sciendo

J Vet Res 68, 325-336, 2024
DOI:10.2478/jvetres-2024-0050

Biomarkers of oxidative stress, biochemical changes, and the activity of lysosomal enzymes in the livers of rainbow trout (*Oncorhynchus mykiss* Walbaum) vaccinated against yersiniosis before a *Yersinia ruckeri* challenge

Natalia Kurhaluk[1]✉, Joanna Grudniewska[2], Agnieszka Pękala-Safińska[3], Joanna Pajdak-Czaus[4], Elżbieta Terech-Majewska[4], Aleksandra Platt-Samoraj[4], Halina Tkaczenko[5]

[1]Department of Animal Physiology, [5]Department of Zoology, Institute of Biology, Pomeranian University in Słupsk, 76-200 Słupsk, Poland
[2]Department of Salmonid Research, Stanislaw Sakowicz Inland Fisheries Institute, 83-330 Rutki, Żukowo, Poland
[3]Department of Preclinical Sciences and Infectious Diseases, Faculty of Veterinary Medicine and Animal Sciences, Poznań University of Life Sciences, 60-637 Poznań, Poland
[4]Department of Epizootiology, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, 10-718 Olsztyn, Poland
natalia.kurhaluk@upsl.edu.pl

Received: April 6, 2024 *Accepted: September 4, 2024*

Abstract

Introduction: This study aimed to evaluate biomarkers of oxidative stress (2-thiobarbituric acid reactive substances, aldehyde and ketone derivatives of oxidatively modified proteins and total antioxidant capacity), the activity of antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase), that of lysosomal enzymes (alanyl aminopeptidase, leucyl aminopeptidase, β-N-acetylglucosaminidase and acid phosphatase) and changes in biochemical parameters (alanine aminotransferase, aspartate aminotransferase, de Ritis ratio, lactate dehydrogenase activity, lactate and pyruvate levels and their ratio) in the liver tissue of fish that were vaccinated against enteric redmouth disease and challenged with its causative agent, the bacterium *Yersinia ruckeri*. **Material and Methods:** The vaccine was administered orally to trout, some of which were challenged with *Y. ruckeri* 61 days later. For comparison, unvaccinated and unchallenged trout and unvaccinated and challenged trout were also evaluated. **Results:** In the unvaccinated fish, infection with *Y. ruckeri* disrupted the pro-oxidant/antioxidant balance, led to a significant increase in lipid peroxidation and oxidative modification of proteins, decreased total antioxidant capacity and significantly increased the activity of lysosomal enzymes. In vaccinated fish, the *Y. ruckeri* challenge increased the activity of glutathione-related enzymes and decreased lipid peroxidation, anaerobic metabolism and the activity of lysosomal enzymes in fish livers relative to the unvaccinated and challenged group. In contrast, these parameters increased after the *Y. ruckeri* challenge in unvaccinated trout relative to those in the untreated group. **Conclusion:** Vaccination exerted a protective effect during the *Y. ruckeri* challenge and had no adverse effect on fish livers.

Keywords: *Yersinia ruckeri*, challenge model, rainbow trout, oxidative stress, antioxidant defences, lysosomal enzymes.

Introduction

Yersiniosis (enteric redmouth disease, ERM), a septic disease affecting salmonids and particularly rainbow trout, has been reported in the Baltic region, most countries of Eastern and Western Europe and the USA. The disease also affects whitefish, largemouth bass, sturgeon, fathead minnow, perch and crayfish (1, 35). Yersiniosis is caused by *Yersinia ruckeri*, a Gram-negative bacterium of the Enterobacteriaceae family which possesses peritrichous flagella that are very motile in fresh cultures. Juvenile trout with a length of

6–8 cm are most susceptible to the disease, while fish longer than 12.5 cm display a chronic form of the disease and are carriers of the pathogen (22).

Yersiniosis can manifest as a fulminant, acute, subacute or chronic infection. Clinical signs are absent in fulminant infections. Acute yersiniosis is characterised by darkening of the skin, and rainbow trout turn nearly black with clinical signs of septicaemia. Diagnostic symptoms include inflammation and erosion of the mouth ("red mouth"), gill covers and the base of the fin rays. Dotted and spotted haemorrhages appear on the lower part of the abdomen. Crescent-shaped haemorrhages and bilateral exophthalmia are observed in the eyeball, sometimes with rupture of the eyeball. The gills may be anaemic or reddened at the base. Severely infected fish may bleed when pressure is applied to gill covers. These symptoms are less pronounced in subacute and chronic infections. Post-mortem examinations of diseased fish reveal hyperaemia of the peritoneum and adipose tissue. Numerous haemorrhages are observed on the serous membrane of the peritoneal cavity. The liver and hindgut are hyperaemic. In some cases, the liver is yellowish with numerous haemorrhages. The spleen is enlarged and dark red in colour. Numerous haemorrhages are also present on the surface of the swim bladder. The muscle tissue near the spine turns red. The blood may be watery due to a significant decrease in the number of red blood cells. In a dying fish, the abdomen is swollen, the stomach may be filled with colourless watery fluid and the intestines may turn yellow (16, 33).

Prevention of yersiniosis involves the protection of healthy farms and strict compliance with fish farm stocking density, water quality and reclamation, veterinary inspection and sanitary requirements. Vaccination against yersiniosis is one of the most important and successful health practices in the aquaculture industry, demonstrating that antibiotics are not always necessary to control bacterial diseases (5). A farm is considered healthy if *Y. ruckeri* is not detected in water and fish one year after an outbreak has been eliminated. Prophylaxis against ERM includes vaccination and is strongly recommended in rainbow trout to avoid economic losses due to mortality (34). The ERM vaccine was one of the first fish vaccines and was developed before 1970. It contains formalin-inactivated bacteria and was administered orally when first launched. Research demonstrated that the vaccine was more effective when administered by injection, but this route of administration was difficult to implement in fish farms breeding large numbers of small fish. Fish for oral vaccination with this preparation should weigh >20 g, and anaesthesia is required to reduce stress. Immersion vaccines, which are less time consuming and less stressful for fish, were developed in the years following 1970. In this route of administration, fry are immersed in diluted bacterin for 30–120 seconds. Immersion vaccines are recommended only for small fish (5–20 g), and if booster vaccination is required, it is usually administered by injection (10).

Oral vaccination has been suggested as a way to increase protection, but the effect of this route of administration on the physiological state of fish has not been researched extensively to date. The aim of this study was to determine the protective effect of oral immunisation against yersiniosis on liver biochemistry in rainbow trout. The efficacy of vaccination against *Y. ruckeri* in fish challenged with live *Y. ruckeri* bacteria was evaluated by analysing the following cellular defence parameters in liver tissue: biomarkers of oxidative stress (2-thiobarbituric acid reactive substances (TBARS), aldehyde and ketone derivatives of oxidatively modified proteins (OMP) and total antioxidant capacity (TAC)); the activity of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx)); the activity of lysosomal enzymes (alanyl aminopeptidase (AAP), leucyl aminopeptidase (LAP), β-N-acetylglucosaminidase (NAG) and acid phosphatase (AcP)); and changes in biochemical parameters (alanine aminotransferase (ALT – Enzyme Commission (EC) 2.6.1.2), aspartate aminotransferase (AST – EC 2.6.1.1) and lactate dehydrogenase (LDH – EC 1.1.1.27) activity de Ritis ratio, and lactate and pyruvate levels and their ratio (L/P ratio)). The present study evaluated the levels of oxidative stress and antioxidant defence biomarkers, biochemical changes, and the activity of lysosomal enzymes in the livers of rainbow trout neither vaccinated against yersiniosis nor challenged with *Y. ruckeri*, in the livers of vaccinated and unchallenged fish, in those of unvaccinated fish challenged with *Y. ruckeri*, and in those of vaccinated rainbow trout after the *Y. ruckeri* challenge. Four groups of fish were used in the study to fully and independently characterise and evaluate the biochemical processes occurring in fish livers. It should be noted that oxidative stress biomarkers, biochemical changes, and the activity of lysosomal enzymes in the vaccinated and unchallenged and unvaccinated and challenged groups were compared with the untreated control group, whereas the vaccinated group challenged with *Y. ruckeri* was compared independently with the vaccinated and unchallenged group and the unvaccinated and challenged group. This experimental design supported the identification of biochemical changes both during the *Y. ruckeri* infection and after vaccination against *Y. ruckeri*, as well as in the vaccinated group subjected to the *Y. ruckeri* challenge.

