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EFFECTS OF ORAL VACCINATION AGAINST *YERSINIA RUCKERI* ON OXIDATIVE STRESS BIOMARKERS IN GILLS, LIVER AND HEART OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM)

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Abstract

The aim of this study was to assess the effect of oral vaccination against *Yersinia ruckeri* based on oxidative stress biomarkers in different tissues of rainbow trout (*Oncorhynchus mykiss* Walbaum). Vaccine consisted of three *Y. ruckeri* strains (O1 serotype) that originated from rainbow trout cultured on the different farms, where fish exhibited clinical signs of enteric redmouth disease. Concentrated vaccine was incorporated in the fish food; treatment was delivers three times at one day intervals. One month after immunization, gills, hepatic and cardiac tissues were sampled. The vaccinated trout showed tissue-specific oxidative stress responses in the gills, liver, and heart. The gill tissue was the most sensitive to oxidative damage among the samples. Accumulation of oxidative stress biomarkers in the rainbow trout was tissue-specific with following accumulation: gills > heart > liver. These results suggest that the trout expressed tissue-specific oxidative stress mechanisms due to anti-*Yersinia* vaccine treatment. There were no statistically significant alterations in the activities of antioxidant defenses instead superoxide dismutase activity in the hepatic and cardiac tissue and glutathione peroxidase activity in the gills of vaccinated trout. Correlative analysis confirmed the role of catalase in the antioxidant defense in vaccinated trout. The oxidative stress biomarkers, i.e. content of oxidative modified proteins in the gills, and liver, and 2-thiobarbituric acid reactive substances level in the gills, and total antioxidant capacity in the liver, were sensitive to vaccination of trout against *Y. ruckeri* and may potentially be used as biomarkers in evaluating vaccine toxicity in rainbow trout.

Key words: rainbow trout *Oncorhynchus mykiss*, *Yersinia ruckeri*, immunization, oxidative stress, tissues

INTRODUCTION

The coastal waters of the Baltic Sea are constantly threatened by metal contamination in water, surface sediments, accumulated algal biomass, oil spills, due to the extensive transportation of oil products across the sea, by influence of Nuclear Power Plant etc. The complex of microbial communities in the Baltic Sea appeared to be correlated with the concentration of the pollutants in sediments (Gubelit et al. 2016). Over-intensive microbial contamination in coastal water increases the chance of fish infections, particularly among fry and immunocompromised population. Excessive aquaculture and heavy rain precipitation greatly increase the concentration of fish pathogens in coastal waters (Santhiya et al. 2011). Pathogen distribution may also have an extension effect along coastlines. Learning how pathogens distribute and circulate near coast lines will help to partition areas with different levels of contamination to facilitate pollution administration and to submit prophylactic approaches to defense of microbial infections of fish (Schippmann et al. 2013, Xie et al. 2017).
Enteric redmouth disease (ERM) is an acute or chronic generalized bacterial septicaemia in fish caused by *Yersinia ruckeri* (Barnes 2011). The causative agent, a Gram-negative enteric bacterium, which was first isolated in the Hagerman Valley, Idaho, USA, in the early 1950s, was described fully by Ross and co-workers (1966) and defined as a new species, *Y. ruckeri*, in 1978 (Ewing et al. 1978). *Y. ruckeri* is a member of the family *Enterobacteriaceae* within the gamma-proteobacteria subdivision. Generally of coccoid-rod cell morphology, *Y. ruckeri* cells are slightly curved, 1.0 μm in diameter and 2-3 μm in length, though culture for 48 h or longer results in long filamentous cells (Barnes 2011).The signs of disease include exophthalmia and darkening of the skin, and subcutaneous hemorrhages in and around the mouth and throat, which give the disease its common name. Petechial hemorrhages may occur on the surfaces of the liver, pancreas, pyloric caeca, swim bladder and in the lateral muscles. The spleen is often enlarged and can be almost black in color, and the lower intestine can become reddened and filled with an opaque, yellowish fluid (Kumar et al. 2015).
The virulence in rainbow trout of 32 isolates of *Y. ruckeri*, representing a range of biotypes, serotypes, and the outer membrane protein (OMP)-types, was examined by Davies (1991). Two of the six serotype O1 clonal groups of *Y. ruckeri*, clones 2 and 5, were virulent, whereas the other four clonal groups, clones 1, 3, 4 and 6, as well as all serotype O2, O5, O6 and O7 isolates examined, were avirulent (Barnes 2011). The virulent serotype O1 clonal groups were serum resistant, whereas the avirulent serotype O1 clonal groups and other serotypes were, with some exceptions, serum sensitive (Davies 1991). The host innate immune factors in normal serum include complement (activated via the alternative or lectin pathways), lysozyme, a range of proteases, α2 macroglobulin and a range of lectins (e.g. MBL, ladderlectin and the pentraxins such as serum amyloid protein P), among other factors (Barnes 2011). *Y. ruckeri* has been shown to bind ladderlectin from trout serum in relatively low amounts (Young et al. 2007). Findings of Young and co-workers (2007) indicate that rainbow trout have variable amounts of plasma ladderlectin capable of binding to the surfaces of several relevant bacterial targets.

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 and immersion and is suitable for vaccinating large numbers of fish of all sizes. It avoids the handling stress experienced by the fish with the other two methods. The major disadvantage with 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 proves that fish possess gut associated lymphoid tissue (GALT) in the second gut segment (Quentel and Vigneulle 1997). 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, therefore the antigen may 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 obtained, but varying degrees of success have been reported in the literature. These variations are believed to be due to differences in experimental design between studies, including antigen preparation, the age and species of fish, water temperature at the time of vaccination, duration of vaccine feeding and antigen integrity when it reaches the hind gut (Thompson and Adams 2004).

