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Effects of a β-glucan-enriched diet on biomarkers of oxidative stress, energy metabolism and lysosomal function in muscle tissue of European grayling (*Thymallus thymallus* L.)

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Abstract

Introduction: The grayling (*Thymallus thymallus* L.) has several advantages over other fish species that make it attractive for aquaculture and invest it with importance for food security. The study assessed the effects of a β-glucan-enriched diet on biomarkers of oxidative stress, energy metabolism and lysosomal function in muscle tissue of European grayling (*Thymallus thymallus* L.). **Material and Methods:** Sixty-six grayling weighing approximately 34 g were divided into equal control and experimental groups. A basal diet was fed to the control group and a β-glucan-enriched one was fed to the experimental group for 45 d. Lipid peroxidation (LP) level; oxidative protein modification (OPM); total antioxidant status (TAS); and superoxide dismutase (SOD), catalase (CAT), glutathione reductase, glutathione peroxidase (GPx), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate (LDH), succinate dehydrogenase (SDH), alanyl aminopeptidase, leucyl aminopeptidase, acid phosphatase (AcP) and β-N-acetylglucosaminidase (NAG) activities were assessed in the muscle tissue of fish euthanised after 15, 30 and 45 d of feeding. **Results:** The β-glucan supplementation reduced LP, attenuated OPM and improved the TAS in muscle tissue. Increased SOD and CAT activity and maintenance of GPx activity in muscle tissue were the main mediators of these effects. They also affected energy metabolism through modulation of key enzymes and metabolites, including ALT, AST, LDH, SDH, AcP and NAG activity, and altered lactate and pyruvate levels. Multivariate analysis of variance, supported by high F-values and low P-values indicating statistical significance, highlighted the significant effect of β-glucans and feeding duration on markers of oxidative stress, antioxidant defences and TAS. **Conclusion:** B-glucans altered the balance between aerobic and anaerobic metabolism, reduced OPM and modulated the transaminase response, affecting amino acid metabolism and the production of Krebs cycle intermediates.

Keywords: anaerobic and aerobic metabolism, antioxidant status, muscle, oxidative protein modification, lysosomal enzymes.

Introduction

Aquaculture is central to today's economy, contributing significantly to food security, economic growth, and environmental sustainability worldwide. The grayling (*Thymallus thymallus* L.) has several advantages over other species that make it attractive for aquaculture (10). As a freshwater fish, the grayling thrives in a wide range of environmental conditions and is resilient to fluctuations in water temperature, hardness and pH, which enhances its adaptability in aquaculture environments. Grayling exhibit relatively fast growth rates in optimal culture conditions, contributing to shorter production cycles and increased efficiency (16). However, there is relatively little research on the biochemical and physiological parameters of grayling. Therefore, these studies were undertaken to contribute to the understanding of this species and its potential for aquaculture.

B-glucans have been shown to play a critical role in mitigating stress responses in aquacultured fish (29). They can spare fish some of the negative effects of stressors such as handling, transport and environmental fluctuations. By promoting stress resilience, β-glucans

help maintain fish health and performance, ensuring stable production even in challenging conditions (26). There are promising opportunities to improve aquaculture practices through the use of β-glucans (12). Known for their immunostimulatory properties, β-glucans can enhance the immune response of fish (34). By activating immune cells and increasing the production of immune factors, β-glucans help fish resist pathogens more effectively. This can lead to reduced disease incidence and improved overall health of fish stocks in aquaculture systems. In addition, β-glucans have been shown to enhance growth performance in several fish species by improving nutrient absorption and utilisation (19, 24). Incorporating β-glucans into aquaculture therefore represents a holistic approach to improving immune function, growth performance, feed efficiency and stress management, promoting sustainable practices and increasing the profitability of aquaculture operations.

Many studies analysed oxidative stress parameters and antioxidant defence mechanisms in response to environmental stressors, such as water pollution, temperature changes or oxygen deprivation, and showed these stressors' impact on fish farming and aquaculture production (11, 31). Because of the important impact of oxidative stress on the health and performance of farmed organisms, surveillance of it is crucial to efficiency gains in aquaculture. Parameters for free radical levels provide valuable information on the efficiency of nutrition, the quality of the culture environment, and the effectiveness of the health management strategies used, which is crucial for optimising culture conditions and reducing losses in aquaculture (15, 31).

Investigating the effects of β-glucans on the function of lysosomal enzymes in farmed fish species such as grayling may have important implications for fish health and overall condition. As dietary components, β-glucans can affect the activity of lysosomal enzymes, *e.g.* proteases and glycosidases, which play key roles in digestion, metabolism and immune defence in fish (5, 7). Research on the effects of β-glucans on lysosomal enzyme function in farmed fish species in different conditions contributes importantly because of its potential to optimise fish nutrition, improve health and immunity and support the sustainability of aquaculture (7).

Muscle tissue is the main site of both aerobic and anaerobic energy metabolism, and its condition is an important indicator of fish health and performance. Therefore, research into the effects of β-glucans on its structure and function can provide important information on the overall health of farmed fish. When grayling muscle tissue comes under research, it benefits the fish farming industry, as it helps to assess the quality of fish meat, its nutritional composition, and organoleptic properties, which can be modified by a diet containing β-glucans. This research is therefore essential for full understanding of the effects of β-glucans on the health, performance and meat quality of farmed fish species and for optimisation of farming practices and feeding strategies.

The aim of this study was to investigate in detail the effects of β-glucans on the levels of oxidative stress markers such as lipid peroxidation and oxidative protein modification on energy metabolism (involving both anaerobic and aerobic mechanisms) and on lysosomal function in grayling muscle tissue. Multivariate analysis of variance (MANOVA) was used to simultaneously assess the effects of β-glucans on these different aspects, with the aim of better understanding the biological mechanisms regulated by β-glucans in grayling muscle tissue.

