (Fig. 1B, C). In our study, a statistically significant decrease in OMP AD levels in muscle tissue was noted

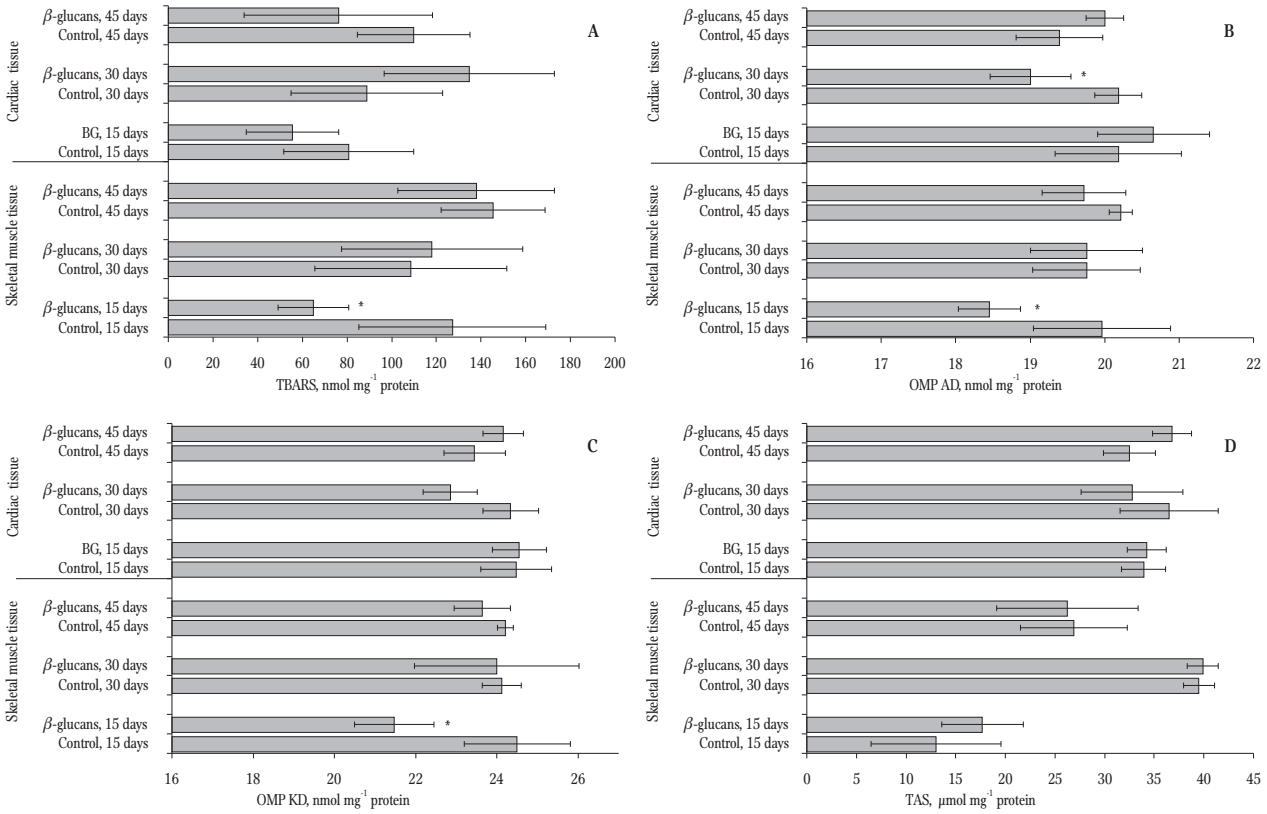

Figure 1. Levels of TBARS (A), aldehydic (B), and ketonic derivatives of oxidatively modified proteins (C) and TAS (D) in skeletal muscle and cardiac tissues of European whitefish fed a diet supplemented with β-glucans for three study periods (15, 30, and 45 days). Results are means ± standard deviation SD (n = 11). *Differences between the unhandled control group and the experimental group were analyzed using three-way ANOVA followed by Bonferroni's post-hoc test. Differences were considered significant at $P < 0.05$.