Material and Methods

Fish. The experiments involved rainbow trout (*Oncorhynchus mykiss* Walbaum) weighing approximately 320 g. The study was conducted in the Department of Salmonid Research of the Stanisław Sakowicz Inland Fisheries Institute in Olsztyn (Poland). The water

temperature was 14.5 ± 0.5°C and its pH was 7.5. The dissolved oxygen level was approximately 12 ppm, and supplemental oxygen was provided by water aeration at 25 L/min^{-1}. A 12 h/12 h photoperiod was established. Commercial pelleted diets were provided at optimal levels with the use of 12-h fish belt feeders. Daily dietary allowances were calculated according to current dietary guidelines. All enzymatic assays were performed at the Department of Zoology and the Department of Animal Physiology of the Institute of Biology of the Pomeranian University in Słupsk (Poland).

Characteristics of the study groups. For vaccination purposes, the fish were divided into two parts, each part numbering 150 fish. Each part was kept in a square 1,000 L tank under the same environmental conditions. One part was left unvaccinated, and the other was vaccinated with a concentrate added to feed containing inactivated *Y. ruckeri*. The vaccine was produced in the Department of Fish Diseases of the National Veterinary Research Institute in Puławy (Poland) according to a patented procedure (Polish patent No. 236777) and was orally administered three times at one-day intervals at a concentration of 1×10^9 cells per mL. The trout were maintained for 60 d after vaccination at a water temperature of 14.5 ± 0.5°C and pH 7.5.

Twenty fish from each part were used for the experiment. At 60 d, 20 vaccinated fish were divided into two groups of 10 and each was moved to a separate aquarium. Also at this time, 20 unvaccinated fish were divided into two groups of 10 and each was moved to a separate aquarium. For the challenge test at 61 d after vaccination, 20 fish were used, 10 of them being the trout in one aquarium of unvaccinated specimens and the other 10 being the trout in one aquarium of vaccinated fish. The experimental infection was induced with *Y. ruckeri* serotype O1, biotype 2, isolated from a disease outbreak on a rainbow trout farm. Prior to the experimental infection, *Y. ruckeri* was cultured on tryptone soy agar with 5% horse blood (Oxoid, Basingstoke, UK) at 25 ±1°C for 24 h. Fish were infected intraperitoneally at a dose of 10^7 colony-forming units per mL of suspension (17).

The resulting four experimental groups were as follows: the control group of unvaccinated and unchallenged fish, the group of unvaccinated and challenged fish, the group of vaccinated and unchallenged fish, and the group of vaccinated and challenged fish. The experiment lasted 10 d, during which the fish were monitored for mortality. The trout were observed three times daily for behavioural and clinical changes, and mortalities in each group were counted to assess cumulative survival. Swabs of the head kidney were taken from dead fish to determine whether a specific bacterium was the cause of death. Mortality was expressed as a percentage (n = 10 = 100%).

Sampling. Fish were trapped and sacrificed 10 d after the challenge test. The liver was excised *in situ*. The organs were perfused with cold isolation buffer and homogenised using a glass Waverly H500 homogeniser (SoCal BioMed, Waverly, IA, USA) with a motor-driven pestle immersed in an ice-water bath to obtain a 1:9 (weight/volume) homogenate. The isolation buffer contained 100 mM tris-HCl; the pH of the homogenate and buffer solution was adjusted to 7.2 with HCl. The homogenates were centrifuged at 3,000 rpm for 15 min at 4°C. After centrifugation, the supernatant was collected and used for biochemical assays. Protein content was determined according to the method proposed by Bradford (4) with bovine serum albumin as the standard. Absorbance was recorded at 595 nm. All assays were performed in duplicate at 22 ± 0.5°C. Enzymatic reactions were initiated by adding tissue supernatant.

For lysosomal enzyme assays, liver tissue was rinsed with 0.15 M KCl cold isolation buffer to remove blood, and it was homogenised on ice in a Potter-Elvehjem glass homogeniser with a motorised Teflon pestle. The isolation buffer contained 0.25 M sucrose and 2 mM ethylenediaminetetraacetic acid; the pH of the homogenate and buffer solution was adjusted to 7.0 with KOH. Liver tissue homogenates were prepared at 20% (w/v) for differential centrifugation according to the method described by DeMartino and Goldberg (9). After centrifugation, the supernatant fractions were resuspended in 50 mM acetic acid/sodium acetate buffer, pH 5.0, before storage and use. The isolated fractions were homogenised and subjected to two freeze-thaw cycles (23).

Determination of 2-thiobarbituric acid reactive substances. An aliquot of the homogenate was used to determine the lipid peroxidation status of the sample by measuring the concentration of TBARS according to Kamyshnikov (18). These concentrations were expressed in nmol of malonic dialdehyde (MDA) per mg protein.

Determination of the carbonyl groups of oxidatively modified proteins. Carbonyl groups were determined as indicators of oxidative damage to proteins according to the method described by Levine *et al.* (24) with some modifications. Carbonyl content was measured spectrophotometrically at 370 nm (for aldehyde derivatives, OMP_{370}) and 430 nm (for ketone derivatives, OMP_{430}) using a molar extinction coefficient of 22,000 $M^{-1} \cdot cm^{-1}$ and expressed in nmol per mg protein.

Determination of superoxide dismutase activity. Superoxide dismutase activity was determined based on its ability to dismutate superoxide generated during quercetin auto-oxidation in an alkaline medium (pH 10.0), according to the method described by Kostiuk *et al.* (20). The result was expressed in units of SOD per mg of tissue protein.

Determination of catalase activity. Catalase activity was determined spectrophotometrically by measuring the reduction of H_2O_2 in the reaction mixture at a wavelength of 410 nm, according to the method described by Koroliuk *et al.* (19). One unit of CAT activity was defined as the amount of enzyme required to degrade 1 nmol of H_2O_2 per minute per mg of protein.

Determination of glutathione reductase activity. Glutathione reductase activity in the sample was measured according to the method described by Glatzle *et al.* (14) with some modifications. Glutathione reductase activity was expressed as nmol of $NADPH_2$ per minute per mg of protein.

Determination of glutathione peroxidase activity. Glutathione peroxidase activity was determined by measuring the non-enzymatic reduction of glutathione (GSH – the substrate) at an absorbance of 412 nm after incubation with 5,5-dithiobis-2-nitrobenzoic acid, according to the method proposed by Moin (28). Glutathione peroxidase activity was expressed in nmol of GSH per minute per mg protein.

Total antioxidant capacity assay. Total antioxidant capacity was estimated spectrophotometrically at 532 nm according to the Tween 80 oxidation method (12). Levels of TAC were expressed in %.

Determination of alanine aminotransferase and aspartate aminotransferase activity. Alanine aminotransferase and AST activity was determined spectrophotometrically by the standard enzymatic method (29). The results were expressed in nmol of pyruvate per minute per mg protein.

Determination of lactate dehydrogenase activity. Lactate dehydrogenase activity was determined by the colorimetric method described by Sevela and Tovarek (30). The result was expressed in nmol of pyruvate per minute per mg protein.

Determination of lactate and pyruvate concentrations. Lactate and pyruvate concentrations were measured according to the method described by Herasimov and Plaksina (15). Absorbance was measured at 430 nm. The calibration curves of lactate (0.1–5 mM) and pyruvate (0.1–5 mM) were plotted, and the results were expressed in nmol per mg protein.