Measurement of innate immune responses to primary and secondary infection by *Y. ruckeri* suggested activation of pathways via Toll-like receptors (Barnes 2011). This leads to high up regulation of genes expressing acute phase proteins including the pentraxin serum amyloid protein A and pro-inflammatory cytokines interleukin 1 (IL-1β), IL-6 and tumour necrosis factor alpha (TNFα). Many complement factors are down-regulated, including the critical opsonin C3. Secondary infection leads to a diminished response and lower infection loads in the liver, suggesting that onset of adaptive immunity may reduce the infection by lowering the innate response to secondary infections (Barnes 2011). Raida and Buchmann (2008a) demonstrated that gene expression in fish vaccinated against *Y. ruckeri* was up-regulated with regard to the pro-inflammatory cytokines IFN-γ, TNF-α, IL-6 and the anti-inflammatory cytokines IL-10 and TGF-β, the cell receptors TcR, CD8α, CD4, C5aR and the teleost specific immunoglobulin IgT (Raida and Buchmann 2008a). They quantified expression of cytokine and receptor genes and found that upregulation of cytokines and T-cell receptors was high during primary infection and associated with high levels of *Y. ruckeri* in the spleen. Humoral factors such as Ig and complement are less important in the protection induced by bath vaccination. Expression of cellular factors such as CD8α was significantly increased in the protected trout and this suggests that cellular factors including cytotoxic T-cells could play a role in immunity against *Y. ruckeri* (Raida and Buchmann 2008a).

Reactive oxygen species (ROS) production contributes to elimination of pathogens and induces activation of immune defense mechanisms (Paiva and Bozza 2014). However, excessive ROS formation can induce oxidative stress, leading to cell dam-

age and cell death may follow (Poljsak et al. 2013). ROS comprise both free radical and non-free radical oxygen containing molecules such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), and the hydroxyl radical (·OH) (Poljsak et al. 2013). Lipid peroxidation of polyunsaturated lipids is a preferred method for marking oxidative stress levels (Rahal et al. 2014). The product of lipid peroxidation, malondialdehyde, is easily detected in blood/plasma and has been used as a measure of oxidative stress (Rahal et al. 2014). In addition, the unsaturated aldehydes produced from these reactions have been implicated in modification of cellular proteins and other constituents (Marnett 2000). The oxidative modification of proteins by ROS is implicated in the etiology or progression of diseases (Levine 2002). An increase in the rate of their production or a decrease in their rate of scavenging will increase the oxidative modification of cellular molecules, including proteins, and will disrupt cellular function either by loss of catalytic and structural integrity or by interruption of regulatory pathways (Stadtman and Levine 2000). For the most part, oxidatively modified proteins are not repaired and must be removed by proteolytic degradation, a process that normally proceeds very efficiently from microorganisms to mammals (Levine 2002). The level of these modified molecules can be quantitated by measurement of the protein carbonyl content. It is the most widely used marker of oxidative modification of proteins (Chevion et al. 2000).
Therefore, exploring the effects of vaccination against *Y. ruckeri* on health condition of trout in general, and levels of oxidative stress biomarkers in different tissues would be of value. The present study aims to clarify the effects of 4 weeks post-vaccination against *Y. ruckeri* on gills, liver, and heart function, as well as the oxidative mechanism underlying those effects, by detecting relevant lipid peroxidation and protein oxidation biomarkers as well as antioxidant defenses.

MATERIALS AND METHODS

Experimental animals. Rainbow trout (*Oncorhynchus mykiss* Walbaum) with a mean body mass of (107.9 ±3.1) g were used in the experiments. The study was carried out in a Department of Salmonid Research, Inland Fisheries Institute in Rutki (Poland). Experiments were performed at a water temperature of 14.5 ±0.5°C and the pH was 7.5. The dissolved oxygen level was about 12 ppm with additional oxygen supply with a water flow of 25 L per min, photoperiod of 12 hours per day. The fish were fed with commercial pelleted diet at an optimal level, using 12-hour belt feeders for fish. The daily dose of feed calculated in accordance with the applicable table feed (From and Rasmussen 1984). All enzymatic assays were carried out at Department of Zoology and Animal Physiology, Institute of Biology and Environmental Protection, Pomeranian University in Słupsk (Poland).

Experimental design. The fish were divided into two groups: I) control, II) immunized by vaccine against *Y. ruckeri*. Fish were held in 1000-L square tanks (150 fish per tank) in the same environmental conditions. The vaccine was produced in Department of Fish Diseases, National Veterinary Research Institute in Puławy (Poland). The vaccine against Yersiniosis was prepared from formalin-inactivated cells of *Y. ruckeri*.

Vaccine contains three *Y. ruckeri* strains originating from rainbow trout cultured on the different farms, where fish exhibited clinical signs of Yersiniosis. All bacteria isolates belonged to O1 serotype biotype 2 that are pathogenic in fish and showed some differences in their biochemical properties. The bacterial strains were inoculated onto trypticase soya broth (TSB, BioMerieux) and incubated for 48 h at 24°C ±1°C. After incubation the purity of the culture was checked. The density of the vaccine was estimated spectrophotometrically, and formalin was added to the final concentration of 0.4% in order to inactive the bacterial strains. Vaccine sterility control was performed after 48h incubation period. Prepared vaccine at the concentration of $1x10^9$ cells per mL was used to inoculate fish *per os*. Vaccine concentrate was added to fish feed and administered three times with one-day interval in between feedings.
The fish were kept for 30 days after vaccination at a water temperature 14.5 ±0.5°C and the pH 7.5. In our study, 15 rainbow trout from unhandled control and 15 vaccinated trout were used at first month after immunization. One month after immunization, samples from rainbow trout were collected.