on day 15 of β-glucan dietary supplementation. Similar effects of OMP AD reduction compared to the values obtained in the control group were noted in cardiac tissue on day 30 of β-glucan dietary supplementation. The level of OMP KD decreased in muscle tissue on day 15 of β-glucan dietary supplementation compared to the data obtained in the control group, but no changes in this parameter were noted in cardiac tissue. The effects of β-glucan dietary supplementation on OMP and lipoperoxidation processes were not accompanied by changes in TAS values (Fig. 1D). The analysis of variance for the TAS data in this study was $F_{11.82} = 53.95$, $P < 0.001$. Thus, the assessment of the overall oxidative status of the fish after β-glucan dietary supplementation revealed the stabilization of cellular structure and function.

We used the sum-of-squares (SS) MANOVA test in our statistical analysis of the full model to assess total variability in the data explained by the model, which captured the variation in lipid peroxidation and oxidative protein modification associated with β-glucan dietary supplementation and its duration. The high SS test result indicated that a large proportion of this variability was explained by the model, suggesting the strong effects of β-glucan dietary supplementation and its duration. The results of this series of statistical analyses are shown below. For TBARS levels, the results of this test were as follows: $R = 0.688$, $R^2 = 0.473$, and $R^2_{adj} = 0.402$. The results for OMP AD ($R = 0.711$, $R^2 = 0.506$, $R^2_{adj} = 0.440$, $P < 0.001$) and OMP KD ($R = 0.717$, $R^2 = 0.514$, $R^2_{adj} = 0.449$, $P < 0.001$) showed higher dependencies in relation to the TBARS data. For the TAS value, the statistical analysis showed the

highest correlation between the data analyzed and the SS test value as follows: R = 0.937, R^2 = 0.877, and R^2_{adj} = 0.862, P < 0.001.

Thus, the improvement of immune function from the application of β-glucans was shown to reduce oxidative stress and effectively manage oxidative damage. Consequently, by boosting the immune system with β-glucans, better overall health and increased resistance to oxidative stress was achieved, which promoted improved growth and higher survival rates of the fish in aquaculture environments.

Lysosomal enzymes

The next part of our study was to investigate four lysosomal enzymes: alanyl aminopeptidase (AAP), leucyl aminopeptidase (LAP), acid phosphatase (AcP), and β-N-acetylglucosaminidase (Fig. 2). Lysosomal enzymes play a crucial role in the body by participating in digestion, recycling cellular components, and defending against pathogens. It is well known that lysosomal enzymes are key components of lysosomes, which are responsible for degrading proteins, lipids, carbohydrates, and other complex macromolecules that are essential for maintaining cellular homeostasis and supporting immune system function (McDonald and Barrett 1986).

Figure 2A shows data for AAP ($F_{11.82}$ = 6.90, P < 0.001), which facilitates protein breakdown by hydrolysing peptide bonds at the N-terminus, specifically removing alanine residues, which are critical for protein digestion, and nutrient assimilation in fish. The results of this study showed that the AAP activity in the muscle tissue was lower on day 15 of β-glucan dietary supplementation, while this activity at the end of the study (after 45 days of β-glucan dietary supplementation) was increased compared to the data obtained in the untreated control group. In the same fish, the AAP activity in cardiac tissue increased significantly compared to the control group at the beginning of the study (day 15 of β-glucan dietary supplementation) (Fig. 2A).

LAP activity ($F_{11.82}$ = 5.21, P = 0.001), which acts as an enzyme removing leucine residues from the N-terminus of proteins, plays an important role in protein turnover, peptide processing, and the regulation of hormonal and signalling molecules in fish tissues (Fig. 2B). LAP activity measured in muscle tissue on day 15 of β-glucan dietary supplementation was statistically elevated compared to the control group. In turn, a decrease in the activity of this enzyme was observed on day 45 of β-glucan dietary supplementation. LAP activity in cardiac tissue was reduced only with 15 days of β-glucan dietary supplementation (Fig. 2B).