Material and Methods

Fish and experimental design. Sixty-six healthy grayling weighing 33.8 ± 1.7 g at the start of the experiment were used in the research. The fish were kept in an indoor system with a fresh water supply, adequate aeration and an internal power filter. The water quality parameters were as follows: temperature 16 ± 2°C, dissolved oxygen 12 ± 0.5 ppm and pH 7.4–7.6. During the acclimation period of 14 d, the fish were fed a commercial basal diet at a rate of 1.5% of body weight four times a day. After acclimatisation, the fish were randomly divided into two groups and housed in aerated square 250 L tanks containing dechlorinated tap water (70 fish per tank). One tank contained one group. Natural photoperiod conditions were maintained throughout the feeding trial. The experimental part of the study was carried out at the Department of Salmonid Research of the Stanisław Sakowicz Institute of Inland Fisheries, Rutki, Poland.

The groups were fed for 15, 30, and 45 d as follows: the control group (n = 33) received a control basal diet and the β-glucan group (n = 33) received the basal diet supplemented with Yestimun powder at a dose of 1% of the basal diet, this being a supplement containing 85% β-1,3/1,6 glucans (Leiber, Bramsche, Germany). This insoluble and highly purified preparation derives its natural polysaccharides from brewer's yeast (*Saccharomyces cerevisiae*), where cell walls typically contain about 30% of dry weight β-glucans. At the end of the 15-, 30- and 45-day feeding periods, 22 fish each time were sacrificed by decapitation and muscle tissue was taken from the sides near the centre of the back. Because fish development occurs over a relatively long time and relates to changes in metabolism, 22 untreated control group fish were also sacrificed and sampled at each period. The experiments were performed in duplicate.

Preparation of tissue homogenates. Blood was removed from the muscles using a cold isolation buffer. Muscle tissue samples were homogenised in ice-cold buffer (100 mM tris-HCl, pH 7.2) in a Waverly H500 homogeniser with a motor-driven pestle (SoCal BioMed, Waverly, IA, USA). Homogenates were

centrifuged at 3,000 × *g* for 15 min at 4°C. After centrifugation, the supernatant was collected and frozen at −22°C for further analysis. Protein content was determined with the Bradford method (2) using bovine serum albumin as a standard. Absorbance was recorded at 595 nm. Enzymatic reactions were initiated by the addition of the tissue supernatant.

Lipid peroxidation level. Peroxidation of lipids in the homogenate was determined as 2-thiobarbituric acid reactive species (TBARS) according to the method proposed by Buege and Aust (4) with some modifications. Briefly, the homogenate was mixed with a 2-thiobarbituric acid-trichloroacetic acid reagent, shaken thoroughly and heated to 85°C for 20 min. The samples were then allowed to cool to room temperature. After centrifugation at 1,200 × *g* for 10 min at room temperature, the absorbance of the pink chromogen present in the clear supernatant was measured at 532 nm using a spectrophotometer. Tetraethoxypropane was used as a standard. The values were expressed as nmol TBARS per mg protein.

Determination of carbonyl groups in oxidatively modified proteins (OMP*).* Oxidation of proteins can result in additional aldehyde and ketone groups of amino acid residues that can interact with 2,4-dinitrophenylhydrazine (2,4-DNPH). It is known that the maximum absorption for neutral aliphatic aldehyde dinitrophenyl hydrazones is in a range from 260 to 558 nm, and for alkaline aldehyde hydrazones it is in a range from 258 to 520 nm. For neutral aliphatic ketone dinitrophenyl hydrazones, the maximum absorption is in a 363–367 nm range, and for alkaline ketone hydrazones it is between 430 and 535 nm (28). Neutral aliphatic aldehyde dinitrophenylhydrazones (OMP ADn) were analysed at 356 nm, neutral aliphatic ketone dinitrophenylhydrazones (OMP KDn) at 370 nm, alkaline aliphatic aldehyde dinitrophenylhydrazones (OMP ADa) at 430 nm, and alkaline aliphatic ketone dinitrophenylhydrazones at 530 nm (20, 28).

Superoxide dismutase activity assay. The Randox kit method (RANSOD, Cat. No. SD 125; Randox Laboratories, Crumlin, UK) was used to measure superoxide dismutase (SOD) activity, following the protocols developed by Woolliams *et al.* (37) and Suttle and McMurray (32). This method is based on the principle that xanthine and xanthine oxidase generate superoxide radicals which then react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The results were calculated and expressed as units per gram of protein.

Catalase activity assay. Catalase (CAT) activity was measured using the method described by Koroliuk *et al.* (17), which involves monitoring the reduction in the concentration of hydrogen peroxide (H_2O_2) in the reaction mixture. One unit of catalase activity is defined as the amount of enzyme required to degrade 1 μmol of H_2O_2 per minute per milligram of protein.

Glutathione reductase activity assay. The Glut Red Assay Kit (RX Monza, GR 2368; Randox Laboratories) was used to determine the activity of glutathione reductase (GR) in the fish muscle tissue. This method is based on the principles outlined by Goldberg and Spooner (9) and Melissinos *et al.* (21). The assay measures reduced glutathione colorimetrically at 340 nm using the RX Monza analyser. The initial absorbance values were expressed in units per milligram of protein.

Glutathione peroxidase activity assay. Glutathione peroxidase (GPx) activity in the tissue samples was assessed using the Ransel Glutathione Peroxidase Assay Kit (RX Monza, RS 504; Randox Laboratories). This assay involves the enzyme-catalysed oxidation of glutathione by cumene hydroperoxide. The procedures followed those described by Paglia and Valentine (25) and Kraus and Ganther (18) with our modifications. The decrease in absorbance at 340 nm was monitored using an RX Monza analyser with a 1 cm cuvette light path at 37°C. The results were expressed in units per milligram of protein.

Total antioxidant status assay. For these studies, total antioxidant status (TAS) was determined using the Randox kit (Cat. No. NX 2332; Randox Laboratories). A 2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) assay was used for TAS measurement. This method, based on the absorbance of the $ABTS^+$ radical cation, follows the procedure proposed by Miller *et al.* (22). The assay involves incubation of ABTS with peroxidase (metmyoglobin) and hydrogen peroxide (H_2O_2) to generate the $ABTS^+$ radical cation. The results were calculated and expressed as micromoles per milligram of protein.