Sampling. The animals were captured and killed 31 days post vaccination (n = 15 in each group). Gills, liver, and heart were removed *in situ*. The organs were rinsed clear of blood with cold isolation buffer and homogenized using a glass homogenizer H500 with a motor-driven pestle immersed in an ice water bath to a yield a homogenate in proportion 1:9 (weight/volume). The isolation buffer contained 100 mMtris-HCl; a pH of 7.2 was adjusted with HCl. Homogenates were centrifuged at 3,000g for 15 min at 4°C. After centrifugation, the supernatant was collected and frozen at -20°C until analyzed. Protein contents were determined using the method of Bradford (1976) with bovine serum albumin as a standard. Absorbance was recorded at 595 nm. All enzymatic assays were carried out at 22 ±0.5°C using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany) in duplicate. The enzymatic reactions were started by the addition of the tissue supernatant. The specific assay conditions were as follows.

Oxidative stress biomarkers assay

Assay of 2-thiobarbituric acid reactive substances (TBARS). An aliquot of the homogenate was used to determine the lipid peroxidation status of the sample by measuring the concentration of 2-thiobarbituric-acid-reacting substances (TBARS), according to Kamyshnikov (2004). Reaction mixture consisted of a sample homogenate (2.1 mL, 10% w/v) in tris-HCl buffer (100 mM, pH 7.2), 2-thiobarbituric acid (TBA; 0.8%, 1.0 mL), and trichloracetic acid (TCA; 20%, 1.0 mL). The total volume was kept in a water bath at 100°C for 10 min. After cooling, mixture was centrifuged at 3,000 g for 10 min. The absorbance of the supernatant was measured at 540 nm. TBARS values were reported as nmoles malondialdehyde (MDA) per mg protein.

Assay of carbonyl groups of oxidatively modified protein. Carbonyl groups were measured as an indication of oxidative damage to proteins according to the method of Levine and co-workers (1990) in modification of Dubinina co-workers (1995). Samples were incubated at room temperature for 1 h with 10 mM 2,4-dinitrophenylhydrazine (DNTP)

in 2M HCl. Blanks were run without DNTP. Afterwards, proteins were precipitated with TCA and centrifuged for 20 min at 3,000 g. The protein pellet was washed three times with ethanol:ethylacetate (1 : 1) and incubated at 37°C until complete resuspension. The carbonyl content could be 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 protein.

Assay of superoxide dismutase activity. Superoxide dismutase (SOD, E.C. 1.15.1.1) 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 by Kostiuk and co-workers (1990). Briefly, 1.0 mL of C reagent was mixed with 0.1 mL of sample (dilution in water, 1 : 1000). C reagent was made ex tempore (mixture of equal volumes of 0.1-M K, Na-phosphate buffer, pH 7.8, and 0.08-M EDTA solution); pH of C reagent was adjusted to 10.0 by adding tetramethylenediamine. Distilled water (0.1 mL) was added to blank vials instead of sample. The total volume of all samples was brought up to 2.4 mL using distilled water. The reaction was initiated by adding 0.1 mL of quercetin (1.4 mM dissolved in dimethyl sulfoxide). Absorbance at 406 nm was measured immediately and 20 min after addition of quercetin solution. Activity was expressed in units of SOD per mg of tissue protein.

Measurement of catalase activity. Catalase (CAT, E.C. 1.11.1.6) activity was determined by measuring the decrease of H_2O_2 in the reaction mixture using a spectrophotometer at the wavelength of 410 nm using the method described by Koroliuk and co-workers (1988). The reaction was initiated by adding 0.1 mL of the sample into the incubation medium (2 mL of 0.03% H_2O_2 solution) and to 1.0 mL of 4% ammonium molybdate dissolved in 12.5 mM H_2SO_4 solution (blank sample). The duration of reaction was 10 min at room temperature. The reaction was terminated by adding 1.0 mL of 4% ammonium molybdate dissolved in 12.5 mM H_2SO_4 solution to incubation medium and 1mL of 125 mM H_2SO_4 to all samples. The samples were centrifuged at 3,000 g for 5 min. The absorbance of the obtained solution was measured at 410 nm and compared with that of the blank sample. One unit of CAT activity was defined as the amount of enzyme required for decomposition of 1 μmol H_2O_2 per min per mg of protein.

Measurement of glutathione reductase activity. Glutathione reductase (GR, EC 1.6.4.2) activity in the sample was measured according to the method described by Glatzle and co-workers (1974) with some modifications. The enzymatic activity was assayed spectrophotometrically by measuring $NADPH_2$ consumption. In the presence of GSSG and $NADPH_2$, GR reduces GSSG and oxidises $NADPH_2$, resulting in a decrease in the absorbance at 340 nm. The enzyme assay mixture contained 2.4 mL of 67 mM sodium phosphate buffer (pH 6.6), 0.2 mL of 7.5 mM oxidised glutathione, and 0.1 mL of sample. The rate of $NADPH_2$ oxidation was measured spectrophotometrically at 340 nm. Quantification was performed based on a molar extinction coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$ of $NADPH_2$. The GR activity was expressed as μmol of $NADPH_2$ per min per mg of protein.

