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RESEARCH ARTICLE

Time-dependent changes in oxidative stress biomarkers and activities of lysosomal and antioxidant enzymes in hepatic tissue of rainbow trout (*Oncorhynchus mykiss* Walbaum) following vaccination against *Yersinia ruckeri*

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Abstract. This study analyzed time-dependent effects of vaccination against *Y. ruckeri* on the oxidative mechanism underlying those effects by detecting relevant lipid peroxidation (2-thiobarbituric acid reactive substances, TBARS) and protein oxidation biomarkers [aldehydic and ketonic derivatives of oxidatively modified proteins (OMP)], antioxidant defenses [activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), total antioxidant capacity (TAC)], as well as activities of lysosomal functioning [alanyl aminopeptidase (AAP), leucyl aminopeptidase (LAP), acid phosphatase (AcP), and β-N-acetylglucosaminidase (NAG)] in hepatic tissue of rainbow trout, *Oncorhynchus mykiss* (Walbaum) following anti-*Y. ruckeri* vaccination in the first, second, and sixth months. A concentrated vaccine with *Y. ruckeri* strains was enclosed in fish feed and was administered three times every other day. Rainbow trout from each group were euthanized 31, 61, and 181 days following vaccination, and hepatic tissue was sampled for analysis. In the current study, vaccination against *Y. ruckeri* resulted in a no statistically significant change in TBARS levels, while aldehydic and ketonic derivatives of OMP in hepatic tissue decreased, especially after the first and second months following immunization. Moreover, the activities of glutathione-dependent enzymes increased, especially after the first and sixth months. The highest TAC levels were observed two and six months after vaccination. It has been shown that vaccination-related oxidative stress in hepatic tissue is involved in adaptive responses through the temporary mobilization of antioxidant and lysosomal enzymes in rainbow trout. The present study showed the effect of vaccination on lysosome membrane permeability for carbohydrate cleavage after the development of immunity against *Yersinia*, whereas antioxidant defence was reduced. Our results confirmed that the concept of preserving antioxidant enzyme function after vaccination was also

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evident when CAT, GR, and GPx activities either increased or were unchanged following vaccination.

Keywords: Yersiniosis, oral vaccination, aquaculture, immunization, oxidative stress, antioxidant defense, lysosomal enzymes

Introduction

Yersinia ruckeri is a ubiquitous finfish pathogen capable of causing major mortalities in farmed fish stocks (Ghosh et al. 2016). This bacterium is the etiological agent of enteric redmouth disease (ERM) of farmed salmonids (Ormsby et al. 2016; Wangkahart et al. 2019). The name of the disease comes from the characteristic symptom of petechiae around the mouth (hence, redmouth disease) (Kumar et al. 2015). Subcutaneous hemorrhages can form at the corners of the mouth and in the gums and tongue. Other clinical signs include exophthalmia, darkening of the skin, splenomegaly, and inflammation of the lower intestine with an accumulation of thick yellow fluid. The bacterium enters the fish via the secondary gill lamellae, and from there, it spreads to the blood and internal organs (Kumar et al. 2015). Gills are regarded as the entry route of *Y. ruckeri* rods, but the likelihood of the disease depends on the virulence of the given strain. Characteristic clinical signs of yersiniosis, such as hemorrhages around the oral cavity, are caused by extracellular products (ECPs) of *Y. ruckeri* (Pękala and Antychowicz 2010).

Y. ruckeri is a bacterium with a global distribution. Rainbow trout are considered the most susceptible to this disease, but it has also been isolated from infected specimens of other fish species and from clinically healthy fish (Pajdak-Czaus et al. 2019). Its importance is also increasing as a pathogen of Atlantic salmon (*Salmo salar*), which causes a disease called salmon blood spot, or yersiniosis, which is usually milder than ERM (Austin and Austin 2016, Ormsby and Davies 2017). ERM is successfully controlled with commercial vaccines and, in fact, represents one of the first diseases to be controlled by immunization. The success of the vaccine has been reported to be variable under field conditions and often does not completely prevent disease outbreaks when the level of infection is high, as seen when fish are stressed (Horne and Barnes 1999, Nguyen et al. 2018, Jaafar et al. 2019, Yang et al. 2021). A greater understanding of the fish response against *Y. ruckeri* and during vaccination against ERM will help improve this situation.

Vaccinating rainbow trout against ERM by immersion in *Y. ruckeri* bacterin confers a high degree of protection to the fish (Raida et al. 2011). ERM has been controlled successfully using immersion-applied bacterin vaccines for several decades (Welch and LaPatra 2016). On the other hand, oral administration is the ideal method for administering vaccines to fish, whereby the vaccine is incorporated into fish feed. It is less labor-intensive than injection or immersion and is suitable for vaccinating large numbers of fish of all sizes, and it avoids the handling stress experienced by the fish with the other two methods. The major disadvantage of this route of administration is that lower levels of protection are achieved, and the duration of protection elicited is shorter (Thompson and Adams 2004).

It was shown that anal intubation resulted in a protective response in salmonids against *Y. ruckeri* and *Vibrio anguillarum* (Johnson and Amend 1983). This fact suggests that immune cells in the posterior gut are capable of antigen uptake and processing. In fact, research has shown that fish possess gut-associated lymphoid tissue (GALT) in the second gut segment (Quentel and Vigneulle 1997). Since one of the major problems associated with oral vaccination is the degradation of antigen by the gastric fluid in the stomach and anterior gut of the fish, the antigen can be inactivated by the time it reaches the posterior part of the intestine (Thompson and Adams 2004). Many studies have been carried out to examine the efficacy of oral vaccines in fish (Quentel and Vigneulle 1997). These studies have looked at the types of immune responses stimulated by oral vaccination and the levels of protection conferred, but varying degrees of success have been reported in the literature. These variations are believed to stem from differences in experimental design among studies, including

antigen preparation, the age and species of fishes, the water temperature at the time of vaccination, the duration of vaccine feeding, and antigen integrity when it reaches the posterior gut (Thompson and Adams 2004).