AcP is important in the metabolism and immune response of fish. Assessment of AcP activity was the next step in our investigation (Fig. 2C). Measurement of AcP activity can serve as an indicator of fish health, allowing monitoring of their health status and responses to dietary and environmental changes ($F_{11.82}$ = 6.41, P < 0.001). The activity of this enzyme was reduced in muscle tissue on day 30 of β-glucan dietary supplementation and in cardiac tissue on day 15 of β-glucan dietary supplementation. AcP activity may indicate disease states or inappropriate aquaculture conditions. In conclusion, this enzyme plays an important role in fish metabolism and immune response, and its changes after β-glucan dietary supplementation may have a complex effect on fish health and production efficiency in aquaculture.

It is known that the activity of polysaccharide-containing β-glucans may be linked to lysosomal enzymes and that lysosomal enzymes can break down these complex sugars in the body. One of the lysosomal enzymes that may be involved in the degradation of polysaccharides contained in β-glucans is β-N-acetylglucosaminidase (NAG) (Fig. 2D). NAG is responsible for the hydrolysis of N-acetylglucosamine, a component of many polysaccharides that may be involved in the metabolism of β-glucans and their utilization by the body as shown in the statistical analysis ($F_{11.82}$ = 8.44, P = 0.001). There was a statistically significant increase in NAG activity in muscle tissue on days 15 and 30 of β-glucan dietary supplementation and in cardiac tissue on days 15 and 45 of β-glucan dietary supplementation. Therefore, our results highlight the important role NAG plays in the metabolism and