Assay of glutathione peroxidase activity. Glutathione peroxidase (GPx, EC 1.11.1.9) activity was determined by detecting the nonenzymaticutilization of 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). The assay mixture contained 0.8 mL of 0.1 M tris-HCl buffer with 6 mM EDTA and 12 mM sodium azide (pH 8.9), 0.1 mL of 4.8 mM GSH, 0.2 mL of sample, 1 mL of 20 mM t-butyl hydroperoxide, and 0.1 mL of 0.01M DTNB. The rate of GSH reduction was measured spectrophotometrically at 412 nm. Glutathione peroxidase activity was expressed as μmol GSH per min per mg of protein.

Assay of total antioxidant capacity (TAC). The TAC level was estimated spectrophotometrically at 532 nm following the method with Tween 80 oxidation (Galaktionova et al. 1998). Briefly, 0.2 mL of tissue homogenate was added to 2 mL of 1% Tween 80. Blank assay instead of sample included 0.2 mL of distilled water. The mixture was incubated during 48 hours at 37°C. After cooling, 1 mL of 40% TCA was added. The mixture was centrifuged at 3,000 g for 10 min. After centrifugation, 2 mL of supernatant and 2 mL of 0.25% TBA reagent was mixed. The mixture was heated in boiling water bath at 100°C for 15 minutes. The absorbance of the obtained solution was measured at 532 nm and was compared with the blank. TAC level was expressed in %.

Statistical analysis. Data were presented as the mean ±S.E.M. and were checked for assumptions of normality using the Kolmogorov–Smirnov one-sample test and Lilliefors tests ($p > 0.05$). In order to find significant differences (significance level, $p < 0.05$) between control and vaccinated groups, Mann–Whitney *U* test was applied to the data (Zar 1999). Differences were considered significant at $p < 0.05$. The relationships between oxidative stress biomarkers of all individuals were evaluated using Spearman's correlation analysis. All statistical analysis was performed by STATISTICA 8.0 software (StatSoft, Kraków, Poland).

RESULTS

The level of lipid peroxidation in liver and the heart of trout treated by vaccine did not significantly differ from that in the control sample. In the gills of the vaccinated group, the TBARS level on 4 weeks post-vaccination were significantly higher than the TBARS obtained in unhandled control (by 109%, $p = 0.000$) (Fig. 1).
Highly reactive oxygen species that are formed during normal metabolism and under conditions of oxidative stress are able to oxidize proteins or convert lipid and carbohydrate derivatives to compounds that react with functional groups on proteins. Among other changes, these ROS-mediated reactions lead to the formation of protein carbonyl derivatives, which serve as a marker for ROS-mediated protein damage (Stadtman and Berlett 1998). Aldehydic derivatives of oxidatively modified proteins in the gills on 4 weeks post-vaccination were significantly increased (by 56.8%, $p = 0.023$) compared to unhandled group (Fig. 2A).

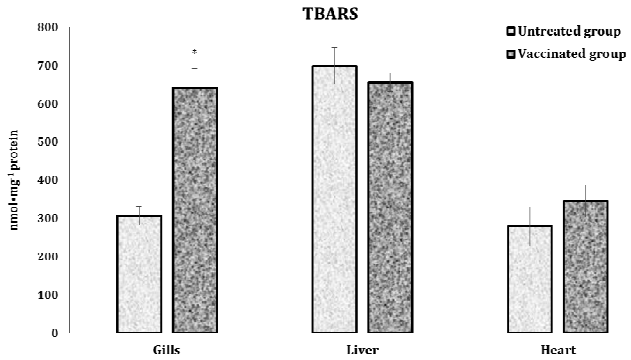

Fig. 1. The level of lipid peroxidation (nmol TBARS per mg protein) in gills, liver, and heart of the trout treated by vaccine against *Y. ruckeri* on 4 weeks post-vaccination
Data are represented as mean ±S.E.M. (n = 15)
* the significant difference was shown as $p < 0.05$ when compared unhandled group and vaccinated group values on 4 weeks post-vaccination

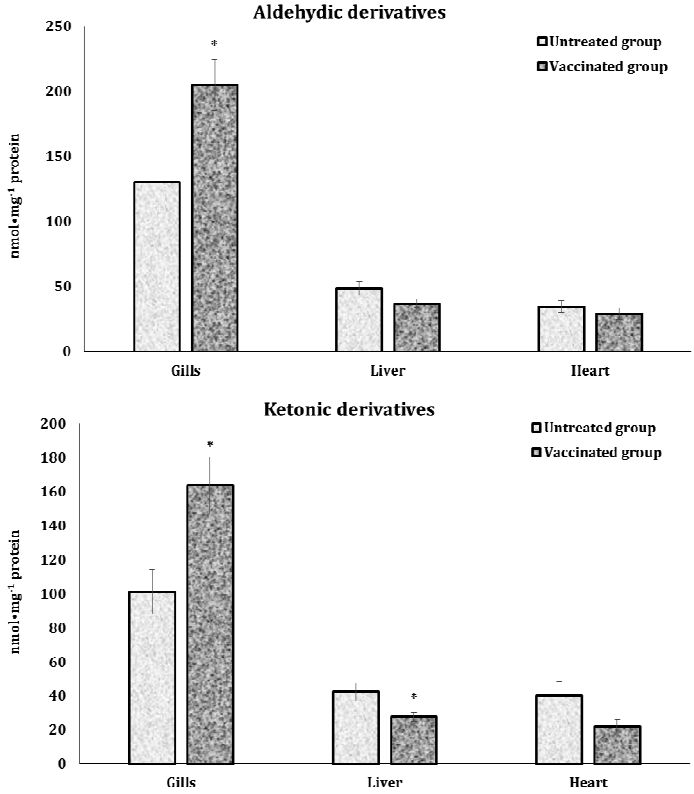

Fig. 2. Aldehydic and ketonic derivatives of oxidatively modified proteins in gills, liver, and heart of the trout treated by vaccine against *Y. ruckeri* on 4 weeks post-vaccination
Data are represented as mean ±S.E.M. (n = 15)
* the significant difference was shown as $p < 0.05$ when compared unhandled group and vaccinated group values on 4 weeks post-vaccination