Alanine aminotransferase and aspartate aminotransferase activity assays. The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined spectrophotometrically using a standard enzymatic method described by Reitman and Frankel (27). The substrates in the reaction were both used at pH 7.4 and were α-ketoglutarate (2 mM) plus L-aspartate (200 mM) for AST and α-ketoglutarate (2 mM) plus L-alanine (200 mM) for ALT. The levels of the keto-groups were determined using 2,4-DNPH. The intensity of the colour was related to the enzymatic activity of the respective enzymes. A blank sample was prepared in the same way as the experimental sample but with the addition of distilled water. For the measurement of AST and ALT activities, pyruvate sodium formation was used as a standard to calibrate the graph. The formation of one micromole of pyruvate per hour at 37°C incubation per milligram of protein was defined as one unit of AST or ALT activity.

Lactate dehydrogenase activity assay. The colourimetric method described by Sevela and Tovarek (30) was used for the determination of lactate dehydrogenase (LDH) activity. The principle of the method is based on the reduction of pyruvate to lactate

catalysed by LDH in the presence of nicotinamide adenine dinucleotide (NAD^+). In the calculation of LDH activity, pyruvate was used as a standard to construct the calibration curve. One unit of LDH activity was defined as the formation of one micromole of pyruvate per hour per milligram of tissue protein when incubated at 37°C.

Succinate dehydrogenase activity. The activity of succinate dehydrogenase (SDH) was determined spectrophotometrically according to the method proposed by Eschenko and Volski (8). One unit of SDH activity was defined as the amount of enzyme required to degrade one nanomole of succinic acid per minute per milligram of tissue protein.

Lactate and pyruvate levels. The amount of lactate was determined by reaction with hydroquinone, while the amount of pyruvate was determined with dimethylaminobenzaldehyde (13), both amounts being estimated by measurement of the absorbance of the samples at 420 nm and 430 nm. The calibration curves for lactate (0–5 mM) and pyruvate (0.1–5 mM) were used. The results were expressed as nanomoles per milligram of tissue protein.

Lysosomal enzyme assay. Homogenates (20%, w/v) were prepared by differential centrifugation. The activities of alanyl aminopeptidase and leucyl aminopeptidase were measured spectrophotometrically according to DeMartino and Goldberg (6). The reaction was initiated by incubating 50 µL of the sample with 500 µL of substrate incubation medium containing dimethylformamide (Serva, Heidelberg, Germany) for 60 min at 37°C and pH 6.0. After incubation, 500 µL of buffer containing Fast Blue BB salt in 2% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) was added. The absorbance was then measured at 540 nm. L-alanyl-2-naphthylamine in 0.1 M phosphate-buffered saline (PBS) buffer was used as a substrate for alanyl aminopeptidase, and L-leucyl-2-naphthylamine in 0.1 M PBS (pH 7.0) was used to measure leucyl aminopeptidase activity. Acid phosphatase (AcP) and β-N-acetylglucosaminidase (NAG) activities were determined spectrophotometrically at 420 nm using 4-nitrophenyl derivatives as substrates according to the method proposed by Barrett and Heath (1). Enzyme activities were expressed as nanomoles per hour per milligram of protein.

Statistical analysis. The Statistica 13.3 package (TIBCO, Palo Alto, CA, USA) was used for basic statistics (the significance of regression slopes, analysis of variance for significance between groups and a distribution test). The data were tested for homogeneity of variance using Levene's test for equality of error variances. Normality was tested using the Kolmogorov–Smirnov test. The results are expressed as mean ± standard deviation. Significant differences between means were determined using a multiple range test at minimum P-value < 0.05. Non-normally distributed data were logarithmically transformed. Student's *t*-tests with 95% confidence intervals ($\alpha = 0.05$) were used to determine the significance of differences between study types and between groups. Pearson's regression analysis using the multiple regression module was used to correlate parametric values. The correlation and regression analysis included the correlation coefficient (r), regression equation and the significance of these relationships (P-value). Arithmetic mean concentrations of biomarkers and enzyme activity in muscle tissue were estimated using two-way ANOVA. Statistically significant relationships were obtained for all values using multivariate significance tests of main effects (the study type and β-glucan effect). In the model approach, a two-way classification model for the combination of the effects of two factors was used. To describe the full model, the coefficients of the multiple correlation analysis (R) and of determination (R^2) and the corrected form of the latter reduced by random errors in data analysis (R^2_{adj}) were used. The sum of squares (SS) test was used to describe the proportions of all analysed biomarkers of oxidative stress and biochemical parameters with the F test and their significance.

Results

Oxidative stress biomarkers. The study of the level of lipid peroxidation processes in the grayling muscle tissue carried out over three study periods showed that the application of dietary β-glucans resulted in a significant reduction in the level of TBARS, both at the beginning (on day 15 of the study) and at the end of the study (on day 45), compared with the data obtained in the untreated control group. The analysis of variance value in the statistical analysis for TBARS was $F_{5.60} = 12.96$, P-value = 0.000. These changes are shown in Fig. 1A.

Feeding the fish with β-glucans was associated with a reduction in the level of LP processes during two periods of the study (at 15 and 45 d). The results of measurement of the second important biomarker of oxidative stress, TAS, which provide a general background of the antioxidant capacity of this biological system, are shown in Fig. 1B. A statistically significant increase in the value of TAS ($F_{5.60} = 63.45$, P-value = 0.000) was observed in the grayling muscles only in the third period of the study (45 d), compared to the untreated control group. In summary, a decrease in LP and an increase in the TAS of muscle tissue were observed during the longer feeding periods.

Oxidative modification of proteins can affect protein structure and function and is integral to several physiological and pathological processes. The effects of β-glucans on the oxidative modification of proteins in grayling muscle (Fig. 2) were studied with statistical dependencies using analysis of variance ($F_{5.60} = 35.61–57.18$, P-value = 0.000). The levels of OMP KDn and OMP ADa were reduced in the muscle tissue after 15 d of treatment with β-glucans compared to the untreated control group.