Lysosomal enzyme assay. The activity of AAP and LAP was determined spectrophotometrically according to DeMartino and Goldberg (9). The reaction was initiated by incubating 50 µL of the sample and 500 µL of the substrate incubation medium with dimethylformamide (Serva Electrophoresis, Heidelberg, Germany) for 60 min at 37°C, pH 6.0, followed by the addition of 500 µL of the stop buffer containing Fast Blue BB salt dissolved in 2% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA). Absorbance was measured at 540 nm. L-alanyl-2-naphtylamine in 0.1 M phosphate-buffered saline (PBS) 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 a substrate to determine leucyl aminopeptidase activity. The activity of acid phosphatase and NAG was determined spectrophotometrically at 420 nm with 4-nitrophenyl derivatives as the substrate, according to the method proposed by Barrett and Heath (2). Enzyme activities were expressed in nmol per hour per mg protein.

Statistical analysis. The results were expressed as the mean ± standard deviation. Data from each individual were processed separately in Statistica 13.3 (TIBCO Software, Palo Alto, CA, USA). The normality of distribution was assessed with the Kolmogorov–Smirnov test (P-value > 0.05), and the homogeneity of variance was assessed with Levene's test. Significant differences in the analysed parameters within and between groups were determined by one-way analysis of variance (ANOVA) with unequal sample sizes and Tukey's post-hoc test. Correlation and regression analyses were also carried out. Differences were considered significant at P-value < 0.05 (32).

Results

Oxidative stress. Thiobarbituric acid reactive substances, the end products of lipid peroxidation, are easily detected in various animal tissues as a reliable indicator of oxidative stress. Their levels were determined in the first step of the experiment. Significant differences in TBARS values between groups were noted in ANOVA ($F_{3.27}$ = 27.38, P-value = 0.000). The data are shown in Fig. 1A. This parameter was highest in the liver tissue of unvaccinated rainbow trout challenged with *Y. ruckeri*. The levels were 61.6% higher (P-value < 0.05) in the liver tissue of these trout than in the liver tissue of the untreated control group. Levels of TBARS in the liver tissue of vaccinated rainbow trout challenged with *Y. ruckeri* were the lowest of all groups', and were 31.4% lower (P-value < 0.05) than in the vaccinated group and unchallenged group and 53.3% lower (P-value < 0.05) than in the vaccinated group challenged with *Y. ruckeri* (Fig. 1A).

The concentrations of aldehyde derivatives of OMP in the liver tissue of rainbow trout challenged with *Y. ruckeri* increased by 20.4% (P-value < 0.05) relative to the untreated control group. The concentrations of these derivatives were highest ($F_{3.27}$ = 3.07, P-value = 0.044) in the unvaccinated *Y. ruckeri*-challenge group and in vaccinated rainbow trout after the *Y. ruckeri* challenge (Fig. 1B). The highest concentrations of ketone derivatives of OMP ($F_{3.27}$ = 4.71, P-value = 0.001) were also noted in the unvaccinated group challenged with *Y. ruckeri* and in vaccinated rainbow trout after the *Y. ruckeri* challenge. The concentrations of these derivatives were 42.1% higher (P-value < 0.05) in the liver tissue of vaccinated rainbow trout challenged with *Y. ruckeri* than in this tissue in the vaccinated and unchallenged group (Fig. 1C).

The highest TAC levels ($F_{3.27}$ = 8.55, P-value = 0.000) were noted in the liver tissue of vaccinated rainbow trout challenged with *Y. ruckeri*. This parameter was 15.5% lower (P-value < 0.05) in the liver tissue of unvaccinated rainbow trout challenged with *Y. ruckeri* than in the untreated control group. Total antioxidant capacity was similar in the remaining groups (Fig. 1D). The regression analysis of the dependent variable for TAC produced the following results: R = 0.633, R^2 = 0.401 and R^2_{adj} = 0.380, with a regression coefficient of $\beta = -0.633 \pm 0.13$.

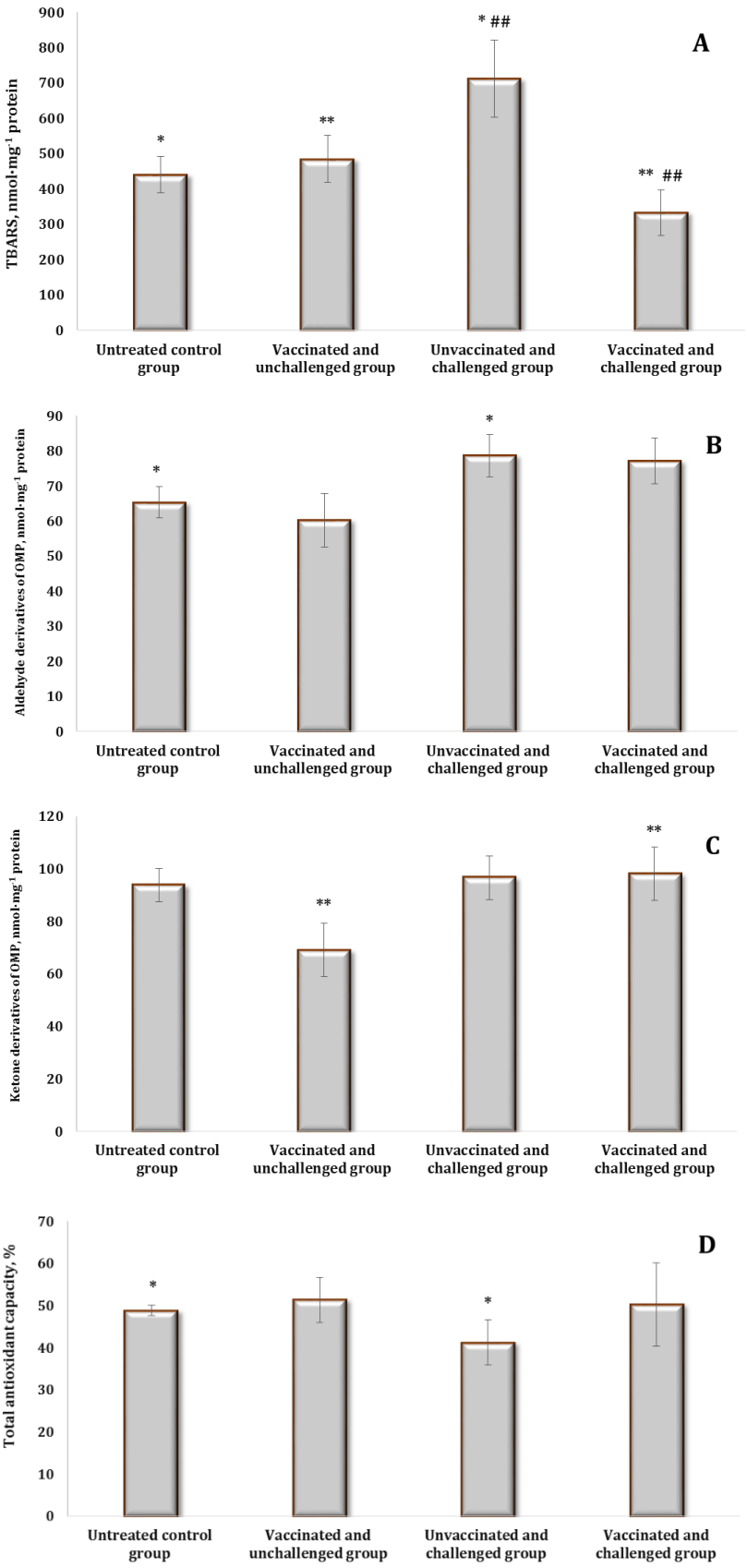

Fig. 1. Oxidative stress biomarkers estimated based on the levels of 2-thiobarbituric acid reactive substances (TBARS, A), aldehyde derivatives (B) and ketone derivatives (C) of oxidatively modified proteins, and total antioxidant capacity (D) in the liver tissue of rainbow trout orally immunised with *Y. ruckeri* vaccine and challenged with *Y. ruckeri*. Data are presented as means ± standard deviation (n = 10); * – significant differences (P-value < 0.05) between the untreated control group and the unvaccinated group challenged with *Y. ruckeri*; ** – significant differences (P-value < 0.05) between the vaccinated group subjected to the *Y. ruckeri* challenge and the vaccinated and unchallenged group; ## – significant differences (P-value < 0.05) between the vaccinated group subjected to the *Y. ruckeri* challenge and the unvaccinated group challenged with *Y. ruckeri*