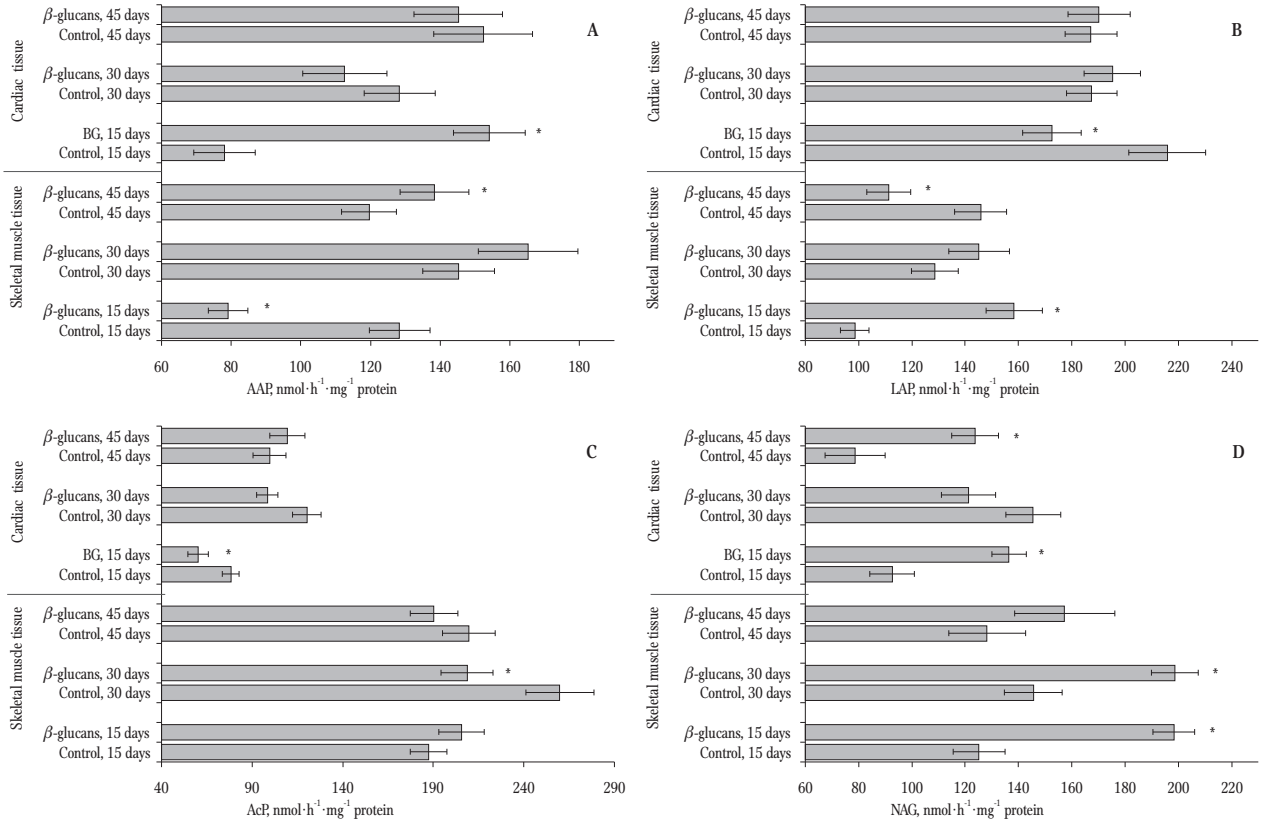

Figure 2. Activities of alanyl aminopeptidase (AAP, A), leucyl aminopeptidase (LAP, B), acid phosphatase (AcP, C), and β-N-acetylglucosaminidase (NAG, D) in skeletal muscle and cardiac tissues of European whitefish fed a diet supplemented with β-glucans for three study periods (15, 30, and 45 days). Results are means ± standard deviation SD (n = 11). *– Differences between the unhandled control group and the experimental group were analyzed with three-way ANOVA followed by Bonferroni's post-hoc test. Differences were considered significant at $P < 0.05$.

utilisation of β-glucans in biological systems, such as fish tissues, when used as a feed additive. The SS MANOVA test in our statistical analysis showed the following dependencies for the lysosomal enzymes analyzed: NAG > AAP > AcP > LAP.

MANOVA analysis

Multivariate significance tests, MANOVA, allowed us to assess significant differences among the groups of fish or the duration of dietary supplementation in statistical analyses that simultaneously included several independent (tissue type predictor, β-glucan supplementation) and dependent (oxidative stress biomarkers and lysosomal enzyme activity) variables. We used sigma-constrained parameterization; this analysis technique takes into account the limitations of statistical models, such as conditional inequalities or other restrictions. Additionally, it may involve the incorporation of specific data structures that require a specialized approach to parameter estimation, aiming to improve the accuracy of the model by taking into account additional conditions or data structures that are not addressed by traditional statistical methods. We used the technique of effective hypothesis decomposition. This refers to techniques that allow for complex statistical hypotheses to be broken down into more fundamental and measurable components. They are often used to reveal elements of a hypothesis that have the greatest impact on study results. Hypothesis decomposition provides a better understanding of how different variables or factors

influence statistical results, potentially leading to more precise conclusions from data analysis. In the statistical analysis, we used four univariate MANOVA tests and obtained statistically significant values between 0.171 and 0.854 (P = 0.008) for the tissue type factor, between 0.191 and 0.840 (P = 0.004) for the experimental conditions factor, and between 0.193 and 4.018 (P < 0.001) for the three stages. Thus, it can be concluded that the long-term effect of β-glucans has a more pronounced influence than the other main factors analyzed.

MANOVA regression analysis of dependent variables

Since β-regression coefficients are indicators of the strength and direction of relationships among independent variables (predictors) and the dependent variable in regression analysis, we also performed this analysis. It is well known that β-regression coefficients are crucial for understanding and analyzing regression results, as they allow inferences to be made about the significance of the predictors and their contribution to explaining variability in the dependent variable. In our study, the statistically significant value of the β coefficient for the tissue type predictor was calculated as β = -0.278 ± 0.092 (t = -3.017, P = 0.003) for lipid peroxidation biomarkers and β = 0.354 ± 0.092 (t = -3.836, P < 0.001) for the duration of β-glucan dietary supplementation. For OMP AD it was β = 0.221 ± 0.100 (t = 2.211, P = 0.029) for the tissue predictor and β = -0.231 ± 0.100 (t = -2.308, P = 0.023) for the effect of β-glucans. The analysis of the dependent variables by MANOVA regression for the OMP KD values was β = -0.334 ± 0.098 (t = -3.427, P < 0.001). For this purpose, the estimation of the TAS value via the MANOVA regression analysis showed the highest dependencies on the dietary supplementation duration predictor, which was β = 0.646 ± 0.080 (t = 8.084, P < 0.001). In our study, relative to lysosomal enzymes, the highest value of the β-coefficient for NAG activity was obtained as a function of tissue type and β-glucan effect at β = 0.432 ± 0.080 (t = 5.034, P < 0.001) and β = 0.342 ± 0.020 (t = 4.021, P = 0.001).