Exploring the time-dependent effects of vaccination against *Y. ruckeri* on the health condition of trout, in general, and oxidative stress and antioxidant defense biomarkers in different tissues, specifically, would be valuable. It is hypothesized that the oxidative stress biomarkers and lysosomal enzymes might be sensitive to vaccination against *Y. ruckeri* and could potentially be used as biomarkers for assessing the health and welfare of rainbow trout. The present study aims to analyze the effects of vaccination against *Y. ruckeri* on the oxidative mechanisms and the lysosomal function underlying those effects by detecting relevant antioxidant defenses in trout that were vaccinated orally against *Y. ruckeri* in the first, second, and sixth months after immunization. Thus, the current study used biomarkers of lipid peroxidation (2-thiobarbituric acid reactive substances, TBARS) and protein oxidation (aldehydic and ketonic derivatives of oxidatively modified proteins [OMP]), antioxidant defenses (activities of superoxide dismutase [SOD], catalase [CAT], glutathione reductase [GR], glutathione peroxidase [GPx], total antioxidant capacity [TAC]), as well as activities of lysosomal functioning (alanyl aminopeptidase [AAP], leucyl aminopeptidase [LAP], acid phosphatase [AcP], and β-N-acetylglucosaminidase [NAG]) in hepatic tissue of rainbow trout to evaluate the effects of immunization against *Y. ruckeri* in the first, second, and sixth months after oral vaccination.

Materials and methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum) with body weights ranging from 105–135 g were used in the experiments. The study was conducted at the Department of Salmonid Research, Stanisław Sakowicz Inland Fisheries Institute in Olsztyn (Poland). The experiments were performed at a water temperature of 14.5 ± 0.5°C, and a pH of 7.5. The dissolved oxygen level was about 12 ppm with an additional oxygen supply at a water flow rate of 25 L per min, and a photoperiod of 12 hours per day. The fish were fed a commercial pelleted diet at the optimal level using 12-hour belt feeders for fish. The daily dose of feed was calculated according to the applicable table feed (From and Rasmussen 1984). All enzymatic assays were carried out at the Department of Zoology and Animal Physiology, Institute of Biology and Earth Sciences, Pomeranian University in Słupsk (Poland).

Experimental design

The fish were divided into two groups: I) control, II) vaccinated with a *Y. ruckeri* vaccine. The fish were held in 1,000 L square tanks (150 fish per tank) under the same environmental conditions. The vaccine was produced at the Department of Fish Diseases, National Veterinary Research Institute in Pulawy (Poland) according to the procedure in patent no. P.428259. The vaccine at a concentration of $1 \cdot 10^9$ cells per mL was used to inoculate fish orally. Vaccine concentrate was added to fish feed and administered three times at one-day intervals between feedings.

The fish were kept for 30, 60, and 180 days after vaccination at a water temperature of 14.5 ± 0.5°C and pH 7.5. In the current study, 15 rainbow trout from the unhandled control group and 15 vaccinated trout were used before vaccination, as well as after the first, second, and sixth months after vaccination. Liver samples from rainbow trout were collected.

Sampling

The animals were caught and sacrificed before vaccination, as well as 31, 61, and 181 days following vaccination. The liver was removed *in situ*. The organs were rinsed clear of blood with cold isolation buffer

and homogenized with a H500 glass homogenizer with a motor-driven pestle immersed in an ice water bath to yield a homogenate in a proportion of 1:9 (weight/volume). The isolation buffer contained 100 mM Tris-HCl, and pH 7.2 was adjusted with HCl. Homogenates were centrifuged at 3,000 rpm for 15 min at 4°C. After centrifugation, the supernatant was collected and frozen at −25°C until analysis. The protein content was determined using the Bradford method (1976) with bovine serum albumin as the standard. Absorbance was recorded at 595 nm. All assays were carried out at 22 ± 0.5°C in duplicate. The enzymatic reactions were started by adding the tissue supernatant. The specific assay conditions were as follows. For lysosomal enzyme assays, hepatic tissue was removed, weighed, washed in ice-cold buffer, and minced. The minced tissues were rinsed with cold 0.15 M KCl isolation buffer to remove blood and then homogenized in a glass Potter-Elvehjem homogenizer with a motor-driven Teflon pestle on ice. The isolation buffer consisted of 0.25 M sucrose and 2 mM EDTA; the pH value was adjusted to 7.0 with KOH. The homogenates of hepatic tissue 20% (w/v) were prepared for the next differential centrifugation according to the method described in DeMartino and Goldberg (1978). After centrifugation, the supernatant fractions were saved and used after resuspension in 50 mM acetic acid/sodium acetate buffer, pH 5.0. The isolated fractions were homogenized and subjected to two freeze-thaw cycles (Kurhaluk and Tkachenko 2021).

Oxidative stress biomarkers assay

For the assay of 2-thiobarbituric acid reactive substances (TBARS), an aliquot of the homogenate was used to determine the lipid peroxidation status of the sample according to Kamyshnikov (2004). TBARS values were reported as nmoles of malonic dialdehyde (MDA) per mg of protein. Carbonyl groups of oxidatively modified proteins were measured as an indication of oxidative damage to proteins according to the method in Levine et al. (1990) and modified in Dubinina et al. (1995). The carbonyl content was measured spectrophotometrically at 370 nm (aldehydic derivatives, OMP_{370}) and at 430 nm (ketonic derivatives, OMP_{430}) (molar extinction coefficient 22,000 $M^{-1} \cdot cm^{-1}$) and expressed as nmol per mg of protein.