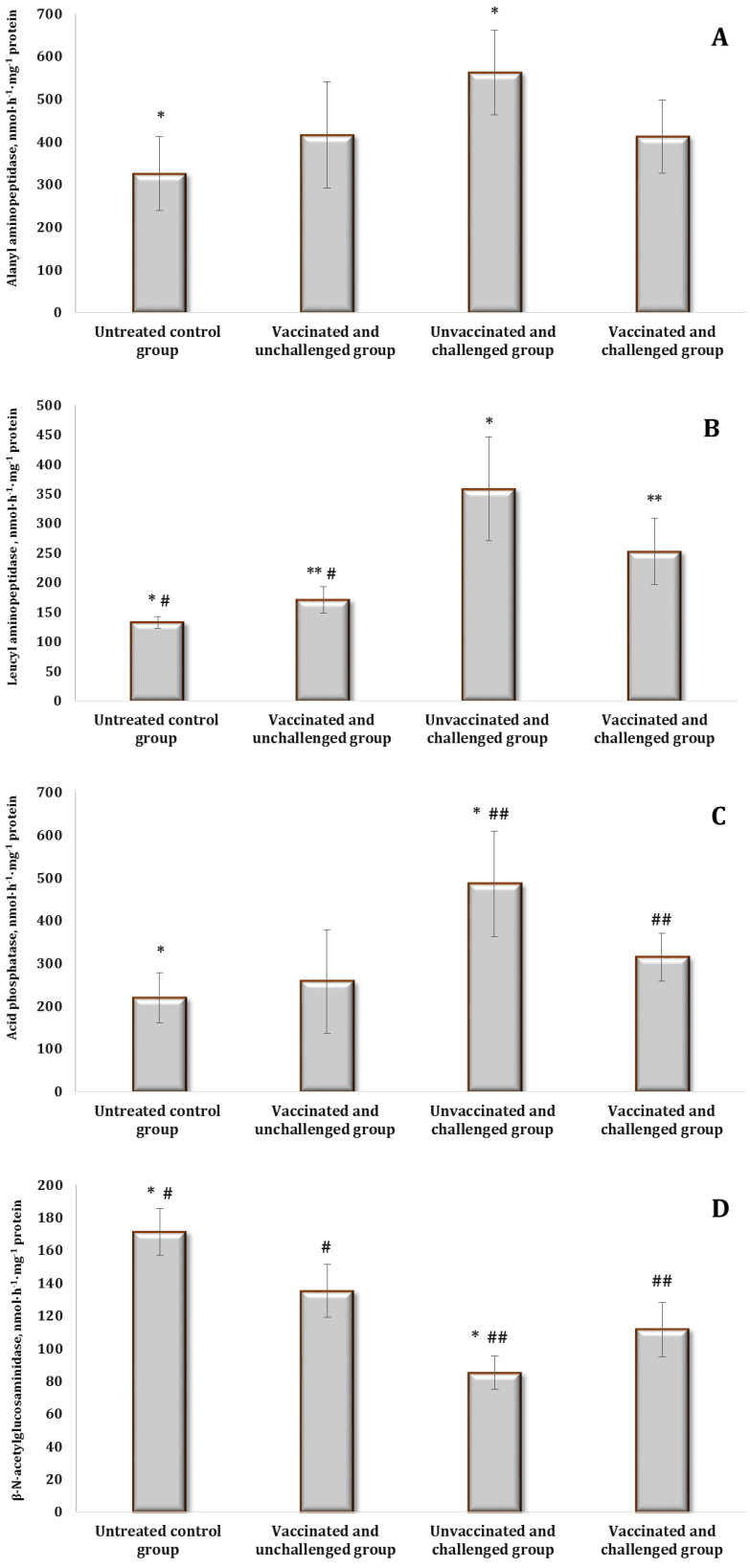

Fig. 2. Activity of the alanyl aminopeptidase (nmol·h^{-1}·mg^{-1} protein), leucyl aminopeptidase (nmol·h^{-1}·mg^{-1} protein), acid phosphatase (AcP, nmol·h^{-1}·mg^{-1} protein), and β-N-acetylglucosaminidase (nmol·h^{-1}·mg^{-1} protein) in the liver tissue of rainbow trout orally immunised against *Y. ruckeri* and challenged with this bacterium. Data are presented as means ± standard deviation (n = 10); * – significant differences (P-value < 0.05) between the untreated control group and the unvaccinated group challenged with *Y. ruckeri*; ** – significant differences (P-value < 0.05) between the vaccinated group subjected to the *Y. ruckeri* challenge and the vaccinated and unchallenged group; # – significant differences (P-value < 0.05) between the untreated control group and the vaccinated and unchallenged group; ## – significant differences (P-value < 0.05) between the vaccinated group subjected to the *Y. ruckeri* challenge and the unvaccinated group challenged with *Y. ruckeri*

Activity of antioxidant enzymes. Changes in the activity of the major antioxidant enzymes in the liver tissue of vaccinated rainbow trout after the *Y. ruckeri* challenge are shown in Table 1. The *Y. ruckeri* challenge suppressed enzymatic activity in the liver tissue of vaccinated trout by decreasing that of SOD and CAT relative to the unvaccinated and challenged group. The activity of antioxidant enzymes involved in glutathione metabolism (GR and GPx) increased considerably in the vaccinated and challenged group relative to the unvaccinated *Y. ruckeri*-challenged group.

In the statistical analysis of SOD, the F-value was determined at $F_{3.27} = 0.960$ (P-value = 0.426). Superoxide dismutase activity was 43.2% higher (P-value < 0.05) in the liver tissue of unvaccinated rainbow trout challenged with *Y. ruckeri* than in the untreated control group. The activity of SOD was 28.8% lower (P-value < 0.05) in vaccinated rainbow trout after the *Y. ruckeri* challenge than in the unvaccinated group challenged with *Y. ruckeri* (Table 1).

Similarly, CAT activity ($F_{3.27} = 29.96$, P-value = 0.000) was 70.9% higher in the unvaccinated group challenged with *Y. ruckeri* relative to the untreated control group (P-value < 0.05). The regression analysis of the dependent variable for CAT produced the following results: R = 0.749, $R^2 = 0.651$ and $R^2_{adj} = 0.546$, with a regression coefficient of $\beta = -0.75 \pm 0.13$. Catalase activity was 23.2% lower (P-value < 0.05) in vaccinated rainbow trout than in the untreated control group. Its activity increased by 16% (P-value < 0.05) in the liver tissue of vaccinated rainbow trout challenged with *Y. ruckeri* compared to the vaccinated and unchallenged group (Table 1).

Glutathione reductase activity ($F_{3.27} = 4.71$, P-value = 0.001) was 65.4% higher in the vaccinated and unchallenged group (25.13 ± 2.17 nmol·min^{-1}·mg^{-1} protein) than in the untreated control group (P-value < 0.05). In challenged and unvaccinated rainbow trout, GR activity was 25.8% higher (P-value < 0.05) than in the untreated control group. Its activity was 114.6% higher (P-value < 0.05) in vaccinated rainbow trout challenged with *Y. ruckeri* than in the unvaccinated and challenged group.

The highest GPx activity ($F_{3.27} = 7.084$, P-value = 0.001) was observed in vaccinated trout challenged with *Y. ruckeri* (357.86 ± 70.71 nmol·min^{-1}·mg^{-1} protein) relative to the vaccinated and unchallenged group and the challenged and unvaccinated group. The regression analysis of the dependent variable for GPx activity produced the following results: R = 0.409, $R^2 = 0.167$ and $R^2_{adj} = 0.138$, with a regression coefficient of $\beta = -0.41 \pm 0.17$. The activity of GPx in the vaccinated and challenged group was 42.8% greater than that in the vaccinated and challenged group and 91.4%greater than the activity in the unvaccinated and challenged group (P-value < 0.05) (Table 1).