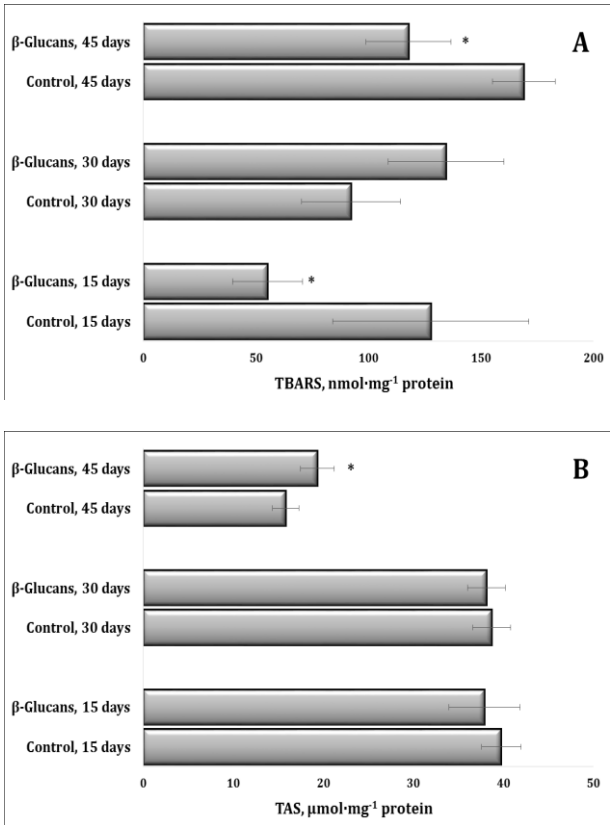

Fig. 1. Levels of thiobarbituric acid reactive species (TBARS) (A) and total antioxidant status (TAS) (B) in the muscle tissue of grayling fed a diet supplemented with β-glucans or fed a basal diet over three study periods (15, 30, and 45 days of feeding). Results are expressed as mean ± standard deviation (n = 11). * – significant difference between the untreated control group and the experimental group (P-value < 0.05)

Antioxidant enzymes. In the analysis of the evidence of enzymatic antioxidant defence in the grayling muscles, the results of which are given in Table 1, the activity of the enzymes of the first and second levels of this defence was evaluated. Statistically significant changes were noted mainly for SOD, considered to be an enzyme of the first level of antioxidant defence ($F_{5.60}$ = 53.43, P-value = 0.000), and for CAT, associated with the second level ($F_{5.60}$ = 6.85, P-value = 0.000). With regard to SOD, the β-glucan treatment significantly reduced its activity in all three study periods (15, 30 and 45 d of feeding) compared to the values obtained in the untreated control group. However, with regard to CAT, its activity changed ambiguously, *i.e.* in the first study period, it increased significantly on day 15, whereas it was significantly decreased in the following periods, compared to the values obtained in the untreated control group. While there was no change in the activity of GR, which is involved in the metabolism of glutathione as one of the major tissue antioxidants ($F_{5.60}$ = 4.70, P-value = 0.001), the activity of GPx was twice as high on day 30 of feeding as it was in the untreated control group ($F_{5.60}$ = 24.37, P-value = 0.000). The effects of β-glucans were mainly observed to be achieved through the distribution of SOD/CAT activity. However, these effects are maintained by GPx in muscle tissue.

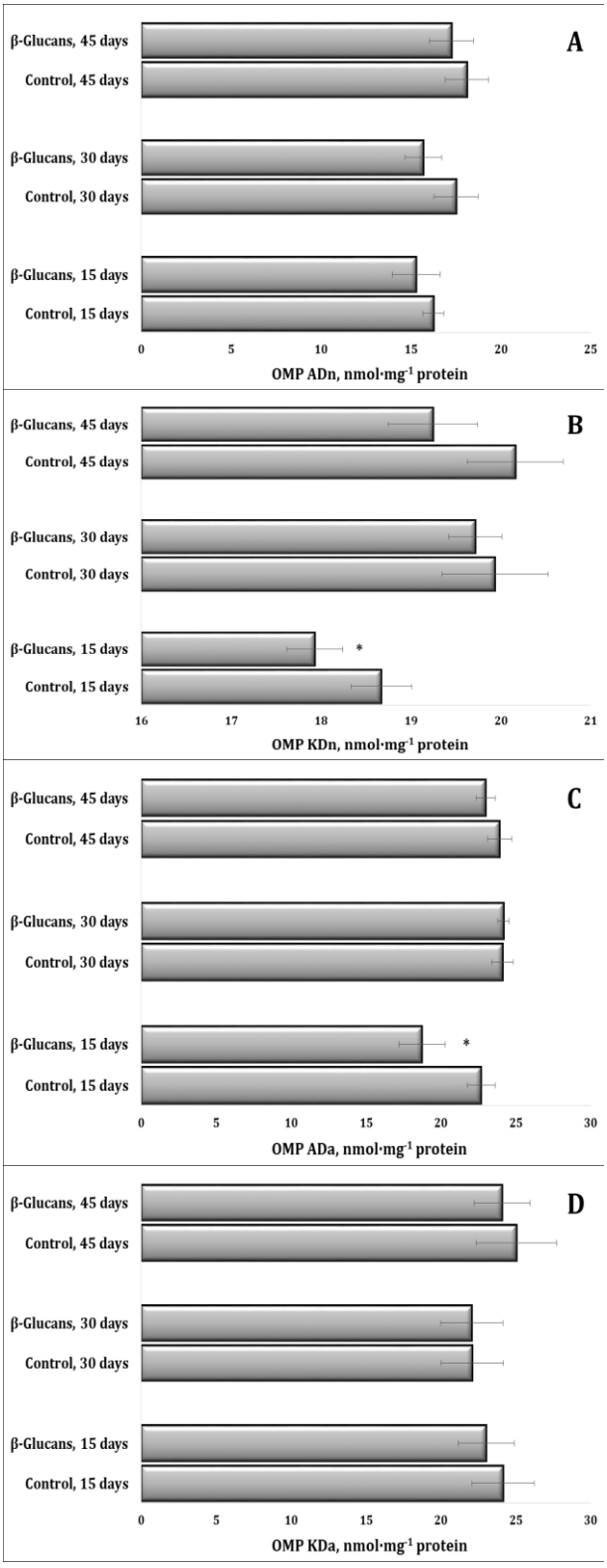

Fig. 2. Levels of neutral aliphatic aldehyde dinitrophenyl hydrazones (OMP ADn, A), neutral aliphatic ketone dinitrophenyl hydrazones (OMP KDn, B), alkaline aliphatic aldehyde dinitrophenyl hydrazones (oxidatively modified protein (OMP) ADa, C), and alkaline aliphatic ketone dinitrophenyl hydrazones (OMP KDa, D) in the muscle tissue of grayling fed a diet supplemented with β-glucans or a basal diet over three study periods (15, 30, and 45 days of feeding). Results are expressed as mean ± standard deviation (n = 11). * – significant differences between the untreated control group and the experimental group (P-value < 0.05)