Superoxide dismutase (SOD) activity was assessed by its ability to dismutate superoxide produced during quercetin auto-oxidation in an alkaline medium (pH 10.0) using the method described in Kostiuk et al. (1990). The activity was expressed in units of SOD per mg of tissue protein. Catalase (CAT) activity was determined by measuring the decrease of H_2O_2 in the reaction mixture using a spectrophotometer at a wavelength of 410 nm using the method described in Koroliuk et al. (1988). One unit of CAT activity was defined as the amount of enzyme required to decompose 1 μmol H_2O_2 per min per mg of protein. Glutathione reductase (GR) activity was measured according to the method described in Glatzle et al. (1974) with some modifications. GR activity was expressed as nmol of $NADPH_2$ per min per mg of protein. Glutathione peroxidase (GPx) activity was determined by detecting the non-enzymatic utilization of reduced glutathione (GSH – the reacting substrate) at an absorbance of 412 nm after incubation with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) according to the method of Moin (1986). Glutathione peroxidase activity was expressed as nmol GSH per min per mg of protein. The total antioxidant capacity (TAC) level was estimated spectrophotometrically at 532 nm following the method with Tween 80 oxidation (Galaktionova et al. 1998). TAC level was expressed in %.

Lysosomal enzyme assays

The activity of lysosomal alanyl aminopeptidase (AAP) and leucyl aminopeptidase (LAP) was determined spectrophotometrically according to McDonald and Barrett (1986) as Fast Blue BB salt (4-benzoyloamino-2,5-diethoxybenzene-diazonium chloride) derivatives at 540 nm. L-alanyl-2-naphtylamine in 0.1M PBS buffer was used as the substrate for determinations of AAP activity, and L-leucyl-2-naphtylamine in 0.1M

PBS pH 7.0 buffer was used as the substrate for determinations of LAP activity. The activities of other lysosomal enzymes, such as acid phosphatase (AcP) and β-N-acetylglucosaminidase (NAG), were determined spectrophotometrically as 4-nitrophenyl derivatives at 420 nm with the method of Barrett and Heath (1977). The activities of enzymes were expressed in nmol per h per mg of protein.

Statistical analysis

The results were expressed as mean ± SD. Statistical analysis was conducted with STATISTICA 13.3 (TIBCO Software Inc.). Significant differences among means were measured using a multiple range test at min. $P < 0.05$. Data without a normal distribution were log-transformed. Statistical tests with 95% confidence intervals ($\alpha = 0.05$) were applied to determine the significance of differences among the parameters studied (Stanisz 2006). The data were tested for homogeneity of variance using Levene's test, and normality was checked with the Kolmogorov-Smirnov test. Student's *t*-test was used for paired comparisons for data with normal distribution. ANOVA was performed to compare values in the untreated and vaccinated groups before vaccination, as well as in the first, second, and sixth months after vaccination. We should note that as can be seen from the objectives of our study and the detailed study protocol presented, we investigated four groups (before vaccination, as well as in the first, second, and sixth months after vaccination) relative to their controls in two versions (unvaccinated and vaccinated groups). It was this study design that helped us later to assess the level of oxidative stress and lysosomal destruction processes in each specific time interval in pairwise comparisons using Student's *t*-test.

Results

Oxidative stress biomarkers

The data from this study indicated that the level of lipid peroxidation in hepatic tissue of trout vaccinated with the anti-*Yersinia* vaccine was not significantly different compared to the untreated groups six months after vaccination (Fig. 1).

The highest TBARS value was noted in the first month after vaccination (by 62.7%, $P < 0.05$), with a decrease in the second month (by 46.8%, $P < 0.05$), and a subsequent increase after six months (by 24.9%, $P > 0.05$) compared to the value before vaccination (0 months). Similarly, to the vaccinated group, the highest TBARS value in hepatic tissue of the untreated control group was recorded in the first month (by 59.2%, $P < 0.05$), with a decrease in the second month (by 47.9%, $P < 0.05$), and a subsequent increase after six months (by 5.2%, $P > 0.05$) compared to the value before vaccination (Fig. 1). A statistically significant decrease in TBARS values between the untreated and vaccinated groups was noted after one month (by 67.3% and 67.2%, $P < 0.05$, respectively) compared to values two months after vaccination. In contrast, a statistically significant increase in TBARS values between untreated and vaccinated groups was noted one month after vaccination (by 51.3% and 30.3%, $P < 0.05$, respectively) compared to values after six months. Similarly, a statistically significant increase in TBARS values between untreated and vaccinated groups was noted two months after vaccination (by 101.9% and 134.9%, $P < 0.05$, respectively) compared to values after six months (Fig. 1).

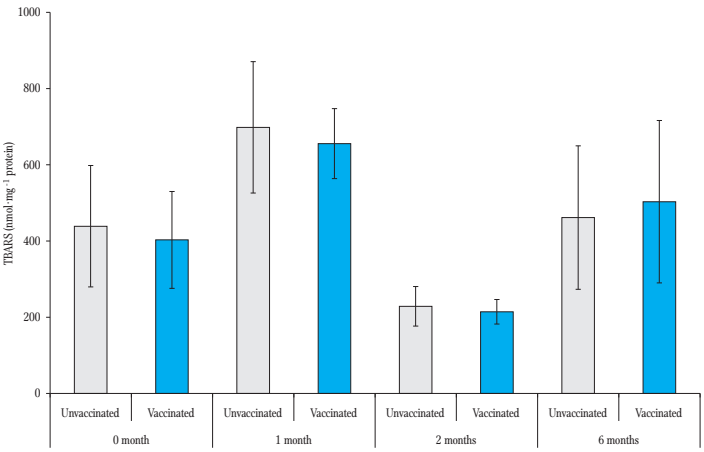

Fig. 1. Level of lipid peroxidation (nmol TBARS•mg^{-1} protein) in hepatic tissue of rainbow trout (*O. mykiss*) treated with a vaccine against *Y. ruckeri* in the first, second, and six months after vaccination. Data are presented as mean ± SD, n = 15.