Biochemical parameters. The values of ALT and AST and the AST/ALT ratio during the experiment, which indicate cellular metabolism changes, are shown in Table 2. Significant decreases (P-value < 0.05) in AST and ALT activity and lactate and pyruvate levels were noted in vaccinated fish after the *Y. ruckeri* challenge compared with the *Y. ruckeri*-challenged and unvaccinated group. In contrast, these parameters were elevated in the latter group relative to the untreated group.

Alanine aminotransferase activity ($F_{3.27} = 5.56$, P-value = 0.004) was 27.1% higher (P-value < 0.05) in the unvaccinated challenged group than in the untreated control group (P-value < 0.05). Its activity was 25.8% lower (P-value < 0.05) in vaccinated trout challenged with *Y. ruckeri* than in the unvaccinated challenged group. Similarly, AST activity was 20.9% lower (P-value < 0.05) in the liver tissue of vaccinated rainbow trout challenged with *Y. ruckeri* than in the unvaccinated challenged group.

Aspartate aminotransferase activity was 22.6% higher (P-value < 0.05) in trout challenged with *Y. ruckeri* than in the untreated group. No significant differences in the AST/ALT ratio ($F_{3.27} = 4.52$, P-value = 0.011) were observed between groups (Table 2). The regression analysis of the dependent variable for AST produced the following results: R = 0.383, $R^2 = 0.147$ and $R^2_{adj} = 0.117$, with a regression coefficient of $\beta = 0.38 \pm 0.17$.

No significant differences in LDH activity were observed between groups. Lactate levels ($F_{3.27} = 7.19$, P-value = 0.001) were 62.2% higher in the unvaccinated *Y. ruckeri*-challenged group than in the untreated control group (P-value < 0.05). Lactate levels decreased by 36.9% (P-value < 0.05) in vaccinated trout challenged with *Y. ruckeri* relative to the unvaccinated challenged group. Pyruvate levels were highest in unvaccinated trout challenged with *Y. ruckeri* and were 53.5% higher (P-value < 0.05) than in the untreated control group. These levels decreased by 26.6% (P-value < 0.05) in vaccinated trout challenged with *Y. ruckeri* relative to the former group. No significant differences in the lactate/pyruvate ratio were observed between groups (Table 2).

Activity of lysosomal enzymes. Alanyl aminopeptidase activity ($F_{3.27} = 6.19$, P-value = 0.002) was 72.8% higher (P-value < 0.05) in the unvaccinated challenged group than in the untreated control group (Fig. 2A). Leucyl aminopeptidase activity ($F_{3.27} = 4.35$, P-value = 0.004) was also 170.5% higher (P-value < 0.05) in the unvaccinated challenged group and 29% higher (P-value < 0.05) in the vaccinated unchallenged group relative to the untreated control group. The activity of this enzyme was 47.7% higher (P-value < 0.05) in the liver tissue of vaccinated rainbow trout challenged with *Y. ruckeri* than in the vaccinated unchallenged group (Fig. 2B).

Acid phosphatase activity ($F_{3.27} = 7.32$, P-value = 0.000) was 112.8% higher (P-value < 0.05) in unvaccinated trout challenged with *Y. ruckeri* than in the untreated group. Its activity was also 35.3% higher (P-value < 0.05) in the liver tissue of vaccinated rainbow trout challenged with *Y. ruckeri* than in the unvaccinated challenged group (Fig. 2C). The regression analysis of the dependent variable for AcP produced the following results: R = 0.383, $R^2 = 0.147$ and $R^2_{adj} = 0.117$, with a regression coefficient of $\beta = -0.36 \pm 0.14$.

Table 1. Activity of superoxide dismutase (SOD, U·mg^{-1} protein), catalase (CAT, nmol·min^{-1}·mg^{-1} protein), glutathione reductase (GR, nmol·min^{-1}·mg^{-1} protein) and glutathione peroxidase (GPx, nmol·min^{-1}·mg^{-1} protein) antioxidant enzymes in the liver tissue of rainbow trout orally vaccinated against *Yersinia ruckeri* and challenged with this bacterium

Parameter	Untreated control group	Vaccinated unchallenged group	Unvaccinated group challenged with *Yersinia ruckeri*	Vaccinated group challenged with *Yersinia ruckeri*
SOD, U·mg^{-1} protein	643.90 ± 99.08*	627.65 ± 38.09	921.76 ± 139.25*$^{\#\#}$	656.44 ± 49.11$^{\#\#}$
CAT, nmol·min^{-1}·mg^{-1} protein	12.92 ± 0.67*$^{\#}$	9.93 ± 0.59$^{\#}$**	22.08 ± 1.88*$^{\#\#}$	11.52 ± 0.98**$^{\#\#}$
GR, nmol·min^{-1}·mg^{-1} protein	15.19 ± 1.84*$^{\#}$	25.13 ± 2.17$^{\#}$	11.27 ± 3.70*$^{\#\#}$	24.19 ± 2.12$^{\#\#}$
GPx, nmol·min^{-1}·mg^{-1} protein	238.89 ± 23.26	250.61 ± 43.82**	186.96 ± 46.39$^{\#\#}$	357.86 ± 70.71**$^{\#\#}$

Data are presented as means ± standard deviation (n = 10); * – significant differences (P-value < 0.05) between the untreated control group and the unvaccinated group challenged with *Y. ruckeri*; ** – significant differences (P-value < 0.05) between the vaccinated group subjected to the *Y. ruckeri* challenge and the vaccinated and unchallenged group; # – significant differences (P-value < 0.05) between the untreated control group and the vaccinated and unchallenged group; ## – significant differences (P-value< 0.05) between the vaccinated group subjected to the *Y. ruckeri* challenge and the unvaccinated group challenged with *Y. ruckeri*

Table 2. Activity of alanine aminotransferase (ALT, nmol·min^{-1}·mg^{-1} protein) and aspartate aminotransferase (AST, nmol·min^{-1}·mg^{-1} protein), AST/ALT ratio, lactate dehydrogenase activity (LDH, nmol·min^{-1}·mg^{-1} protein), lactate and pyruvate levels (nmol·mg^{-1} protein) and the L/P ratio in the liver tissue of rainbow trout orally immunised against *Yersinia ruckeri* and challenged with this bacterium

Parameter	Group			
	Untreated control group	Vaccinated unchallenged group	Unvaccinated group challenged with *Yersinia ruckeri*	Vaccinated group challenged with *Yersinia ruckeri*
ALT, nmol·min^{-1}·mg^{-1} protein	4.46 ± 0.48*	4.25 ± 0.72	5.67 ± 0.65*$^{\#\#}$	4.21 ± 0.68$^{\#\#}$
AST, nmol·min^{-1}·mg^{-1} protein	3.36 ± 0.23*	3.48 ± 0.34	4.12 ± 0.33*$^{\#\#}$	3.26 ± 0.33$^{\#\#}$
AST/ALT ratio	0.75 ± 0.06	0.83 ± 0.11	0.76 ± 0.10	0.78 ± 0.09
LDH, nmol·min^{-1}·mg^{-1} protein	22.61 ± 0.81	21.87 ± 0.21	22.09 ± 0.42	22.20 ± 0.33
Lactate, nmol·mg^{-1} protein	63.50 ± 17.12*	74.61 ± 19.64	102.97 ± 18.09*$^{\#\#}$	64.94 ± 11.78$^{\#\#}$
Pyruvate, nmol·mg^{-1} protein	29.56 ± 6.13*	37.01 ± 5.96	45.37 ± 11.38*$^{\#\#}$	33.31 ± 5.50$^{\#\#}$
L/P ratio	2.22 ± 0.70	2.07 ± 0.64	2.39 ± 0.91	1.99 ± 0.49

Data are presented as means ± standard deviation (n = 10); * – significant differences (P-value < 0.05) between the untreated control group and the unvaccinated group challenged with *Y. ruckeri*; ## – significant differences (P-value < 0.05) between the vaccinated group subjected to the *Y. ruckeri* challenge and the unvaccinated group challenged with *Y. ruckeri*

β-N-acetylglucosaminidase activity ($F_{3,27}$ = 5.16, P-value = 0.002) decreased by 50.3% (P-value < 0.05) in unvaccinated challenged trout and decreased by 21.1% in vaccinated and unchallenged trout relative to the untreated control group. Its activity was 30.9% higher (P-value < 0.05) in the liver tissue of vaccinated rainbow trout challenged with *Y. ruckeri* than in the unvaccinated challenged group (Fig. 2D).