Markers of energy metabolism. The activities of ALT ($F_{5.60}$ = 134.91, P-value = 0.000) and AST ($F_{5.60}$ = 57.61, P-value = 0.000), key enzymes in amino acid metabolism, and Krebs cycle ketoacids involved in transamination were examined. The results of this series of evaluations of the products of energy-related metabolism in the muscle tissue of grayling fed β-glucans are presented in Table 2. The elevated lactate and pyruvate levels indicated an intensification of anaerobic metabolism. Facilitation of this intensification is implied by the higher activity level of LDH ($F_{5.60}$ = 34.33, P-value = 0.000) in the β-glucan-fed group at the earliest study period, because it catalyses the conversion of pyruvate to lactate. The levels of both of these were statistically significantly increased in the supplemented group after 15 d feeding ($F_{5.60}$ = 43.36, P-value = 0.000 and $F_{5.60}$ = 27.23, P-value = 0.000, respectively).

Table 1. Antioxidant enzyme activity in muscle tissue of grayling fed a diet supplemented with β-glucans or fed a basal diet over three study periods (15, 30, and 45 days of feeding)

Enzyme	Group	Enzyme activity (U mg^{-1} protein/μmol min^{-1} protein (catalase only))		
		15 days' feeding	30 days' feeding	45 days' feeding
Superoxide dismutase	Control	693.32 ± 91.65	932.99 ± 36.67	603.92 ± 95.22
	β-glucans	315.19 ± 86.72*	774.24 ± 50.62*	319.85 ± 74.35*
Catalase	Control	2.67 ± 0.27	4.54 ± 0.48	3.85 ± 1.01
	β-glucans	4.82 ± 1.10*	2.64 ± 0.83*	1.80 ± 0.34*
Glutathione reductase	Control	45.47 ± 2.59	25.40 ± 5.69	25.60 ± 3.83
	β-glucans	39.53 ± 4.14	25.18 ± 3.44	31.69 ± 2.08
Glutathione peroxidase	Control	101.32 ± 13.25	148.69 ± 28.65	274.59 ± 19.76
	β-glucans	111.36 ± 12.47	294.00 ± 29.09*	251.41 ± 15.79

* – statistically significant difference between the β-glucan and control groups (P-value < 0.05)

Table 2. Metabolic enzyme activity and metabolite levels in muscle tissue of grayling fed a diet supplemented with β-glucans or fed a basal diet over three study periods (15, 30 and 45 days of feeding)

Enzyme	Group	Enzyme activity (U mg^{-1} protein (enzymes)/nmol mg^{-1} protein (metabolites))		
		15 days' feeding	30 days' feeding	45 days' feeding
Alanine aminotransferase	Control	24.62 ± 3.72	11.49 ± 2.03	12.37 ± 0.86
	β-glucans	32.93 ± 3.96	12.52 ± 2.15	10.60 ± 1.53
Aspartate aminotransferase	Control	30.61 ± 2.91	19.58 ± 1.30	17.18 ± 1.56
	β-glucans	33.34 ± 5.95	21.13 ± 2.46	17.71 ± 1.08
Lactate dehydrogenase	Control	88.22 ± 6.49	59.05 ± 3.44	56.60 ± 6.90
	β-glucans	122.29 ± 11.11*	63.90 ± 8.28	58.21 ± 5.64
Succinate dehydrogenase	Control	4.26 ± 0.33	2.19 ± 0.22	1.18 ± 1.10
	β-glucans	3.73 ± 0.47*	1.23 ± 0.89	0.98 ± 0.73
Lactate	Control	103.14 ± 18.48	43.39 ± 13.50	93.87 ± 24.00
	β-glucans	175.61 ± 16.52*	86.79 ± 13.31*	80.67 ± 20.29
Pyruvate	Control	54.87 ± 10.21	35.15 ± 4.41	35.56 ± 3.79
	β-glucans	94.79 ± 15.18*	35.10 ± 4.98	41.11 ± 6.50

* – statistically significant difference between the β-glucan and control groups (P-value < 0.05)

Table 3. Lysosomal enzyme activity in muscle tissue of grayling fed a diet supplemented with β-glucans or fed a basal diet over three study periods (15, 30 and 45 days of feeding)

Enzyme	Group	Enzyme activity (nmol min^{-1} mg^{-1} protein)		
		15 days' feeding	30 days' feeding	45 days' feeding
Acid phosphatase	Control	125.11 ± 10.72	141.49 ± 14.03	138.27 ± 16.46
	β-glucans	162.93 ± 23.96*	188.52 ± 18.15*	170.60 ± 11.53*
β-N-acetylglucosaminidase	Control	78.61 ± 2.91	119.58 ± 16.30	127.18 ± 8.56
	β-glucans	99.34 ± 6.95*	231.16 ± 24.46*	147.71 ± 9.08
Alanyl aminopeptidase	Control	108.21 ± 9.49	109.05 ± 7.44	96.60 ± 6.90
	β-glucans	62.29 ± 11.11*	63.81 ± 5.28*	108.21 ± 9.64
Leucyl aminopeptidase	Control	123.14 ± 14.41	113.39 ± 23.50	113.87 ± 14.02
	β-glucans	95.61 ± 26.52	106.79 ± 23.32	81.67 ± 18.29

* – statistically significant difference between the β-glucan and control groups (P-value < 0.05)

Table 4. Regression analysis of biochemical data in the muscle tissue of grayling fed a diet supplemented with β-glucans or fed a basal diet over three study periods (15, 30 and 45 days of feeding)