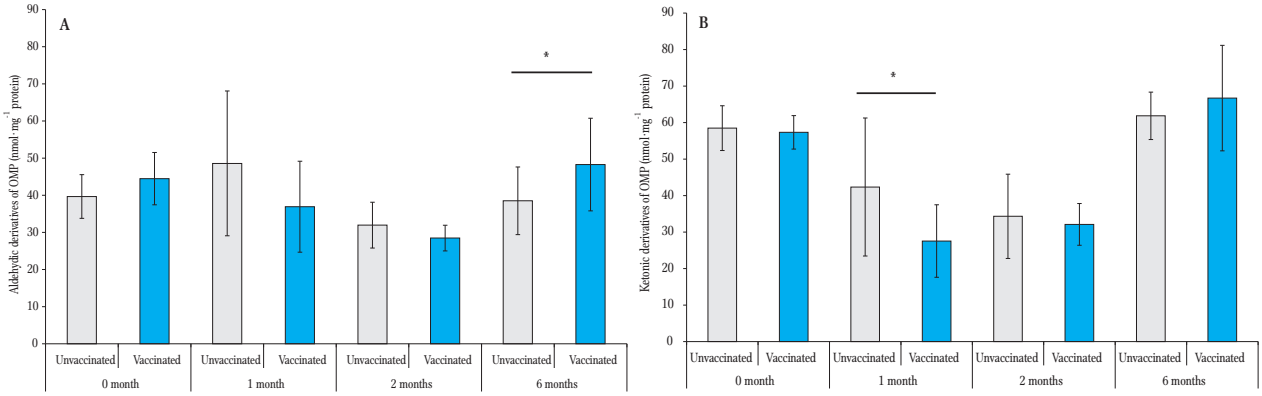

Fig. 2. Levels of aldehydic (A) and ketonic derivatives (B) of oxidatively modified proteins (nmol•mg^{-1} protein) in hepatic tissue of rainbow trout (*O. mykiss*) treated with the vaccine against *Y. ruckeri* in the first, second, and six months after vaccination. Data are presented as mean ± SD, n = 15. ANOVA was used to perform a comparative analysis between values for untreated and vaccinated groups in the first, second, and sixth months after vaccination (*P<0.05).

The levels of aldehydic and ketonic derivatives of oxidatively modified proteins in hepatic tissue of trout treated with the vaccine against *Y. ruckeri* in the first, second, and sixth months after immunization are presented in Fig. 2. Increased levels of aldehydic derivatives of OMP (by 22.4%, P < 0.05) in hepatic tissue of untreated trout were noted before vaccination, while decreases were observed two months after immunization (by 34.2%, P < 0.05) compared to values one month after immunization. Decreased aldehydic derivatives of OMP in hepatic tissue of the vaccinated group were noted one and two months after immunization (by 17% and 28.2%, P < 0.05, respectively) compared to the group before immunization, as well as two months after immunization (by 22.9%, p < 0.05) compared to values one month after immunization. On the other hand, increased aldehydic derivatives of OMP in hepatic tissue of the vaccinated group were observed six months after immunization compared to values one and two months after immunization (by 30.8% and 34.2%, P < 0.05, respectively) (Fig. 2a).

Similar to aldehydic derivatives of OMP, decreased levels of ketonic derivatives of OMP in hepatic tissue of untreated trout were noted one and two months after immunization (by 27.6% and 41.3%, P < 0.05, respectively) compared to the group before immunization, while increased ketonic derivatives of OMP in hepatic tissue of the untreated group were observed six months after immunization compared to values one and two months after immunization (by 46% and 80.2%, P < 0.05, respectively). Decreased ketonic derivatives of OMP in hepatic tissue of the vaccinated group were observed one and two months after immunization (by 52% and 44%, P < 0.05, respectively) compared to the group before immunization, as well as an increase two months after immunization (by 16.5%, P < 0.05) compared to values one month after immunization and six months after immunization compared to values two months after immunization (by 107.8%, P < 0.05) (Fig. 2a).

TAC levels in hepatic tissue of trout treated with the vaccine against *Y. ruckeri* one, two, and six months after immunization are presented in Fig. 3. The highest TAC value was noted in the second and sixth months after immunization (by 264.1% and 232.6%, P < 0.05) compared to that noted before vaccination (0 months). Similar to the vaccinated group, the highest TAC value in hepatic tissue of the untreated control group was noted in the second and sixth months (by 142.2% and 109.4%, P < 0.05) compared to the value recorded before vaccination. Statistically significant increased TAC values were noted in the control and vaccinated groups two (by 136.3% and 253.5%, P < 0.05, respectively) and six months after vaccination (by 104.3% and 222.9%, P < 0.05, respectively) compared to values one month after vaccination (Fig. 3).

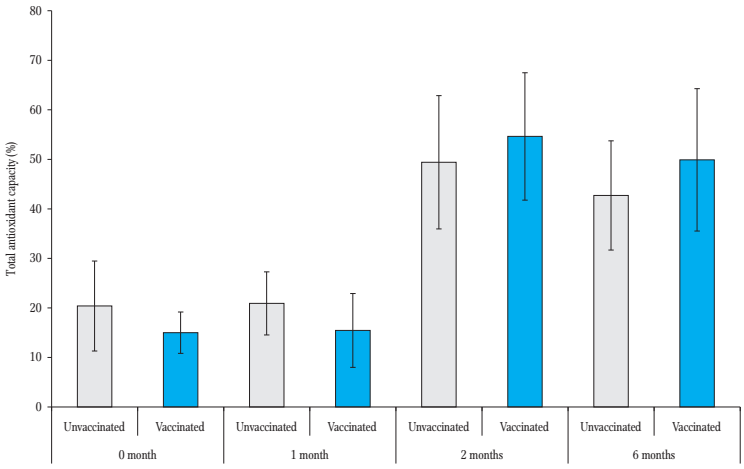

Fig. 3. TAC levels (%) in hepatic tissue of rainbow trout (*O. mykiss*) treated with the vaccine against *Y. ruckeri* in the first, second, and sixth months after vaccination. Data are presented as mean ± SD, n = 15.