Vaccination caused a significant decrease in the ketonic derivatives level in the liver by 35% ($p = 0.033$) compared to the control. Higher level of ketonic derivatives of carbonyl content was noted in gills of fish exposed to vaccine (by 62%, $p = 0.012$) compared to unhandled group (Fig. 2B). Aldehydic and ketonic derivatives of OMB content in cardiac tissue of fish treated by vaccine were non-significantly lower in comparison with unhandled control (Fig. 2B).

Antioxidant defense in the gills, liver, and heart of the trout treated by vaccine against *Y. ruckeri* at first month after immunization are shown in Table 1. There were no statistically significant alterations in the activities of antioxidant defenses instead SOD activity in the hepatic and cardiac tissue and GPx activity in the gills of the trout treated by vaccine against *Y. ruckeri* (Table 1). The SOD activity was decreased in hepatic tissue by 9% ($p = 0.009$) and increased in the heart by 8.5% ($p = 0.019$) after immunization. The GPx activity in the gills of the trout treated by vaccine against *Y. ruckeri* was increased by 113% ($p = 0.000$) compared to the controls (Table 1).

Table 1

Superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase activities in the gills, liver, and heart of the trout treated by vaccine against *Y. ruckeri* on 4 weeks post-vaccination

Antioxidant enzymes	Unhandled control	Vaccinated group
	gills	
SOD, $U \cdot mg^{-1}$ protein	318.34 ±18.82	329.75 ±14.97
CAT, μmol $H_2O_2 \cdot min^{-1} \cdot mg^{-1}$ protein	34.61 ±3.97	44.19 ±6.32
GR, μmol $NADPH_2 \cdot min^{-1} \cdot mg^{-1}$ protein	4.05 ±0.72	4.85 ±0.76
GPx, μmol $GSH \cdot min^{-1} \cdot mg^{-1}$ protein	263.98 ±28.30	563.02 ±41.92*
	liver	
SOD, $U \cdot mg^{-1}$ protein	159.04 ±6.34	145.05 ±2.86*
CAT, μmol $H_2O_2 \cdot min^{-1} \cdot mg^{-1}$ protein	190.09 ±20.27	151.89 ±13.24
GR, μmol $NADPH_2 \cdot min^{-1} \cdot mg^{-1}$ protein	4.65 ±0.82	4.35 ±0.60
GPx, μmol $GSH \cdot min^{-1} \cdot mg^{-1}$ protein	472.88 ±62.86	604.30 ±56.59
	heart	
SOD, $U \cdot mg^{-1}$ protein	280.06 ±9.97	303.90 ±8.22*
CAT, μmol $H_2O_2 \cdot min^{-1} \cdot mg^{-1}$ protein	64.13 ±5.35	45.62 ±5.94
GR, μmol $NADPH_2 \cdot min^{-1} \cdot mg^{-1}$ protein	5.15 ±0.91	3.85 ±0.53
GPx, μmol $GSH \cdot min^{-1} \cdot mg^{-1}$ protein	469.70 ±67.79	428.45 ±64.46

Data are represented as mean ±S.E.M. (n = 15)

* the significant change was shown as $p < 0.05$ when compared values of unhandled and vaccinated groups

The total antioxidant capacity (TAC) is another marker used for indirectly determining the levels of oxidative stress in tissue (Bisogni et al. 2012). TAC was significantly decreased in the liver of vaccinated group compared to those in the control on 4 weeks post-vaccination (by 26.1%, $p = 0.010$) (Fig. 3).

Correlations between oxidative stress markers in the gills, liver and heart of the trout vaccinated against *Y. ruckeri* on 4 weeks post-vaccination are demonstrated in Table 2.

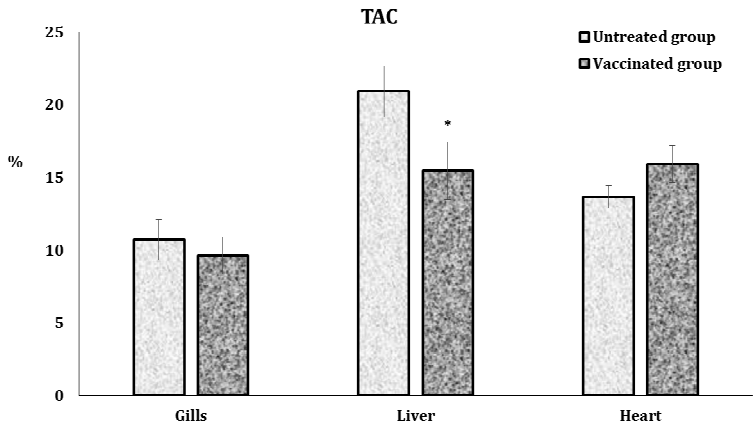

Fig. 3. Total antioxidant capacity (TAC, %) in gills, liver, and heart of the trout treated by vaccine against *Y. ruckeri* on 4 weeks post-vaccination
Data are represented as mean ±S.E.M. (n = 15)
* the significant difference was shown as $p < 0.05$ when compared unhandled group and vaccinated group values on 4 weeks post-vaccination