Biomarker	Regression analysis data type	β ± standard error
Thiobarbituric acid reactive species	β-glucans	−0.275 ± 0.108
	Duration	0.430 ± 0.108
Oxidatively modified protein (aldehyde dinitrophenyl hydrazone, neutral)	β-glucans	−0.349 ± 0.086
	Duration	0.640 ± 0.086
Oxidatively modified protein (ketone dinitrophenyl hydrazone, neutral)	β-glucans	−0.358 ± 0.066
	Duration	0.538 ± 0.094
Oxidatively modified protein (aldehyde dinitrophenyl hydrazone, alkaline)	β-glucans	−0.285 ± 0.078
	Duration	0.430 ± 0.088
Oxidatively modified protein (ketone dinitrophenyl hydrazone, alkaline)	β-glucans	−0.375 ± 0.086
	Duration	0.458 ± 0.099
Superoxide dismutase	β-glucans	−0.544 ± 0.105
Glutathione reductase	Duration	−0.382 ± 0.116
Glutathione peroxidase	β-glucans	0.228 ± 0.089
	Duration	0.664 ± 0.089
Total antioxidant status	Duration	0.262 ± 0.121
Alanine aminotransferase	Duration	−0.805 ± 0.072
Aspartate aminotransferase	Duration	−0.850 ± 0.064
Lactate dehydrogenase	β-glucans	0.243 ± 0.083
	Duration	−0.705 ± 0.083
Succinate dehydrogenase	Duration	−0.537 ± 0.105
Lactate	β-glucans	0.358 ± 0.103
	Duration	−0.446 ± 0.103
Pyruvate	β-glucans	0.312 ± 0.091
	Duration	−0.615 ± 0.091

Lysosomal enzymes. The activity of AcP, a lysosomal enzyme involved in the degradation of various substrates, was also analysed. There was an increase in AcP activity ($F_{5.60}$ = 53.15, P-value = 0.000), which may indicate an increased rate of degradation and recycling of cellular components in lysosomes (Table 3). Another lysosomal enzyme the activity of which was analysed in the fish muscle tissue, and which is mainly involved in the breakdown of N-acetylglucosamine, is β-N-acetylglucosaminidase ($F_{5.60}$ = 29.71, P-value = 0.000) (Table 3). Its increased activity also indicated an intensification of lysosomal metabolism in the muscles of fish fed β-glucans. The analysis of variance of the data obtained for the lysosomal enzymes involved in the hydrolysis of peptides, namely alanyl aminopeptidase (AAP) and leucyl aminopeptidase (LAP), was as follows: $F_{5.60}$ = 15.44, P-value = 0.001 and $F_{5.60}$ = 7.82, P-value = 0.000, respectively. Their activity was found to be unchanged or reduced. In particular, a significant reduction in the activity of AAP was observed.

Significance of the sum-of-squares test for the full model versus residuals. The test comparing the SS for the full model with the SS for the residuals examined the significance of the model, assessed its quality and identified the key variables. It showed that the effects of β-glucans and feeding duration had a significant effect on the parameters studied, and the coefficient of determination (R^2) showed how the independent variables explained the variation in the parameters studied.

For the TBARS biomarker the results were R = 0.721, R^2 = 0.519, R^2_{adj} = 0.479 and F = 12.96, P-value = 0.000. The results of this test were high for OMP ADa and OMP KDn (R = 0.865, R^2 = 0.748, R^2_{adj} = 0.728 and F = 35.60, P-value = 0.000 and R = 0.909, R^2 = 0.826, R^2_{adj} = 0.812 and F = 57.17, P-value = 0.000, respectively), which made it possible to identify which variables had a significant effect on these markers. The high F-value and the low P-value (<0.05) indicated that the full model explained a significant proportion of the variation in the dependent variable compared to the model with residuals, confirming the statistical significance of the model. The R^2 coefficient indicated the amount of total variation in the dependent variable that was explained by the independent variables in the model: the higher the R^2 value, the better the fit of the model to the data.

The most significant effects in connection with antioxidant enzymes were obtained for SOD activity (R = 0.904, R^2 = 0.827, R^2_{adj} = 0.812 and F = 53.43, P-value = 0.000) and TAS (R = 0.917, R^2 = 0.841, R^2_{adj} = 0.827 and F = 63.44, P-value = 0.000). The SS test

also shed light on lysosomal enzyme activity and levels of metabolic markers. The highest dependencies in this analysis were obtained for AcP, NAG and ALT activity.

Thus, the SS test of the full model against the SS model for the residuals showed that the effects of β-glucans and feeding duration had a significant effect on TBARS, OMP ADa, OMP KDn, SOD and TAS, with high F-values and low P-values indicating statistical significance. The R^2 values showed that a substantial part of the variation in these markers was explained by the independent variables, with high dependencies found for SOD activity and TAS.

Dependent variable regression summary. Regression analysis made it possible to determine the relationships between the effects of β-glucans on enzyme activity and the levels of biomarkers of energy-related metabolism, but also to estimate the strength of these effects. In this way, a more precise elucidation of the phenomena studied could be achieved. The regression analysis of the parameters studied, presented in Table 4, showed that the effects of β-glucans and feeding duration were significant on enzyme activity and substrate levels, with the lengthening of the feeding duration leading to an intensification of enzyme activity. In studying the effects of β-glucans and feeding duration on enzyme activity and substrate levels, the β-coefficient in the regression allowed the influence of these independent variables (two determinants) on the dependent variables (oxidative stress biomarkers, metabolic markers and lysosomal enzyme activity) to be assessed. By comparing the β-coefficients for the effects of β-glucans and feeding duration, it was possible to determine which of these variables had the greatest effect. In the analysis of the effects of β-glucans and feeding duration, the larger absolute values of the β-coefficient indicated that feeding duration had a stronger effect on OMP ADn, OMP KDn, ALT, AST, LDH and GPx activity and pyruvate levels, while the effects of β-glucans had a stronger effect on SOD activity.