Antioxidant enzymes

The activities of the major antioxidant enzymes in hepatic tissue of rainbow trout at the first, second, and sixth months after oral vaccination with anti-*Yersinia* vaccine are presented in Table 1. The antioxidant enzymes that provide cellular defense against reactive oxygen species (ROS) include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), and glutathione reductase (GR). However, an imbalance between the activities of cellular antioxidant enzymes and ROS production results in oxidative stress and cellular damage.

Decreased SOD activity in hepatic tissue of vaccinated trout was noted in groups before vaccination, and also one and six months after immunization (by 13.4%, 8.8%, and 14.6%, $P < 0.05$, respectively) compared to the untreated group. In the untreated group, SOD activity in hepatic tissue decreased after the first month following immunization (by 51.2%, $P < 0.05$), which was followed by an increase at six months after immunization (by 242.5%, $P < 0.05$) compared to the values before vaccination. On the other hand, an increase in SOD activity in hepatic tissue of the untreated group was observed two and six months after immunization compared to values after one month (by 128.5% and 601.2%, $P < 0.05$, respectively). The highest SOD activity was observed six months after immunization, and an increase in SOD activity was noted six months after immunization (207%, $P < 0.05$) compared to values two months after immunization. The results for the vaccinated group were similar. A decrease in SOD activity in hepatic tissue was observed two and six months after immunization compared to values from before immunization (by 41.6% and 238.1%, $P < 0.05$,

Table 1

Activities of the superoxide dismutase (SOD, $U \cdot mg^{-1}$ protein), catalase (CAT, $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein), glutathione reductase (GR, $nmol \cdot min^{-1} \cdot mg^{-1}$ protein), and glutathione peroxidase (GPx, $nmol \cdot min^{-1} \cdot mg^{-1}$ protein) in hepatic tissue of rainbow trout 1, 2 and 6 months after oral vaccination with an anti-*Yersinia* vaccine. Data are represented as mean ± SD, n = 15. ANOVA was used to perform a comparative analysis between values for untreated and vaccinated groups in the first, second, and sixth months after vaccination. Data in the same column and the same month that were statistically significantly different are marked with an asterisk ($P<0.05$).

Periods	Groups	SOD	CAT	GR	GPx
0 month	Untreated group (n = 15)	325.80 ± 41.01*	108.57 ± 4.05	1.59 ± 0.30*	681.17 ± 192.06
	Vaccinated group (n = 15)	282.04 ± 29.21*	110.54 ± 3.27	2.71 ± 0.95*,a,f	620.27 ± 189.70
1 month	Untreated group (n = 15)	159.04 ± 22.84*	190.08 ± 73.09	4.05 ± 2.59	472.88 ± 226.62
	Vaccinated group (n = 15)	145.04 ± 10.71*	151.89 ± 49.55	4.35 ± 2.22	604.30 ± 211.74
2 months	Untreated group (n = 15)	363.46 ± 76.343	63.30 ± 17.04*	3.96 ± 1.97	226.37 ± 11.17
	Vaccinated group (n = 15)	399.37 ± 70.67	47.21 ± 8.98*	3.43 ± 2.44	251.58 ± 23.46
6 months	Untreated group (n = 15)	1115.89 ± 140.47*	21.98 ± 0.82	14.34 ± 3.98*	247.11 ± 99.80
	Vaccinated group (n = 15)	953.50 ± 125.82*	22.48 ± 0.41	29.42 ± 10.39*	245.91 ± 81.35

respectively). Moreover, an increase in SOD activity was noted two and six months after immunization (by 175.4% and 557.4%, $P < 0.05$, respectively) compared to values one month after immunization. Six months after immunization, SOD activity increased (by 138.8%, $P < 0.05$) compared to values two months after vaccination (Table 1).

The results of the study revealed decreased CAT activity in hepatic tissue of vaccinated trout two months after vaccination compared to the values for the untreated group (by 25.4%, $P < 0.05$). In the untreated group, CAT activity in hepatic tissue increased in the first month after immunization (by 75.1%, $P < 0.05$) followed by a decrease at two and six months after immunization (by 41.7% and 79.8%, $P < 0.05$) compared to the values before vaccination. Additionally, decreased CAT activity in hepatic tissue of the untreated group was observed two and six months after immunization compared to values one month after immunization (by 66.7% and 88.4%, $P < 0.05$, respectively). Similarly, a decrease in CAT activity in hepatic tissue of the untreated group was observed six months after immunization compared to values after two months (by 65.3%, $P < 0.05$). Results for the vaccinated group were similar. Decreased CAT activity in hepatic tissue was observed two and six months after immunization compared to values before immunization (by 57.3% and 79.7%, $P < 0.05$, respectively). Increased CAT activity was also noted two and six months after immunization (by 68.9% and 85.2%, $P < 0.05$, respectively) compared to values one month after immunization. Six months after immunization, CAT activity decreased (by 52.4%, $P < 0.05$) compared to the values after two months (Table 1).

GR activity levels increased after immunization compared to those in the untreated group, both before (70.4%, $P < 0.05$) and six months after immunization (105.2%, $P < 0.05$). In the untreated group, GR activity in hepatic tissue increased in the first, second, and sixth months after immunization (by 154.7%, 149.1%, and 802.9%, $P < 0.05$) compared to the values before vaccination. In the sixth month after immunization, GR activity in hepatic tissue increased compared to values one (by 254.1%, $P < 0.05$) and two months (by 262.1%, $P < 0.05$) after immunization. In the vaccinated group, increased GR activity in hepatic tissue was observed one and six months after immunization compared to values before vaccination (by 60.5% and 10.9-fold, $P < 0.05$, respectively). Six months after immunization, GR activity in hepatic tissue increased compared to values one (6.8-fold, $P < 0.05$) and two months (8.6-fold, $P < 0.05$) after immunization (Table 1).