Discussion

Selected adaptive mechanisms to infection with *Y. ruckeri* were described by analysing the biochemical response of liver tissue in vaccinated rainbow trout. The activation of adaptive mechanisms in biochemical responses was confirmed, at least as far as the oxidative stress response and lysosomal function were concerned because TBARS levels in the liver tissue of vaccinated fish decreased after the *Y. ruckeri* challenge, and the activity of glutathione-related antioxidant enzymes increased, presumably because the cellular metabolism adapted to the vaccination response. However, the *Y. ruckeri* challenge also induced negative effects in the liver tissue of vaccinated trout by decreasing SOD and CAT activity relative to the challenged group and decreasing CAT activity relative to the vaccinated and unchallenged group. The results of regression and correlation analyses confirmed these observations and revealed significant relationships between the values of CAT and TAC (r = 0.495, P-value = 0.005), CAT and ALT (r = 0.459, P-value = 0.009), CAT and lactate (r = 0.424, P-value = 0.014), and CAT and the AST/ALT ratio (r = -0.462, P-value = 0.009).

Superoxide dismutase is an enzyme that participates in the cellular antioxidant defence system. It catalyses the dismutation (disproportionation) of the superoxide radical to hydrogen peroxide and molecular oxygen (36). Hydrogen peroxide is further converted to water and molecular oxygen through the action of CAT and

GPx (7), which alongside SOD are components of the first line of defence against ROS. The superoxide radical is generated during oxidative energy reactions as a product of one-electron reduction of molecular oxygen. It is produced in nearly all cells that consume oxygen; it can affect all cell components and the intercellular substance, and it also acts as a precursor for the more toxic hydroxyl radical (6). Reactive oxygen species are continuously produced during disease and respiratory burst, which causes oxidative stress and leads to enzyme inactivation, damage to nucleic acids, proteins and lipids, disorganisation of cell membranes, and inactivation of biochemical processes (27).

In multicellular organisms, inflammation is a protective response to damage which aims to localise, destroy and eliminate the pathogen and repair (or replace) damaged tissue (27). There is a growing body of evidence to suggest that ROS are involved in the initiation, development and termination of the inflammatory response. They act as bactericidal agents and as secondary messengers in intracellular signalling. They perform the latter function by post-translational modification of proteins containing redox-sensitive cysteine residues that can be oxidised. Excessive production of ROS can also seriously damage cells and tissues and can increase the activity of antioxidant enzymes (31). Therefore, the observed decrease in SOD and CAT activity in the liver tissue of vaccinated fish challenged with *Y. ruckeri* points to the down-regulation of ROS activity and suggests that ROS generation plays an important role during infection. These results suggest that *Y. ruckeri* inhibits the expression of these antioxidant enzymes *via* a specific mechanism. This observation is supported by recent research which has shown that a TIR domain-containing protein (STIR-2) that enhances virulence in *Y. ruckeri* can disrupt Myd88-mediated signalling to evade the innate immune system and inhibit pre-inflammatory signalling (25).

The analysis of changes in oxidative stress biomarkers in the liver tissue of vaccinated trout challenged with *Y. ruckeri* revealed a significant decrease in TBARS levels relative to the vaccinated and unchallenged group and the group exposed to the *Y. ruckeri* challenge but not vaccinated. No changes were observed in the carbonyl content of oxidatively modified proteins, and the activity of antioxidant enzymes involved in glutathione metabolism (GR – the final first-line-of-defence antioxidant enzyme – and GPx) was distinctly potentiated over this activity in the *Y. ruckeri*-challenged group. Notably lower activity of AST and ALT, which are specific biomarkers of cellular toxicity and liver and heart damage, and fallen lactate and pyruvate levels were noted in vaccinated fish after the *Y. ruckeri* challenge, set against those of the *Y. ruckeri*-challenged and unvaccinated group. In contrast, these parameters were elevated in the *Y. ruckeri*-challenged and unvaccinated group relative to the untreated group. These results suggest that vaccination exerts a protective effect during the *Y. ruckeri* challenge or that vaccination has no adverse effect on fish livers. The results of regression and correlation analyses confirmed these observations and revealed significant relationships between the values of GPx and ketone derivatives of OMP ($r = 0.529$, P-value = 0.002), and between GPx and LDH activity ($r = 0.369$, P-value = 0.041).

Reactive oxygen species play a critical role in both physiological and pathological adaptive immune responses. They are also involved in innate immune cell function by enhancing the killing capacity of phagocytes, which is an important step in the antigen processing and presentation functions of these phagocytes. Oxidative stressors also exert an indirect effect on adaptive immunity by modulating the interaction between innate and adaptive immunity (3). Ghosh *et al.* (13) found that oral vaccination of first-feeding Atlantic salmon (*Salmo salar* L.) confers greater protection against yersiniosis than immersion vaccination. Survival rates were higher in orally vaccinated groups than in immersion groups, which was attributed to adaptive responses in the cells of the innate immune system, in particular non-specific cytotoxic cells. In addition, Bridle *et al.* (5) reported on the expression of six genes in response to a *Y. ruckeri* infection and four genes that are associated with the protective host response to yersiniosis in Atlantic salmon. Real-time PCR confirmed an increase in the expression of three immunologically relevant genes – cathelicidin (47-fold), C-type lectin (19-fold), and the important tissue remodelling and repair enzyme collagenase (17-fold) – in response to yersiniosis. These genes represent three out of the six non-protective and/or pathological responses to yersiniosis. The expression of the immunoglobulin gene and the selenoprotein gene, which are associated with the protective host response, increased 15-fold, which highlights the importance of antibody-mediated protection against yersiniosis (5). The expression of several inflammatory genes as well as IgM and IgT genes increased in the gut of unvaccinated trout after the *Y. ruckeri* challenge and was correlated with a reduction in the severity of bacteraemia (11). The abundance of IgT on the gills of rainbow trout fry infected with *Y. ruckeri* is an indicator of potential antibody-mediated protection in young fish before vaccination (8).

Reactive oxygen species also stimulate phagocytic cells to eliminate pathogens, and oral vaccination against *Y. ruckeri* and the challenge with this bacterium may be critical for respiratory burst and an effective immune response. However, excessive ROS production can cause serious damage to cells and tissues and contribute to oxidative stress. In rainbow trout, the onset of oxidative stress induced by the *Y. ruckeri* challenge was expressed by the initiation of lipid peroxidation processes (TBARS levels), followed by an increase in the oxidative modification of proteins (estimated based on the levels of aldehyde derivatives of OMP) relative to the untreated control group. At the same time, the total antioxidant capacity in the liver of fish challenged with

Y. ruckeri decreased significantly. The results of regression and correlation analyses confirmed these observations and revealed significant relationships between the levels of TBARS and ketone derivatives of OMP ($r = -0.422$, P-value = 0.018).

The *Y. ruckeri* challenge stimulated antioxidant mechanisms in the liver tissue of challenged fish and increased the activity of the SOD and CAT antioxidant enzymes. At the same time, a significant decrease was noted in the activity of enzymes involved in glutathione metabolism. Glutathione reductase activity decreased in the liver of fish challenged with *Y. ruckeri*. In addition, the activity of AST and ALT (specific indicators of cellular toxicity), and lactate and pyruvate levels increased in the livers of fish challenged with *Y. ruckeri*. In fish so challenged, the activity of AST and ALT (which participate in ketone and amino acid metabolism) increased, and metabolic processes shifted towards glycolysis, as manifested by a greater increase in lactate than pyruvate levels. Increased metabolic activity may be required for effective antigen processing and presentation to stimulate a robust immune response during a *Y. ruckeri* challenge. The apparent detrimental effect of the *Y. ruckeri* infection on fish livers corroborates the results reported by Kumar *et al.* (21), who performed a proteomic analysis and demonstrated that lymphoid organs play an important role in rainbow trout infected with *Y. ruckeri*. Thirty-four proteins from the head kidney and 85 proteins from the spleen were found to be differentially expressed in rainbow trout with *Y. ruckeri* infections. These included lysosomal, antioxidant, metalloproteinase, cytoskeletal, tetraspanin, cathepsin B and c-type lectin receptor proteins (21).