DISCUSSION

During the last 10 to 20 years vaccination has become important part of prevention methods against infectious diseases in farmed fish, mainly salmonid species. So far, most commercial vaccines have been inactivated vaccines administered by injection or immersion. Bacterial infections caused by Gram-negative bacteria such as *Vibrio* sp., *Aeromonas* spp., and *Yersinia* spp. have been effectively controlled by vaccination (Gudding et al. 1999). However, protective mechanisms and biochemical changes in fish metabolism activated by this type of immunization are still being debated.
In the present study, the most widely used and accepted markers were utilized to demonstrate the existence of oxidative stress in the tissue (TBARS as marker of lipid peroxidation, aldehydic and ketonic derivatives of OMP, antioxidant defenses and the total antioxidant capacity). Our results clearly demonstrates that immunization by anti-*Yersinia* vaccine not alter the liver and heart of rainbow trout. On the other hand, the gills were more sensitive to vaccination. Oxidative stress parameters were examined in homogenate, and lipid peroxidation as measured by the amount of TBARS were non-significantly ($p > 0.05$) altered in the liver and heart of vaccine-treated fish. Moreover, the present work has showed that the ketonic derivatives of OMP in the hepatic tissue were decreased significantly following vaccination against *Y. ruckeri* (Fig. 1B). All these culminated to an decrease of total antioxidant capacity in liver on 4 weeks after vaccination. In our previously study, TBARS level in the muscle tissue of trout vaccinated against *Y. ruckeri* did not differ significantly from that in controls in the first month after immunization, while aldehydic and ketonic

derivatives of OMB content in the muscle of vaccinated fish was higher in the first month after immunization compared to controls (Tkachenko et al. 2016e).

Table 2

Correlations between oxidative stress markers in gills, and liver of the unhandled trout and trout vaccinated against *Y. ruckeri* on 4 weeks post-vaccination

Correlations	Correlation coefficient, r	Significant level, p
unhandled control, gills		
Aldehydic derivatives of oxidatively modified proteins – TBARS	0.676	0.011
Ketonic derivatives of oxidatively modified proteins – TBARS	0.577	0.039
Aldehydic derivatives of oxidatively modified proteins – TAC	0.604	0.029
Ketonic derivatives of oxidatively modified proteins – TAC	0.610	0.027
Aldehydic derivatives of oxidatively modified proteins – SOD	-0.751	0.003
Ketonic derivatives of oxidatively modified proteins – SOD	-0.826	0.001
vaccinated group, gills		
Aldehydic derivatives of oxidatively modified proteins – TBARS	0.543	0.045
Ketonic derivatives of oxidatively modified proteins – TBARS	0.771	0.001
TBARS – CAT	0.609	0.021
Aldehydic derivatives of oxidatively modified proteins – CAT	0.670	0.009
Ketonic derivatives of oxidatively modified proteins – CAT	0.855	0.000
unhandled control, liver		
Aldehydic derivatives of oxidatively modified proteins – CAT	0.918	0.000
Ketonic derivatives of oxidatively modified proteins – CAT	0.615	0.025
vaccinated group, liver		
Aldehydic derivatives of oxidatively modified proteins – TBARS	0.657	0.011
TBARS–CAT	0.723	0.003
Aldehydic derivatives of oxidatively modified proteins – CAT	0.908	0.000
Ketonic derivatives of oxidatively modified proteins – CAT	0.582	0.029
unhandled control, heart		
Aldehydic derivatives of oxidatively modified proteins – SOD	0.680	0.011
SOD – GPx	0.638	0.019
GPx – TAC	0.610	0.027
vaccinated group, heart		
TBARS – CAT	0.534	0.049
Aldehydic derivatives of oxidatively modified proteins – SOD	-0.583	0.029
Aldehydic derivatives of oxidatively modified proteins – CAT	0.727	0.003
Aldehydic derivatives of oxidatively modified proteins – GPx	0.732	0.003
Ketonic derivatives of oxidatively modified proteins – CAT	0.758	0.002
Ketonic derivatives of oxidatively modified proteins – GPx	0.596	0.025
SOD – GPx	-0.622	0.017
CAT – GPx	0.722	0.003

Our results also suggest that vaccination could promote the activation of the gluconeogenic substrate-providing enzymes, as well as substrates for aerobic metabolism that might in turn contribute to increase of oxidatively modified proteins in the muscle tissue of vaccinated trout (Tkachenko et al. 2016b).