Decreased GPx activity in hepatic tissue of the untreated group was noted in the first (by 30.6%, $P < 0.05$), second (by 66.8%, $P < 0.05$) and sixth months (by 63.7%, $P < 0.05$) after immunization compared to values before immunization, as well as in the second (by 52.1%, $P < 0.05$) and sixth months (by 47.7%, $P < 0.05$) after immunization compared to values from the first month after immunization. In the vaccinated group, GPx activity in hepatic tissue decreased after the first and second months after immunization (by 59.4% and 60.4%, $P < 0.05$) compared to values from before immunization, following a decrease two and six months after immunization (by 58.4% and 59.3%, $P < 0.05$) compared to the values noted one month after vaccination (Table 1).

Lysosomal enzymes

To study the state of the lysosomal apparatus, which plays roles in various processes of adaptation, adaptive reactions to changing environmental conditions, and the development of various pathologies, the activities of four enzymes – alanyl aminopeptidase (AAP), leucyl aminopeptidase (LAP), acid phosphatase (AcP), and β-N-acetylglucosaminidase (NAG) – were evaluated in rainbow trout hepatic tissue one, two, and sixth months after vaccination.

A statistically significant decrease in alanyl aminopeptidase (AAP) activity in hepatic tissue of vaccinated trout was observed in the sixth month after vaccination compared to the first month (by 6.2%, $P < 0.05$) (Fig. 2a). In the untreated group, leucyl aminopeptidase (LAP) activity in hepatic tissue increased in the sixth month after vaccination compared to the first (by 150%, $P < 0.05$) and second

months (by 98.2%, $P < 0.05$) and before vaccination (by 94.7%, $P < 0.05$). In the vaccinated group, LAP activity in hepatic tissue increased in the first (by 253.3%, $P < 0.05$) and sixth months (by 191.7%, $P < 0.05$) compared to the values before vaccination, as well as two months (by 98.8%, $P < 0.05$) compared to six months after vaccination. Moreover, increased LAP activity in hepatic tissue was observed in the vaccinated group (4.6-fold, $P < 0.05$) compared to the untreated group in the first month after vaccination (Fig. 2b).

A statistically significant decrease in acid phosphatase (AcP) activity in hepatic tissue of vaccinated trout was observed in the second (by 18.1%, $P < 0.05$) and sixth months (by 18.4%, $P < 0.05$) after vaccination compared to values before vaccination. Similarly, a decrease in AcP activity in hepatic tissue of vaccinated trout was observed in the second (by 45.1%, $P < 0.05$) and sixth months (by 46.3%, $P < 0.05$) after vaccination compared to the values for the first month after vaccination. In the vaccinated group, AcP activity in hepatic tissue of vaccinated trout increased compared to the values from the first (by 44%, $P < 0.05$) and six months (by 46.2%, $P < 0.05$) after vaccination. Decreased AcP activity in hepatic tissue of vaccinated trout was observed in the second month (by 20.7%, $P < 0.05$) compared to the values from the first month after vaccination. Moreover, an increase in AcP activity in hepatic tissue was observed in the vaccinated group (by 130.8%, $P < 0.05$) compared to the untreated group in the sixth month after vaccination. No statistically significant changes were observed in β-N-acetylglucosaminidase (NAG) (Fig. 2).

Discussion

In the present study the most widely used and accepted biomarkers of oxidative stress and lysosomal functioning were used to monitor oxidative stress in hepatic tissue of rainbow trout immunized with anti-*Yersinia* vaccine after one, two, and six months (TBARS as a biomarker of lipid peroxidation, aldehydic and ketonic derivatives of OMP, enzymes of antioxidant defenses, and the total antioxidant capacity, alanyl aminopeptidase, leucyl aminopeptidase, acid phosphatase, and β-N-acetylglucosaminidase as lysosomal enzymes). The results of the current study on biomarkers of oxidative stress, such as TBARS, OMP, and TAC, clearly demonstrated that immunizing rainbow trout with the anti-*Yersinia* vaccine did not alter their livers. Interestingly, ketonic derivatives of OMP one month after vaccination were statistically lower compared to the untreated group. On the other hand, aldehydic derivatives of OMP six months after vaccination were statistically higher compared to the untreated group (Fig. 2). A statistically significant decrease in ketonic derivatives of OMP in hepatic tissue was observed one month after vaccination and was maintained for the subsequent two months (Fig. 2b). The analysis of changes one month after vaccination compared to all the periods studied indicated that higher lipid peroxidation and carbonyl derivative values resulted in decreased TAC. Two months after vaccination, the lowest TBARS and OMP levels were noted, which were accompanied by increased TAC levels. Six months after vaccination, TBARS and OMP values returned to those from before vaccination, but high TAC levels remained unchanged (Figs. 1–3).

SOD activity was shown to be statistically lower in hepatic tissue of vaccinated trout compared to the untreated groups, both one and six months after immunization. This accompanied lower CAT activity in hepatic tissue of vaccinated trout compared to the untreated groups. The lowest CAT activity was observed six months after vaccination. In this period, high TBARS and OMP values were observed that correlated with increased TAC levels. It was speculated that the maintenance of TAC at high levels resulted from the activation of glutathione-related enzymes, i.e., GR and GPx. Indeed, the highest GR activity was observed six months after vaccination (Table 1). GR is a biologically important enzyme involved in the conversion of oxidized glutathione into GSH, with the catalytic activity of $NADPH_2$ (Harasgama et al. 2020). In the current study, CAT activity was not significantly affected by vaccination against *Y. ruckeri*, except in the first month. Once there is a decrease in CAT activity, the reduction