Thus, we observed significant values of β-coefficients indicating associations between the predictors and the dependent variables across the different experimental conditions. Specifically, tissue type was inversely related to lipid peroxidation and positively related to the duration of dietary supplementation, while OMP AD showed a positive association with tissue type and a negative association with the effects of β-glucan dietary supplementation. MANOVA regression analysis showed significant dependencies of OMP KD on predictor variables, and TAS value showed a strong dependency on the duration of β-glucan dietary supplementation. In particular, lysosomal enzymes, especially NAG activity, showed significant associations with tissue type and β-glucan effects. These findings highlight the complex interplay of predictors in influencing the dependent variables in our study.

Discussion

Studies suggest that β-glucans play an important role in reducing oxidative stress in various fish species (Zeng et al. 2018, Song et al. 2020). The efficacy of β-glucans depends on the species, stress conditions, and duration of treatment (Tkachenko et al. 2022). These findings are essential for optimizing the use of β-glucans in aquaculture to improve fish health and resistance to stress. The dual role of β-glucans in improving immune function and reducing oxidative stress is the reason for the continued interest in their application in aquaculture. These properties are essential for improving fish health and productivity, making β-glucans a valuable component of fish diets in modern aquaculture (Vetvicka et al. 2013, Machuca et al. 2022).

Enhancing the immune system of fish with β-glucans remains an important topic for many reasons (Cárdenas-Reyna et al. 2017, Byrne et al. 2020). Firstly, there is an increasing global demand for fish as a source of protein, which requires more efficient

and sustainable farming methods. β-glucans offer a promising avenue by potentially boosting fish immunity, thereby reducing susceptibility to disease and improving overall health. Therefore, enhancing the immune system of fish with β-glucans is crucial for improving fish health and production efficiency in aquaculture (Rodrigues et al. 2020, Porter et al. 2022). Our study focused on the analysis of phagocytic activity and phagocytic index in European whitefish fed a diet supplemented with β-glucans for 15, 30, and 45 days. The results showed a significant increase in phagocytic activity in the group fed β-glucans compared to the untreated control group. In addition, the phagocytic index was significantly higher in the group that received β-glucan dietary supplementation throughout the study period. These results suggest that β-glucan supplementation effectively enhances immune responses in European whitefish. The improvement of fish immunity from β-glucans may contribute to the reduction of antibiotic use in aquaculture, which is in line with public health concerns about antibiotic resistance and the need for sustainable development practices in fish farming (Meena et al. 2020).

β-glucans activate immune cell surface receptors, triggering the release of cytokines and chemokines (Zhong et al. 2023). These signalling proteins stimulate monocytes, macrophages, and neutrophils in fish, promoting pathogen clearance through phagocytosis, oxidative burst, and cytotoxic activity (Hadiuzzaman et al. 2022). In addition, β-glucans enhance immunological memory and specific antibody production by activating T and B lymphocytes (Ali et al. 2015). Studies show that β-glucans improve key biochemical (e.g., serum hemoglobin and protein levels) and immunological properties (e.g., lysozyme and phagocytic activity), leading to a stronger immune profile in fish and aquatic organisms (Hadiuzzaman et al. 2022). It reduces susceptibility to acute inflammation and enhances disease resistance by balancing beneficial and harmful immune responses (Murphy et al. 2020).

The mechanisms by which β-glucans suppress pro-inflammatory cytokines and promote anti-inflammatory cytokine production are complex and not fully understood. Non-cereal β-glucans have been shown to inhibit lipopolysaccharide (LPS)-induced nitric oxide and tumor necrosis factor-alpha (TNF-α) release *in vitro* and to reduce TNF-α and interleukin-6 (IL-6) secretion in LPS-challenged mice (Jedinak et al. 2011, Xu et al. 2012). The interaction between β-glucans and their primary receptor, dectin-1, involves additional cooperation with Toll-like receptors (TLRs) to facilitate the release of inflammatory cytokines (Brown et al. 2003). Yeast-derived β-glucans have been observed to induce robust expression of the immunomodulatory cytokine interleukin-1 receptor antagonist (IL-1Ra), a process that is independent of the conventional β-glucan receptors dectin-1 and CR3 (Smeekens et al. 2015).