The present study observed a marked alteration in the peroxidative process in different tissues following vaccination of trout against *Y. ruckeri*. There was increase in

lipid peroxidation in gills of fish treated with vaccine. Fish immunization against *Y. ruckeri* enhances also protein oxidation in gills that is directly related to free radical mediated toxicity induced by vaccination. It appears that T-cell activation is critical for immunity development. Raida and Buchmann (2008b) investigated development of adaptive immunity in rainbow trout surviving a primary infection with 5 x 10^5 CFU *Y. ruckeri* O1 (LD_{50} dose) by transcriptome analysis. These fish surviving a primary infection showed also a significantly increased survival following a secondary infection (same dose) when compared to naive trout. The cytokines and chemokines comprised IL-1β, IL-1 receptor antagonist (Ra), IL-6, IL-8, IL-10, IL-11 and IFN-γ, IL-1 receptor I and II (IL-RI and IL-RII). Transcript levels of genes encoding cytokines and receptors were increased during the primary infection but not during the secondary infection. Changes of T cell occurrence or activity in the spleen during the infections were inferred from the transcript level of T cell receptor (TCR), CD4 and CD8α genes. No alteration in the expression of MHC class II and immunoglobulins IgM and IgT was detected. The amount of *Y. ruckeri* O1 in the spleen was correlated to the expression of IL-1β, IL-8 and IL-10 genes with a peak expression (first infection). The low transcript levels of the bacterial gene and the hosts' immune genes during the re-infection can be interpreted as a result of development of adaptive immunity. This would explain the relatively fast elimination of the bacteria during the secondary infection whereby the activation of cytokines becomes less pronounced (Raida and Buchmann 2008b). Interestingly, no protection of naive fish was conferred when plasma from immune animals was transferred passively, strongly suggesting that cellular immune factors, and particularly CD8 T-cells, may be critical for protection against infection by *Y. ruckeri* (Barnes 2011). The generally successful vaccination of salmonids against ERM using *Y. ruckeri* bacterins based on serovar I and the numerous studies reporting cross-protection have perhaps led to a degree of complacency and a general consideration that other serovars are unnecessary in commercial vaccines (McCarthy and Johnson 1982).

In the present study, the most widely used and accepted markers were utilized to demonstrate the existence of oxidative stress in the gills, liver, and heart of trout vaccinated against *Y. ruckeri* (TBARS as a marker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, antioxidant enzymes, and the total antioxidant capacity). TBARS are a highly toxic by-products formed in part by lipid oxidation derived free radicals. Lipid peroxidation products reacts both irreversibly and reversibly with proteins and phospholipids with profound effects (Slatter et al. 2000). In this study, our results clearly demonstrate that immunization by anti-*Yersinia* vaccine do not alter liver and heart functioning in rainbow trout (Figs 1 and 2). Liver is the major site for metabolism (Liss et al. 1985), thus oxidative stress biomarkers are regarded as markers of liver injury. Our results indicated that the liver of immunized trout respond differently compared to gills and cardiac tissue. After 4 weeks after immunization, the ketonic derivatives of oxidatively modified proteins in liver were significantly decreased with alteration of total antioxidant capacity (Figs 2B, 3).

The present work has also demonstrated that the TBARS level as marker of lipid peroxidation as well as aldehydic and ketonic derivatives of oxidatively modified proteins increased significantly in the gills of trout following vaccination against

Y. ruckeri (Figs 1 and 2). The fish gill is the most physiologically diversified vertebrate organ, and its vasculature is the most intricate. Many of the anatomical characteristics of interlamellar vessels are strikingly similar to those of mammalian lymphatic capillaries, with the exception that interlamellar vessels that are directly fed by arteriovenous-like anastomoses. It is likely that gill interlamellar and mammalian lymphatics are physiologically equivalent (Olson 2002). Small and large lymphocytes, macrophages, neutrophils, eosinophilic granulocytes and antibody-secreting cells have been observed in the gill-associated lymphoid tissue of different fish species (Salinas et al. 2011). IgM+ cells (B-cells, plasma cells and IgM-bearing macrophages) are very abundant in the stratified epithelium of the gill arch and filaments (Grove et al. 2006). Study of dos Santos et al. (2001) strongly supports the importance of the route of immunization to locally stimulate antibody-secreting cells in gills and the importance that the gills might have in specific responses. In addition to the lymphoid tissue found within the gill lamellae, an interbranchial lymphoid tissue has been recently described in salmonids (Haugarvoll et al. 2008, Koppang et al. 2010). Therefore, as the first-line encounter towards antigens, the epithelium of the gills and intestines are important locations for immune reactions. It is likely that these sites should display the first phylogenetic signs of immune cell compartmentalization (Haugarvoll et al. 2008).

Immunization against *Y. ruckeri* reported here might play an important role in vaccination-induced oxidative stress in gills of trout due to phagocyte respiratory burst. ROS are used by the immune system as weapons against pathogens (Paiva and Bozza 2014). During the immune processes, the activation of phagocytes and/or the action of bacterial products with specific receptors are capable of promoting the assembly of the multicomponent flavoprotein NADPH oxidase, which catalyzes the production of high amounts of the superoxide anion radical ($O_2^{\cdot-}$). Under these particular circumstances, neutrophils and macrophages are recognized to produce superoxide free radicals and H_2O_2, which are essential for defense against phagocytized or invading microbes (Puertollano et al. 2011). Microbe recognition sets the immune system in motion, and ROS are produced not only in the phagocyte respiratory burst but also in other cell compartments, such as mitochondria, as intermediaries in many signal transduction pathways, such as leukocyte pattern recognition receptor (PRR) signaling. The generation of ROS is a prerequisite to the formation of mechanisms promote microbe clearance, whereas others can potentially contribute to microbe persistence (Paiva and Bozza 2014). At moderate concentrations, ROS play an important role as regulatory mediators in signaling processes. Many of the ROS-mediated responses actually protect the cells against oxidative stress and reestablish "redox homeostasis". At high concentrations, ROS are hazardous for cells and damage all major cellular constituents (Dröge 2002).