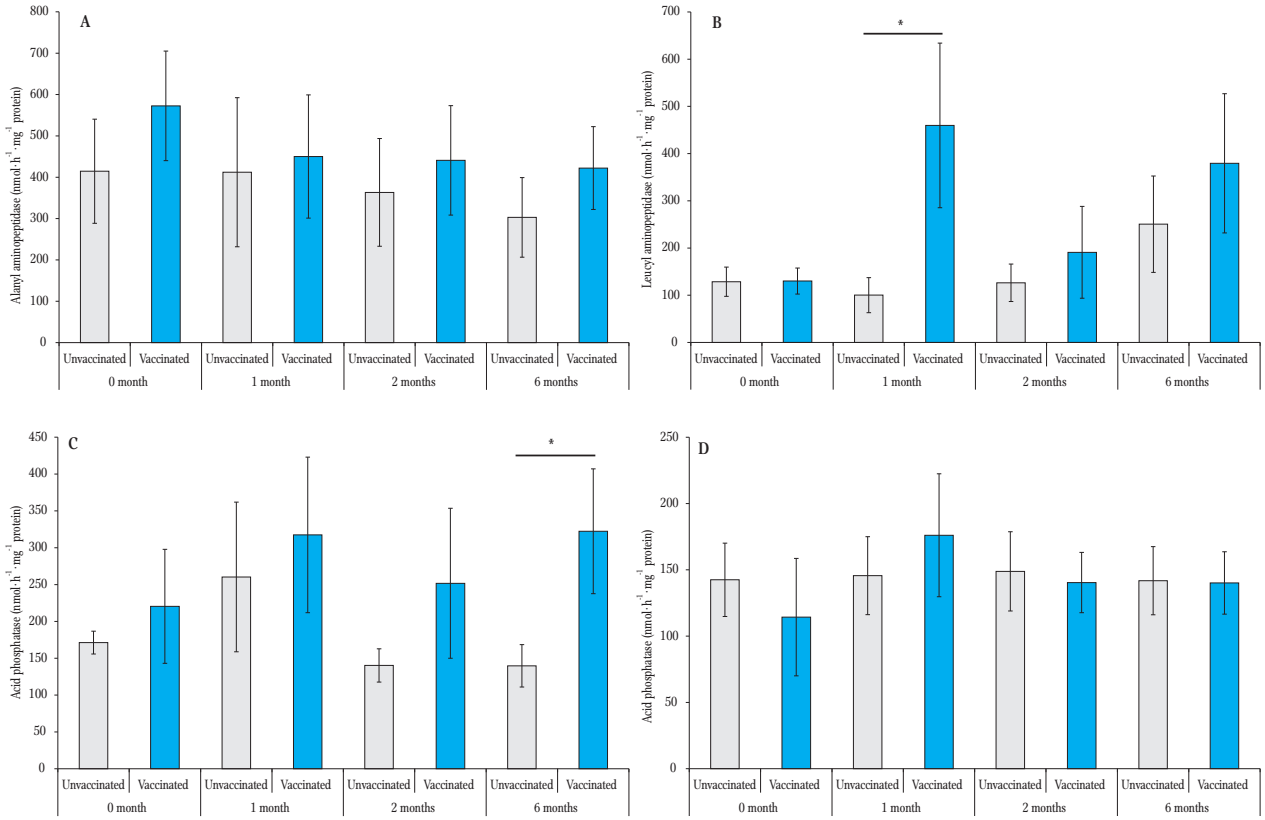

Fig. 4. Activities of alanyl aminopeptidase (AAP, nmol·h^{-1}·mg^{-1} protein), leucyl aminopeptidase (LAP, nmol·h^{-1}·mg^{-1} protein), acid phosphatase (AcP, nmol·h^{-1}·mg^{-1} protein), and β-N-acetylglucosaminidase (NAG, nmol·h^{-1}·mg^{-1} protein) in hepatic tissue of rainbow trout (*O. mykiss*) 1, 2, and 6 months after oral vaccination with an anti-*Yersinia* vaccine. Data are presented as mean ± SD, n = 15. ANOVA was used to perform a comparative analysis between values for untreated and vaccinated groups in the first, second, and sixth months after vaccination (*P<0.05)

reaction of H_2O_2 into H_2O and O_2 is accomplished by GPx (Scandalios 2005). In the current study, CAT and GPx activity decreased in the second and sixth months after vaccination. Thus, the system of antioxidant defenses in hepatic tissue of trout after vaccination modified the direction of antioxidant protection toward the preferential use of enzymes related to glutathione metabolism.

There is evidence suggesting a link between immune response producing pro-oxidants, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), and the activation of oxidative stress (Sorci and Faivre 2009). ROS are produced by NADPH-oxidase in phagocyte respiratory burst in response to microbe invasion and recognition. Microbe recognition sets the immune system in motion. ROS are also produced in other cell compartments, such as mitochondria, as intermediaries in many signal transduction pathways, such as leukocyte pattern recognition receptor signaling. The generation of ROS is a prerequisite to the formation of neutrophil extracellular traps (Paiva and Bozza 2014). Following oxidative burst, the immune responses also increase oxygen consumption by cells (Hasselquist and Nilsson 2012) and might contribute to an increase in the generation of ROS.

Non-specific and specific immune responses of fish against *Y. ruckeri* strains have been studied extensively (Kumar et al. 2015). The immune mechanisms responsible for protection can comprise cellular and humoral elements (Raida et al. 2011). A key hallmark of the vertebrate adaptive immune system is the generation of antigen-specific antibodies from B cells. For example, both O-antigen and

formalin-inactivated *Y. ruckeri* cells induced an immune response in rainbow trout, producing peak antibody levels in the spleen at 14 days post-exposure and an overall maximum titer at 28 days post-exposure (Raida et al. 2011). The neutrophil and macrophage responses that accompany inflammation in the peritoneal cavity of rainbow trout were studied by Afonso et al. (1998). Intraperitoneal injection of casein, Incomplete Freund's Adjuvant (IFA), and live or formalin-killed *Y. ruckeri* resulted in a rapid influx of neutrophils, peaking at 24 to 48 h post-injection.