Discussion

Grayling has a high commercial value as a desirable food fish because of its delicious and nutritious flesh, making it popular in the catering industry and recreational fishing. The adaptability, rapid growth and market desirability of grayling highlight its suitability for aquaculture and appeal to producers wishing to meet the demands of the consumer and the recreational fishing sector. However, research focusing on the biochemical and physiological parameters specific to grayling is relatively scarce in the existing literature. This gap in the knowledge prompted our initiative to undertake studies in this area. By investigating these aspects, we aimed to analyse the biomarkers of oxidative stress, anaerobic metabolism and lysosomal function in the muscle tissue of this fish. The metabolic responses in the muscle tissue of grayling fed β-glucans for 15, 30 and 45 d in our study were investigated, particularly in relation to biomarkers of oxidative stress and antioxidant defences that are related to the activity of lysosomal enzymes and markers of metabolic changes. We elucidated the potential impact of β-glucans, known for their immunomodulatory and antioxidant properties, on these physiological processes.

B-glucans are known for their immunomodulatory effects, stimulating both innate and adaptive immune responses. Long-term supplementation could lead to a sustained improvement in the immune system of fish (12, 14, 38). Sustained exposure to β-glucans may result in improved feed conversion, growth rates and survival, particularly in species prone to stress or disease under intensive farming conditions (34). However, the exact effects may vary between species and environmental conditions. Provision of β-glucans through most of the rearing period may also affect muscle and skeletal development, potentially improving overall fish quality and market value (29). Over time, β-glucans may enhance a fish's antioxidant defences, reducing oxidative stress and associated cellular damage. This could result in healthier fish with improved longevity and overall physiological condition. However, while increased antioxidant activity is generally positive, there is a possibility of an imbalance if antioxidant defences are over-stimulated, which could lead to unintended metabolic consequences. Nonetheless, appropriately dosed long-term β-glucan supplementation may improve energy metabolism, leading to better nutrient utilisation and more efficient energy use. This could be particularly beneficial in stressful conditions where energy demands are high (29).

These studies provide insight into growth-related changes in metabolic processes, which is essential for understanding their survival strategies in natural habitats and aquaculture environments. By examining these metabolic biomarkers, we can infer whether fish are experiencing oxidative stress or using available energy substrates efficiently. B-glucans can improve the health and adaptation of fish, particularly in challenging environmental conditions, by supporting aerobic metabolism and reducing oxidative stress. Our research not only contributes to the fundamental understanding of fish physiology, but also explores practical applications to improve aquaculture practices and support sustainable fish populations in changing aquatic environments.

Oxidative modification of proteins can affect protein stability, activity and interactions with other molecules, thereby influencing various biological pathways (35). In our research, we investigated the effect of β-glucans on oxidative protein modifications, focusing on their potential to alleviate oxidative stress in biological systems. Our results showed that β-glucans were effective early in the experiment in reducing the levels of oxidative protein modification. This observation (after 15 d of feeding) was significant because oxidative modifications can impair protein function and contribute to cellular damage and disease progression. By reducing oxidative protein modification, β-glucans demonstrate their potential as antioxidants protecting proteins from structural changes and preserving their biological

activity. This protective effect is particularly valuable in contexts where oxidative stress is a concern, such as ageing, inflammatory conditions, and environmental stress from the stressors found in aquaculture or other biological systems. Our research highlights the therapeutic potential of β-glucans in combating oxidative damage and underlines their role in promoting cellular health and resilience in salmonids. The observation that β-glucans are effective in reducing oxidative protein modifications within the first 15 d of fish feeding is of particular importance, given the detrimental effects of oxidative modifications on protein function, cellular integrity and disease development. These findings highlight the potential of β-glucans as valuable agents in combating oxidative damage and supporting cellular resilience.

As β-glucans can influence the energy metabolism of fish by modulating the activity of key enzymes and metabolites such as ALT, AST, LDH, SDH, lactate and pyruvate, we analysed these key enzymes and metabolites to understand how these substances can support the health and efficiency of energy metabolism in fish muscle. In particular, as we have already shown, they can reduce oxidative stress, thereby improving mitochondrial function and the efficiency of the Krebs cycle. B-glucans fed to grayling may influence anaerobic metabolism, which is critical for fish under stress, by potentially reducing LDH activity and lactate levels through improved metabolic function and reduced oxidative stress. The reduction of SDH activity noted in β-glucan-supplemented grayling muscle ($F_{5.60}$ = 6.21, P-value = 0.000) may be regarded as improvement to the efficiency of the Krebs cycle, which should lead to better utilisation of pyruvate for ATP production rather than its conversion to lactate and thereby improve the overall energy status of muscle tissue. In summary, β-glucans alter the balance between aerobic and anaerobic metabolism, affecting lactate and pyruvate levels, and modulate the activity of transaminase enzymes, affecting amino acid metabolism and the production of energy intermediates. The elevated lactate and pyruvate levels associated with decreased SDH activity and unchanged or high LDH activity suggest that fish adapt to different conditions by favouring anaerobic metabolism. B-glucans may play a key role in supporting fish adaptation and maintaining metabolic homeostasis in changing aquatic environments.

Our study also demonstrated protection against oxidative stress *via* increased LDH activity in the first 15 days of feeding, which may result from the fish's need for rapid adenosine triphosphate production in oxygen-deficient conditions. B-glucans may influence this situation by reducing oxidative stress, thereby limiting the breakdown of energy substrates and promoting better glycolytic reactions. The results of our study showed that feeding β-glucans consistently benefitted muscle tissue condition. Over the prolonged feeding periods, β-glucans reduced lipid peroxidation and improved antioxidant defences. In particular, they rapidly attenuated oxidative modifications of proteins within the first 15 d of feeding, which is critical for preserving protein functionality.

These beneficial effects were facilitated by increased SOD and CAT activity and maintained by GPx activity in the muscle tissue. The effects of β-glucans were primarily evident through changes in the distribution of SOD/CAT activity, although GPx plays a role in maintaining these effects within muscle tissue. As previously reported (33), this may be due to an enhancement of the phagocytic and immunomodulatory functions of salmonid blood and tissues. B-glucans also exerted a significant influence on energy metabolism in the fish muscle by modulating the key enzymes and metabolites ALT, AST, LDH, SDH, lactate and pyruvate. Alanine aminotransferase catalyses the conversion of alanine to pyruvate, which is essential for energy production *via* gluconeogenesis. Aspartate aminotransferase catalyses the transfer of the amino group from aspartate to α-ketoglutarate, forming oxaloacetate and glutamate, with oxaloacetate being a key intermediate in the Krebs cycle affecting adenosine triphosphate (ATP) production. B-glucans altered the balance between aerobic and anaerobic metabolism, affecting lactate and pyruvate levels, and modulated transaminase enzymes, affecting amino acid metabolism and energy production.