Our results also highlight the important role of lysosomal enzymes in the metabolic changes induced in biological systems such as fish tissues during β-glucan dietary supplementation. Lysosomal enzymes play crucial roles in the body as they are involved in digestion, recycling cellular components, and defense against pathogens. They are the major components of lysosomes, which are responsible for degrading proteins, lipids, carbohydrates, and other complex macromolecules that are essential for maintaining cellular homeostasis and supporting immune system function (Li et al. 2024). β-N-acetylglucosaminidase (NAG) plays a critical role in the cleavage of N-acetylglucosamine residues (Chatham et al. 2021). These are constituents of many polysaccharides, including β-glucans. This enzymatic activity is critical in facilitating the breakdown of β-glucans into smaller oligosaccharides and monosaccharides that can be digested and utilized by the body. By breaking down glycosidic linkages within β-glucans, NAG facilitates the release of glucose units and other sugars that serve as energy sources and building blocks for various metabolic processes in cells. This process not only promotes nutrient absorption but also supports immune modulation and overall health by increasing the bioavailability of the beneficial effects of β-glucans on the immune system and intestinal health in fish

fed diets supplemented with β-glucans. Thus, our results highlight the important role of NAG in the metabolism of fish fed β-glucans.

By participating in the hydrolysis of phosphate esters at acidic pH levels, the enzyme AcP aids in the digestion of nutrients, including polysaccharides, such as β-glucans, releasing the products of hydrolysis such as glucose and other sugars (Anand and Srivastava 2012) that fish can more easily absorb. In addition, β-glucans are known to stimulate the immune system of fish thanks to their immunomodulatory functions, and AcP may help break down β-glucans into smaller fragments that are more bioavailable and can interact more effectively with immune cells, thereby enhancing immune responses and inducing changes in oxidative stress. This is confirmed by our correlation analysis regarding the statistical dependence between NAG and TBARS ($r = 0.587$, $P < 0.001$) in muscle tissue on day 30 after β-glucan dietary supplementation and NAG and TAS in cardiac tissue on day 15 after β-glucan dietary supplementation ($r = 0.654$, $P < 0.001$).

Lysosomes play crucial roles in the cellular immune response, acting as the primary intracellular site for the degradation of pathogens, cellular waste, and other macromolecules (Trivedi et al. 2020). The enhancement of lysosomal activity by β-glucans is often attributed to their ability to stimulate immune cells, particularly macrophages, through the recognition of β-glucan receptors such as dectin-1 and complement receptors (Zhong et al. 2023). When β-glucans bind to these receptors on immune cells, they activate signalling pathways that lead to the production of ROS, pro-inflammatory cytokines, and other immune responses. This activation of immune cells enhances a fish's ability to respond to infection and oxidative stress by promoting phagocytosis and improving the clearance of damaged cells and pathogens (Kim et al. 2011). Lysosomal activity is critical in muscle metabolism for maintaining muscle health and function. Lysosomal enzymes are involved in the turnover of cellular components, including the breakdown of proteins and lipids (Bonam et al. 2019). By enhancing lysosomal function, β-glucans may improve the efficiency of muscle tissue regeneration and repair, which is particularly important during stressful events such as handling, infection, or changes in environmental conditions (Brogi et al. 2021).

Fish are increasingly exposed to different types of stressors in aquaculture, underlining the urgency of boosting their natural defenses. Therefore, β-glucans could play a crucial role in strengthening fish against these challenges, ensuring more resilient aquaculture systems. Importantly, the use of β-glucans in fish farming holds great promise for improving fish health, increasing the efficiency of aquaculture production, and addressing global food security challenges (Byrne et al. 2020). Recent research highlights the benefits of β-glucans (Engstad et al. 1992, Dalmo and Bøgwald 2008, Meena et al. 2013). This confirms the results of our statistical analysis performed using four univariate MANOVA tests, which revealed that β-glucans have a more pronounced and consistent influence than the other factors studied, including tissue type and the duration of dietary supplementation. In the context of the variables analyzed, these results suggest that β-glucans may have significant long-term effects.