The mechanisms involved in the biochemical response of rainbow trout to infection with *Y. ruckeri* were also analysed in the study. In vaccinated rainbow trout challenged with *Y. ruckeri*, oxygen-dependent processes and the resulting oxidation of free radicals were reduced by the directionality of cellular metabolism. This mechanism was also confirmed by the statistical redistribution of lactate and pyruvate levels, with a concomitant decrease in ALT and AST activity. In vaccinated fish challenged with *Y. ruckeri*, vaccination prevented the degradation of cell membranes and led to the activation of glutathione-related antioxidant enzymes (GR and GPx). Vaccination also induced changes in the energy metabolism of liver cells, and the substrate pool of metabolites was distributed in a way that prevented oxidative stress and liver damage. These vaccination effects were accompanied by a decrease in lipid peroxidation processes in the liver of vaccinated fish challenged with *Y. ruckeri* relative to the challenged group.

The liver is subjected to various types of stressors because of its functional and morphological characteristics. These stressors increase the activity of proteolytic enzymes, and their effect varies depending on the type of tissue and stressor and the animal's age. Vaccination against yersiniosis affects liver function and may modulate oxidative stress and the activity of lysosomal enzymes. The activity of AAP, LAP and AcP increased significantly after the *Y. ruckeri* challenge. Leucyl aminopeptidase activity also increased in the livers of vaccinated fish. These stressors could influence the activity of lysosomal enzymes by modifying the permeability of lysosomal membranes and the activity of proteolytic enzyme inhibitors. The results of regression and correlation analyses confirmed this hypothesis and revealed the presence of relationships between AAP and CAT activity ($r = -0.462$, P-value = 0.005), AcP activity and TAC levels ($r = -0.532$, P-value = 0.004), and AcP and CAT activity ($r = 0.542$, P-value = 0.003).

Vaccination affected lysosomal function in the liver tissue of vaccinated fish after the *Y. ruckeri* challenge, as well as the activity of lysosomal enzymes. In this group, LAP activity increased significantly, AcP activity decreased and NAG activity increased relative to the unvaccinated and challenged group (Fig. 2). Oral vaccination against *Y. ruckeri* affects liver function, and it may modulate the rate of lipid peroxidation and the activity of lysosomal enzymes. Significant changes in AAP, LAP and AcP activity were observed after the *Y. ruckeri* challenge, which could have contributed to lysosomal degradation by increasing the permeability of lysosomal membranes. Vaccination affected the lysosomal balance and the activity of lysosomal enzymes after the *Y. ruckeri* challenge. A significant decrease in LAP and AcP activity with a concomitant increase in NAG activity were observed in vaccinated fish after the *Y. ruckeri* challenge.

One of the main limitations of this study is the relatively small sample size, which may limit statistical power and the ability to generalise the results. Variability between individual fish and environmental factors could also influence the results, potentially masking or exaggerating the effects observed. The study was conducted in controlled laboratory conditions, which may not perfectly replicate the natural environment of rainbow trout. Factors such as water temperature, pH and ecological interactions in natural habitats may affect oxidative stress biomarkers and enzyme activity differently. The study focused on the immediate biochemical and enzymatic responses following *Y. ruckeri* challenge. Long-term effects and the potential for delayed responses were not investigated, limiting the understanding of chronic effects and the full recovery process.

Future research should include long-term studies to monitor the chronic effects of *Y. ruckeri* infection and vaccination on oxidative stress and lysosomal enzyme activity. This would provide a more complete understanding of the dynamics of the immune response and recovery. Conducting similar studies in natural or semi-natural environments would help to validate the laboratory findings and assess the real-world applicability of the biomarkers. It would also take into account environmental variability and ecological interactions that affect fish health. Expanding the range of biomarkers to include additional indicators of oxidative stress, inflammatory

cytokines and metabolic enzymes would provide a more holistic view of the physiological changes occurring in vaccinated and infected fish. The results of this study could be used to develop more effective vaccines and immunisation strategies. Understanding the biochemical and enzymatic responses to vaccination and infection may help to optimise vaccine formulations to enhance protective immunity and minimise adverse effects. Investigating the combined effects of environmental stressors (*e.g.* temperature changes and pollution) and bacterial challenges on oxidative stress and enzyme activity could provide insights into how multiple stressors interact to affect fish health. This would be particularly relevant for the management of aquaculture systems under changing environmental conditions.

Conclusion

The *Y. ruckeri* challenge disrupted the pro-oxidant/antioxidant balance and led to a significant increase in lipid peroxidation and oxidative modification of proteins, a decrease in total antioxidant capacity, and a significant increase in the activity of lysosomal enzymes. Vaccination influenced lysosomal function in fish livers and prevented damage to lysosomal membranes by increasing LAP activity, decreasing NAG activity, leading to oxidative stress and cell death. In vaccinated fish subjected to the *Y. ruckeri* challenge, lipid peroxidation decreased, the activity of glutathione-related enzymes increased, anaerobic metabolism decreased, and the activity of lysosomal enzymes decreased relative to the challenged group. The *Y. ruckeri* challenge of vaccinated fish also decreased lipid peroxidation and stimulated antioxidant defences relative to the vaccinated group. The study demonstrated that the analysed biomarkers could be useful for assessing fish welfare after vaccination. As demand grows for alternative treatments to maintain the health of farmed fish, these findings could be important for the provision of veterinary medical care in aquaculture.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This research received no external funding.

Animal Rights Statement: This study was conducted at the Stanisław Sakowicz Inland Fisheries Institute (Olsztyn, Poland). The research complied with the Polish animal welfare regulations and was approved by the Local Ethics Committee for Animal Experimentation of The Stanisław Sakowicz Inland Fisheries Institute in Olsztyn, Poland (approval no. 4/2013).