Lysosomes are membrane-bound organelles responsible for the breakdown and recycling of macromolecules. Lysosomal dysfunction occurs with enzymatic and non-enzymatic deficiencies, leading to abnormal accumulation of materials (Ashtari et al. 2016). AAP is a type II integral membrane protein localized on the plasma membrane as an ectoenzyme (Turner 2004). The enzyme is widely distributed, but it is especially abundant in the membranes of the brush border of the kidneys, small intestine, and placenta, as well as in the liver. Much of the initial characterization of AAP was done with renal or intestinal enzymes (Turner 2004). The current results showed that the AAP activity changed statistically non-significantly during the post-vaccination period, i.e., there was a non-significant increase of AAP activity in hepatic tissue of vaccinated fish (at 0, 1, 2, and 6 months after vaccination) (Fig. 4a).

LAP plays various tissue-specific physiological roles in peptide processing or degradation (Strater and Lipscomb 2013). Human LAP has been demonstrated to catalyze post-proteasomal trimming of the N-terminus of antigenic peptides for presentation on major histocompatibility complex class I molecules (Beninga et al. 1998). It has also been suggested that LAP plays a physiological role in glutathione metabolism and in the degradation of glutathione S-conjugates (Cappiello et al. 2004). These functions are based on the ability of the Mn/Zn form of the enzyme to cleave cysteinylglycine (Jösch et al. 2003). The current results demonstrated that the LAP activity in hepatic tissue of vaccinated fish increased statistically significantly after one month in the post-vaccination period (4.6-fold). Moreover, LAP activity increased non-significantly in hepatic tissue of vaccinated fish compared to the untreated group (at 0, 1, 2, and 6 months after vaccination). The highest LAP activity was noted in hepatic tissue of vaccinated fish one and six months after vaccination (Fig. 4b). This correlated with increased TBARS and OMP values in hepatic tissue of vaccinated fish especially one month after vaccination (Figs 1 and 2). Lysosomal acid phosphatase (AcP) is a tartrate-sensitive enzyme with ubiquitous expression (Saftig et al. 1997). The current results revealed that AcP activity in hepatic tissue of vaccinated fish increased statistically significantly after six months in the post-vaccination period (by 131%). Similar to LAP and AAP activity, AcP activity increased non-significantly in hepatic tissue of vaccinated fish compared to the untreated group (at 0, 1, 2, and 6 months after vaccination). The highest values of AcP activity were noted in hepatic tissue of vaccinated fish after one and six months in the post-vaccination period (Fig. 4c). NAG activity in hepatic tissue of vaccinated fish changed non-statistically significantly compared to the untreated group (at 0, 1, 2, and 6 months after vaccination) (Fig. 4c).

Da Cruz et al. (2012) used acid phosphatase as a biomarker of lysosomal integrity to estimate biodiesel cytotoxicity in liver homogenate from Tilapia juveniles. They evaluated the loss of the lysosomal membrane integrity in liver homogenate of Tilapia juveniles exposed to water-soluble-fraction (WSF) of biodiesels through increased acid phosphatase activity as evidence of cytotoxicity. A higher acid phosphatase activity in relation to the control was associated with the degree of lysosomal membrane fragility, which was a consequence of lysosomal dysfunction in the liver of fish exposed to the WSF of biodiesels (da Cruz et al. 2012).

All enzyme-specific activities of lysosomal enzymes (except NAG activity) measured in the present study were significantly and non-significantly higher in the liver of vaccinated fish than in those of the untreated fish. This increase in specific activities was most likely because of the mobilization of glycogen, lipids, and protein by controlled autolysis after vaccination.

Vaccination reduced the hepatic activity of SOD. On the other hand, the lack of changes or even increases in CAT, GR, GPx, LAP, and AcP activities along with reduced OMP levels suggested these enzymes were being specifically maintained after vaccination. The increase of GR and GPx activities and the maintenance of TAC levels during immunization suggested that antioxidant defenses against ROS were more important after the first and following post-vaccinated months for energy reserve mobilization. The increase in antioxidant defenses might have facilitated a protective role, particularly during a period when the immunization-dependent mobilization of macromolecules might have rendered cellular structures more susceptible to oxidative stress caused by vaccination.

Thus, vaccination against *Y. ruckeri* resulted in non-significant changes in TBARS levels as lipid peroxidation biomarkers, aldehydic, and ketonic derivatives of oxidatively modified proteins, and antioxidant defenses, especially glutathione-dependent enzymes. Lysosomal enzymes might contribute to the stabilization of oxidative stress in hepatic tissue of trout for six months following immunization against *Y. ruckeri* and from the effective mechanisms of immunity formation.

Conclusions

The current results demonstrated a temporal mobilization of antioxidant and lysosomal enzymes following rainbow trout vaccination. Lysosomal enzymes involved in the breakdown of carbohydrates increased in hepatic tissue of vaccinated trout, whereas antioxidant defenses decreased. Further, hepatic TBARS levels were maintained after vaccination. The sparing of antioxidant enzymes was also apparent where hepatic activities of CAT, GR, and GPx were either increased or unchanged following vaccination. The relative rates of mobilization of proteins after vaccination might reflect their abundance in the liver and their critical functional roles following vaccination. Vaccination with anti-*Yersinia* vaccine affected the functional state of the liver, modulating the intensity of lipid peroxidation and protein oxidation associated with changes in lysosomal and antioxidant enzyme activities. Possible mechanisms of immune formation after anti-*Yersinia* vaccination could be increased membrane permeability of lysosomes. Vaccination affected the functional state of the lysosomal balance in vaccinated fish. Understanding the role of oxidative stress and lysosomal functioning in the tissues of vaccinated trout has important implications for understanding the complex physiological changes that occur following immunization as well as for improving aquaculture practices to maximize tissue growth and the health of vaccinated fishes.

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Conflicts of interest. The authors declare that they have no conflict of interest. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship and all those who qualify for authorship are listed.

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