Research on oxidative stress and β-glucans, particularly in rainbow trout (*Oncorhynchus mykiss* Walbaum), has shown promising results, as highlighted by Leal et al. (2019). Their aim was to investigate the effects of dietary β-glucans on the early immune response against viral hemorrhagic septicemia virus (VHSV) in rainbow trout. Results from other studies showed a significant reduction in markers of oxidative stress and an increase in antioxidant enzyme activities (Dietrich-Muszalska et al. 2011, Tkachenko et al. 2022, Kurhaluk et al. 2024). These studies highlighted the potential of β-glucans to mitigate oxidative stress in aquatic organisms, suggesting their role in enhancing antioxidant defenses and overall health in fish species, such as rainbow trout. The results are consistent with our study, which showed that improved immune function from β-glucan dietary supplementation reduced oxidative stress (TBARS, OMP AD, OMP KD, and TAS) and effectively managed oxidative damage. The immune system enhancement from β-glucans in our study may contribute to better overall health and improved

resilience to oxidative stress, thus promoting improved growth and survival rates in aquaculture environments. This was confirmed by our correlation analysis regarding the statistical dependencies between TBARS and OMP KD levels ($r = 0.643$, $P < 0.001$) in muscle tissue on day 30 after β-glucan dietary supplementation, TBARS and OMP AD levels ($r = 0.704$, $P < 0.001$) in cardiac tissue on day 30 after β-glucan dietary supplementation, and TBARS and TAS ($r = 0.721$, $P < 0.001$) in cardiac tissue on day 15 after β-glucan dietary supplementation.

In some studies (Dawood et al. 2020, Waikhom et al. 2024) focusing on Nile tilapia (*Oreochromis niloticus* L.), the aim was to determine the optimal duration of β-glucan supplementation to alleviate toxicological effects caused by different stressors. The researchers fed β-glucans to Nile tilapia over different periods, focusing on the effective reduction of oxidative stress markers, increased antioxidant enzyme activities, and immune responses. This research suggests that the duration of β-glucan supplementation plays a critical role in achieving significant antioxidant benefits, providing valuable insights for optimizing fish health management strategies and sustainable aquaculture practices.

Discussions in the literature of data regarding the effects of β-glucans, which play a pivotal role in enhancing immune defenses of fish through multiple mechanisms, indicate that β-glucans are valuable tools in promoting fish health and increasing aquaculture productivity. Some studies provide important data on how β-glucans activate different components of the fish immune system, namely the activation of phagocytes (Petit et al. 2019, Braian et al. 2023). Phagocytes, such as macrophages and neutrophils, are known to be responsible for engulfing and destroying pathogens, and β-glucans bind to receptors on the surface of these cells, stimulating their phagocytic activity. β-glucan supplementation increased phagocytic activity and the number of phagocytes (Zhang et al. 2022).

The next point concerns the stimulation of cytokine production, as cytokines are proteins that play a crucial role in communication among immune system cells. Research conducted by Jørgensen et al. (2000) suggested that β-glucans stimulate the production of pro-inflammatory cytokines, such as interleukin-1 (IL-1) and interleukin-6 (IL-6), which in turn activate other immune cells, and they showed that β-glucans increased the expression of cytokine genes in Atlantic salmon (*Salmo salar* L.), leading to improved immune responses. Another aspect of the effects related to the enhancement of antibody production is whether β-glucans can enhance the production of antibodies as proteins responsible for neutralizing pathogens. Indeed, research data showed that fish fed diets supplemented with β-glucans had higher antibody levels, which increased their resistance to infection (Kumari and Sahoo 2006). Importantly, a study conducted by Jung-Schroers et al. (2018) showed that β-glucan supplementation improved the protective properties of carp (*Cyprinus carpio* L.) mucus against a bacterial challenge from *Aeromonas hydrophila* intubation and that these effects were shown to strengthen the mucosal barrier, which in fish serves as the first line of defense against pathogens, and that β-glucans may be involved.