References

1. Barnes A.C.: Chapter 13, Enteric Redmouth Disease (ERM) (*Yersinia ruckeri*). In: *Fish diseases and disorders*, Volume 3, Viral, bacterial and fungal infections, Third Edition, edited by P.T.K. Woo, J.F. Leatherland, D.W. Bruno, CAB International Publishing, Wallingford, UK, 2011, pp. 484–511.
2. Barrett A.J., Heath M.F.: Lysosomal enzymes. In: *Lysosomes, a Laboratory Handbook*, edited by J.T. Dingle, North-Holland Publishing Company, New York, NY, USA, 1977, pp. 19–146.
3. Bassoy E.Y., Walch M., Martinvalet D.: Reactive Oxygen Species: Do They Play a Role in Adaptive Immunity? Front Immunol 2021, 12, 755856, doi: 10.3389/fimmu.2021.755856.
4. Bradford M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976, 72, 248–254, doi: 10.1006/abio.1976.9999.
5. Bridle A.R., Koop B.F., Nowak B.F.: Identification of surrogates of protection against yersiniosis in immersion vaccinated Atlantic salmon. PLoS One 2012, 7, e40841, doi: 10.1371/journal.pone.0040841.
6. Cadenas E., Davies K.J.: Mitochondrial free radical generation, oxidative stress, and aging. Free Radic Biol Med 2000, 29, 222–230, doi: 10.1016/s0891-5849(00)00317-8.
7. Chelikani P., Fita I., Loewen P.C.: Diversity of structures and properties among catalases. Cell Mol Life Sci 2004, 61, 92–208, doi: 10.1007/s00018-003-3206-5.
8. Chettri J.K., Raida M.K., Kania P.W., Buchmann K.: Differential immune response of rainbow trout (*Oncorhynchus mykiss*) at early developmental stages (larvae and fry) against the bacterial pathogen *Yersinia ruckeri*. Dev Comp Immunol 2012, 36, 463–474, doi: 10.1016/j.dci.2011.08.014.
9. DeMartino G.N., Goldberg A.L.: Thyroid hormones control lysosomal enzyme activities in liver and skeletal muscle. Proc Natl Acad Sci U S A 1978, 75, 1369–1373, doi: 10.1073/pnas.75.3.1369.
10. Deshmukh S., Raida M.K., Dalsgaard I., Chettri J.K., Kania P.W., Buchmann K.: Comparative protection of two different commercial vaccines against *Yersinia ruckeri* serotype O1 and biotype 2 in rainbow trout (*Oncorhynchus mykiss*). Vet Immunol Immunopathol 2012, 145, 379–385, doi: 10.1016/j.vetimm.2011.12.014.
11. Evenhuis J.P., Cleveland B.M.: Modulation of rainbow trout (*Oncorhynchus mykiss*) intestinal immune gene expression following bacterial challenge. Vet Immunol Immunopathol 2012, 146, 8–17, doi: 10.1016/j.vetimm.2012.01.008.
12. Galaktionova L.P., Molchanov A.V., El'chaninova S.A., Varshavskiĭ B.: Sostoianie perekisnogo okisleniia u bol'nykh s iazvennoĭ bolezn'iu zheludka i dvenadtsatiperstnoĭ kishki (Lipid peroxidation in patients with gastric and duodenal peptic ulcers – in Russian). Klin Lab Diagn 1998, 6, 10–14.
13. Ghosh B., Nguyen T.D., Crosbie P.B.B., Nowak B.F., Bridle A.R.: Oral vaccination of first-feeding Atlantic salmon, *Salmo salar* L., confers greater protection against yersiniosis than immersion vaccination. Vaccine 2016, 34, 599–608, doi: 10.1016/j.vaccine.2015.12.044.
14. Glatzle D., Vuilleumier J. P., Weber F., Decker K.: Glutathione reductase test with whole blood, a convenient procedure for the assessment of the riboflavin status in humans. Experientia 1974, 30, 665–667, doi: 10.1007/BF01921531.
15. Herasimov I., Plaksina O.: Non-enzymatic determination of lactate and pyruvate concentrations in blood sample (in Ukrainian). Laboratorna Diagnostyka 2000, 1, 46–48.
16. Horne M.T., Barnes A.C.: Chapter 13, Enteric Redmouth Disease (*Yersinia ruckeri*) In: *Fish diseases and disorders*, Volume 3, Viral, bacterial and fungal infections, First Edition, edited by P.T.K. Woo, D.W. Bruno, CAB International Publishing, Wallingford, UK, 1999, pp. 445–477.
17. Ingerslev H.C., Strube M.L., von Gersdorff Jørgensen L., Dalsgaard I., Boye M., Madsen L.: Diet type dictates the gut microbiota and the immune response against *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol 2014, 40, 624–633, doi: 10.1016/j.fsi.2014.08.021.

18. Kamyshnikov V.: Determination of the content of TBA active substances (malondialdehyde) in blood erythrocytes. In: *Reference Book for Clinical and Biochemical Research and Laboratory Diagnostics* (in Russian). MED Press-inform, Moscow, 2009, pp. 549–550.
19. Koroliuk M.A., Ivanova L.I., Maĭorova I.G., Tokarev V.E.: Metod opredeleniia aktivnosti katalazy (A method of determining catalase activity – in Russian). Lab Delo 1988, 1, 16–19.
20. Kostiuk V.A., Potapovich A.I., Kovaleva Zh.V.: Prostoĭ i chuvstvitel'nyĭ metod opredeleniia aktivnosti superoksid-dismutazy, osnovannyĭ na reaktsii okisleniia kvertsetina (A simple and sensitive method of determination of superoxide dismutase activity based on the reaction of quercetin oxidation – in Russian). Vopr Med Khim 1990, 36, 88–91.
21. Kumar G., Hummel K., Noebauer K., Welch T.J., Razzazi-Fazeli E., El-Matbouli M.: Proteome analysis reveals a role of rainbow trout lymphoid organs during *Yersinia ruckeri* infection process. Sci Rep 2018, 8, 13998, doi: 10.1038/s41598-018-31982-6.
22. Kumar G., Menanteau-Ledouble S., Saleh M., El-Matbouli M.: *Yersinia ruckeri*, the causative agent of enteric redmouth disease in fish. Vet Res 2015, 46, 103, doi: 10.1186/s13567-015-0238-4.
23. Kurhaluk N., Tkachenko H.: Antioxidants, lysosomes and elements status during the life cycle of sea trout *Salmo trutta* m. *trutta* L. Sci Rep 2021, 11, 5545, doi: 10.1038/s41598-021-85127-3.
24. Levine R.L., Garland D., Oliver C.N., Amici A., Climent I., Lenz A.G., Ahn B.W., Shaltiel S., Stadtman E.R.: Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 1990, 186, 464–478, doi: 10.1016/0076-6879(90)86141-h.
25. Liu T., Wang E., Wei W., Wang K., Yang Q., Ai X.: TcpA, a novel *Yersinia ruckeri* TIR-containing virulent protein mediates immune evasion by targeting MyD88 adaptors. Fish Shellfish Immunol 2019, 94, 58–65, doi: 10.1016/j.fsi.2019.08.069.
26. McDonald J.K., Barrett A.J.: Exopeptidases. In: *Mammalian Proteinases: A Glossary and Bibliography*, edited by A.J. Barrett, J.K. McDonald, Academic Press, London, UK, 1986, pp. 114–144.
27. Mittal M., Siddiqui M.R., Tran K., Reddy S.P., Malik A.B.: Reactive oxygen species in inflammation and tissue injury. Antioxid Redox Signal 2014, 20,1126–1167, doi: 10.1089/ars.2012.5149.
28. Moin V.M.: Prostoĭ i spetsificheskiĭ metod opredeleniia aktivnosti glutationperoksidazy v éritrotsitakh (A simple and specific method for determining glutathione peroxidase activity in erythrocytes – in Russian). Lab Delo 1986 12, 724–727.
29. Reitman S., Frankel S.: A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am J Clin Pathol 1957, 28, 56–63, doi: 10.1093/ajcp/28.1.56.
30. Sevela M., Tovarek J.: Metoda stanovení laktikodehydrogenázy v tělních tekutinách (Method for the estimation of lactic dehydrogenase – in Czech). Cas Lek Cesk 1959, 98, 27, 844–848.
31. Spooner R., Yilmaz O.: The role of reactive-oxygen-species in microbial persistence and inflammation. Int J Mol Sci 2011, 12, 334–352, doi: 10.3390/ijms12010334.
32. Stanisz A.: *An accessible statistics course using STATISTICA PL with examples from medicine;* Volume 1, Basic statistics; Volume 2, Linear and non-linear models; Volume 3, Multivariate analyses, StatSoft Polska, Krakow, 2006, 2007, pp. 532, pp. 868, pp. 500.
33. Tobback E., Decostere A., Hermans K., Haesebrouck F., Chiers K.: *Yersinia ruckeri* infections in salmonid fish. J Fish Dis 2007, 30, 257–268, doi: 10.1111/j.1365-2761.2007.00816.x.
34. Villumsen K.R., Neumann L., Ohtani M., Strøm H.K., Raida M.K.: Oral and anal vaccination confers full protection against enteric redmouth disease (ERM) in rainbow trout. PLoS One 2014, 9, e93845, doi: 10.1371/journal.pone.0093845.
35. Wheeler R.W., Davies R.L., Dalsgaard I., Garcia J., Welch T.J., Wagley S., Bateman K.S., Verner-Jeffreys D.W.: *Yersinia ruckeri* biotype 2 isolates from mainland Europe and the UK likely represent different clonal groups. Dis Aquat Organ 2009, 84, 25–33, doi: 10.3354/dao02039.
36. Younus H.: Therapeutic potentials of superoxide dismutase. Int J Health Sci (Qassim) 2018, 12, 88–93.