Adaptation to β-glucan feeding is associated with high levels of lactate and pyruvate production, which may also be an adaptive strategy in response to fluctuating growth-related conditions, such as increased oxygen demand and intensification of metabolism. B-glucans may support the adaptive response of organisms by modulating immune responses and reducing oxidative stress responses that could lead to further metabolic changes. The elevated lactate and pyruvate levels indicated an intensification of anaerobic metabolism, possibly due to a shift in energy production strategies, while β-glucans, known for their immunomodulatory and antioxidant properties, may help maintain metabolic balance in fish muscle, facilitating adaptation to different challenges, such as oxygen deprivation. Our study demonstrated the dominance of anaerobic metabolism in the fish muscle tissue after the β-glucan supplementation. The increased levels of lactate and pyruvate suggested that fish prefer anaerobic metabolism as the primary source of energy production. This pathway is activated during hypoxia or environmental stress where oxygen availability is limited. The reduced SDH activity, while possible to interpret as an efficiency gainer for the Krebs cycle, may alternatively have indicated limitations in the efficiency of the Krebs cycle, which is critical for aerobic metabolism. It is known that SDH is involved in metabolic processes related to energy production in the mitochondria (36).

The literature data show that β-glucans are known for their ability to stimulate the immune system through interactions with receptors on immune cells, such as macrophages. The mechanism involves activation of dectin-1 receptors by β-glucans, leading to the

production of pro-inflammatory cytokines and increased phagocytic activity, including that of macrophages (3). These processes may involve increased activity of lysosomal enzymes in response to the increased demand for degradation and recycling of cellular components. B-glucans may also activate signalling pathways in cells that regulate the expression of genes responsible for lysosomal enzymes. Studies of the effects of β-glucans on macrophages have shown increased expression of genes encoding lysosomal enzymes, suggesting that activation of these pathways may lead to increased synthesis and activity of lysosomal enzymes (23).

In our study, feeding the fish with β-glucans resulted in increased AcP and NAG activity. The statistical analysis using MANOVA highlighted the significant effect of β-glucans and feeding duration on TBARS, OMP ADa, OMP KDn, SOD and TAS, supported by high F-values and low P-values indicating robust statistical significance. In addition, the R^2 values showed that a significant proportion of the variation in these biomarkers was explained by the independent variables, with the influence on SOD activity and TAS being particularly pronounced.

It has been shown that β-glucans present in the cell walls of fungi and yeasts, where they have structural functions, can be endocytosed by animal macrophages and lysosomal cells. Once internalised, β-glucans can be degraded in lysosomes, thereby increasing lysosome activity when they digest such substrates. In addition, β-glucans can influence glycosylation processes in cells, and perturbations in this process can lead to the accumulation of substrates that can potentially be degraded by lysosomal enzymes, as shown in the AcP and NAG enzymes in our study. In conclusion, AcP may be activated by β-glucans in four mechanisms, which are immunomodulation, activation of signalling pathways, degradation of β-glucan-containing structures and regulation of glycosylation processes. These processes may result in increased lysosomal activity in response to changes in cellular metabolism induced by β-glucans (14).

This study provides concrete evidence of how a diet enriched in β-glucans can alleviate oxidative stress in fish, suggesting a practical approach to improving the health and resilience of farmed European grayling. The inclusion of such dietary supplements could potentially improve the overall growth performance and disease resistance of fish in aquaculture. By improving the energy metabolism of European grayling through dietary intervention, this study demonstrates a sustainable approach to fish farming. Healthier fish with optimised metabolic functions may require less pharmaceutical intervention, thereby reducing the environmental impact associated with the use of chemicals. Supplementing the diet of fish in hatchery programmes with β-glucans could improve their survival and adaptability when released into the wild.

Feeding β-glucans to the grayling reduced lipid peroxidation and improved the antioxidant status in their muscle tissue in our study. In particular, within the first 15 d of feeding, β-glucans effectively attenuated oxidative modification of proteins. These effects were primarily mediated by increased SOD and CAT activity and sustained by GPx activity in the muscle. B-glucans also affected energy metabolism in fish muscle tissue by modulating the activities of the key enzymes and metabolites ALT, AST, LDH and SDH and raising lactate and pyruvate levels. B-glucans altered the balance between aerobic and anaerobic metabolism, affected lactate and pyruvate levels and modulated the activity of transaminase enzymes, thereby affecting amino acid metabolism and the production of energy intermediates. Furthermore, our study demonstrated increased AcP and NAG activity in response to β-glucan supplementation. The MANOVA analysis, supported by high F-values and low P-values indicating statistical significance, confirmed the marked effects of β-glucans and feeding duration on the biomarkers TBARS, OMP ADa, OMP KDn, SOD and TAS. The R^2 values showed that, particularly for SOD activity and TAS, a significant proportion of the variance in these markers was explained by the independent variables.

Conclusion

Further research is needed to elucidate the molecular mechanisms by which β-glucans exert their effects on oxidative stress, energy metabolism and lysosomal function. A better understanding of these processes could lead to the development of more targeted nutritional strategies. The effects observed in European grayling could also serve as a model for other fish species in aquaculture. Comparative studies across species would help to generalise findings and tailor dietary interventions to species-specific metabolic and physiological requirements. Investigating how a β-glucan-enriched diet interacts with environmental stressors such as temperature fluctuations, pollutants and varying oxygen levels could provide insights into the robustness of this dietary intervention under different aquaculture and natural conditions. While the study provides valuable data on the short-term effects of a β-glucan-enriched diet, future research should investigate the long-term effects, optimal dosage and duration of supplementation to maximise benefits while avoiding potential adverse effects.

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