The present study provides practical recommendations for optimizing β-glucan supplementation in aquaculture and highlights its role as a cost-effective strategy to improve fish health and productivity. Effective doses range from 0.5 to 1% of the feed that is administered via consistent delivery methods such as feed pellets. Short-term supplementation (15–45 days) is beneficial during high-risk stress periods, while periodic long-term cycles (e.g., 15 days every two months) can provide sustained benefits. Juveniles, being more responsive, will benefit greatly during early growth phases, while mature fish may use β-glucans for recovery after spawning or prolonged stress. In addition, combining β-glucans with other functional additives such as probiotics or omega-3 fatty acids can enhance health benefits.

In commercial aquaculture, β-glucan supplementation has the potential to improve fish health, increase growth rates, improve feed efficiency, and reduce disease management costs. These health benefits translate directly into economic benefits, including increased profitability, reduced environmental impact, and improved farm sustainability.

By incorporating β-glucans into aquaculture feeding strategies, fish farmers can optimize production efficiency while improving fish welfare, making this supplement a valuable tool in the future of sustainable aquaculture.

This study provides valuable insights into the role of β-glucans in alleviating oxidative stress and promoting muscle tissue health in European whitefish. However, several limitations must be considered when interpreting the results and assessing their generalizability. Firstly, the research focused exclusively on European whitefish and its findings may not be directly applicable to other fish species. Inter-species differences in physiology, metabolism, and immune response could significantly influence the efficacy of β-glucan supplementation. Secondly, the study was conducted under controlled laboratory conditions, which may not fully replicate the complex environmental stressors found in commercial aquaculture systems. Validating these findings through field studies in diverse aquaculture environments is therefore required. In addition, while the study examined the effects of β-glucan supplementation over specific intervals (15, 30, and 45 days), the optimal dosage and duration for long-term application in aquaculture remains to be determined. Furthermore, although several biomarkers of oxidative stress and immune function were analyzed, other relevant indicators such as cytokine levels and specific immune cell populations were not assessed. Finally, while this study effectively elucidated the short-term and intermediate effects of β-glucan supplementation, its long-term effects on fish health, growth, reproduction, and resilience to chronic stressors remain unexplored. Addressing these gaps in future research will be essential to fully understand the potential benefits of β-glucans in aquaculture.

Conclusions

The analysis of phagocytic activity and phagocytic index in the blood of European whitefish fed diets supplemented with β-glucans for 15, 30, and 45 days suggests that β-glucans may boost the immune system of fish to improve their immunity. The improvement of immune function induced by β-glucans has been shown to reduce the levels of biomarkers of oxidative stress measured as lipid peroxidation and protein damage (TBARS, aldehyde, and ketone derivatives of OMP) and to effectively manage oxidative damage estimated by total antioxidant status. We demonstrated a statistically significant increase in β-N-acetylglucosaminidase activity in muscle tissue on days 15 and 30 after β-glucan dietary supplementation and in cardiac tissue on days 15 and 45 after β-glucan dietary supplementation. Our results highlight the important role of NAG in the metabolism of different muscle tissues in fish fed β-glucans. Furthermore, as shown by regression analysis, NAG activity showed significant associations with tissue type and the duration of β-glucan dietary supplementation. The long-term effect of β-glucans was confirmed as a more pronounced influence by MANOVA analysis.

Future research should focus on elucidating the specific molecular mechanisms by which β-glucans modulate immune responses and oxidative stress pathways. Investigating the potential synergistic effects of β-glucans with other immune-enhancing or growth-promoting compounds could lead to more refined supplementation strategies. In addition, broader studies across different fish species, aquaculture systems, and environmental conditions are needed to confirm the applicability and scalability of β-glucan supplementation in different commercial settings. Long-term effects on growth, reproduction and resistance to chronic stressors should also be investigated to fully assess the economic and environmental viability of β-glucans as a sustainable aquaculture solution.

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