The antioxidant enzymes that provide the first line of cellular defense to ROS include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), glutathione S-transferease (GST) etc. However, an imbalance between the activities of cellular antioxidant enzymes and ROS production results in oxidative stress and cellular damage. If the antioxidant system is not able to eliminate or neutralize the excess of ROS, there is an increased risk of oxidative damage (Banaee 2013). The observed rise of GPx is certainly the result of an

increased *de novo* synthesis. This increased GPx activity is really close to the tendency observed in the case of oxidative stress induced by vaccination in the gills. Since GPx (using reduced glutathione as a cofactor) is the first line of defense against H_2O_2, it is likely that liver is facing increased levels of this reactive oxygen species. SOD activity also appeared to be significantly decreased in liver. The role of this enzyme is the containment of superoxide ions; it is a classical biomarker of oxidative stress (Dussauze et al. 2015).

Correlative analysis between oxidative stress biomarkers confirms our conclusions (Table 2). Immunization against *Y. ruckeri* in our study is related with alteration in oxidative stress biomarkers. Correlations between carbonyl content of oxidatively modified protein and TBARS level confirm that polyunsaturated fatty acid alkoxyl radicals formed during degradation of lipid hydroperoxides are likely involved in the formation of protein carbonyl derivatives (Refsgaard et al. 2000) in the liver and gills of *Yersinia ruckeri*-vaccinated animals on 4 weeks vaccination. Modification of proteins in condition of oxidative stress can contribute to protein dysfunction or tissue damage and disease progression. Bifunctional, most often α,β-unsaturated carbonyl compounds such as 4-hydroxy-2-nonenal, 4-oxo-2-nonenal and acrolein, generated from oxidation of polyunsaturated fatty acids, readily bind to protein nucleophiles. Modification by bifunctional aldehydes can also lead to intramolecular or intermolecular protein crosslinking (Sayre et al. 2006).

In all tissues, correlations between TBARS, aldehydic and ketonic derivatives of oxidatively modified proteins and CAT activity were observed (Table 2). The SOD-CAT system provides the first line of defense against oxygen toxicity and is usually used as a biomarker of ROS production (Li et al. 2011). Results of the present investigation indicated that the SOD and CAT activity was correlated with oxidative stress biomarkers in vaccinated trout. The alteration of SOD and CAT activity revealed that tissues might suffer from oxidative stress. This result is consistent with a previous study performed with fish exposed to carbamazepine (Li et al. 2011). Similarly, Zhang and co-workers (2004) found that SOD activity decreased gradually as the concentration of 2,4-dichlorophenol increased. In addition, the alterations of SOD may be the result of adaptation or loss in compensatory mechanisms (Jifa et al. 2006).

CAT is mainly located in the peroxisomes and, along with glutathione peroxidase, is responsible for the reduction of H_2O_2 produced from the metabolism of long chain fatty acids in peroxisomes (Filipak Neto et al. 2008). CAT has one of the highest turnover rates of all enzymes: one molecule of CAT can convert millions of molecules of hydrogen peroxide to water and oxygen per second (El-Gendy et al. 2009). In the current study, muscle CAT activity in vaccinated trout was significantly correlated with oxidative stress biomarkers (Table 2). In our previously study, correlations between CAT activity, lipid peroxidation and TAC confirmed the pivotal role of catalase in antioxidant defense in the muscle tissue (Tkachenko et al. 2016e).

Taking the results outlined above into account, it can be concluded that disturbances in redox homeostasis in gills, i.e. lipid peroxidation and protein oxidation, are induced by vaccination against *Y. ruckeri*. High levels of small and large lymphocytes, macrophages, neutrophils, eosinophilic granulocytes and antibody-secreting cells in the gill-associated lymphoid tissue (Salinas et al. 2011) as well as IgM+ cells (B-cells, plasma cells and IgM-bearing macrophages) in the stratified epithelium of

the gill arch and filaments (Grove et al. 2006) and vaccine-induced activation of immune system of trout can exert oxidative effects sensitively and thus the gills should pay the more caution during vaccination against *Y. ruckeri*. The present study revealed that levels of oxidatively modified proteins in liver of vaccinated trout were decreased compared with other tissues. We speculate that lower level of carbonyl derivatives in the hepatic tissue of vaccinated trout resulted in alteration of total antioxidant capacity (Figs 2 and 3).

Fish exposed to vaccination exhibit a variety of physiological responses, including oxidative metabolism imbalances (Tkachenko et al. 2015, 2016 a-e, Tkachenko and Grudniewska 2016). The alterations in oxidative stress biomarkers may indicate a compensatory response of the fish to vaccination. Differences observed in oxidative stress biomarkers obtained in our previous study (Tkachenko et al. 2015, 2016 a-e, Tkachenko and Grudniewska 2016) could reflect variation in the antioxidant mechanisms of vaccinated fish, duration of exposure, and the vaccine tested. Previous studies have shown that oxidative stress indices in fish may vary depending on the tissue and duration of immunization assessed. In our previous study (Tkachenko et al. 2015), we have analyzed the levels of oxidative stress biomarkers [TBARS, aldehydic and ketonic derivatives of OMP, TAC] and metabolic alterations in the liver of juvenile rainbow trout determining the effectiveness of the vaccine against *Y. ruckeri*. A statistically significant reduction in lipid peroxidation between the mean in groups immunized after first and second months after vaccination indicated an effective adaptive antioxidant defense mechanisms of fish for the immunity against *Y. ruckeri*. A similar reduction of lipid peroxidation between the mean in the control group of fish after first and second months of the study was observed. Reducing aldehydic and ketonic derivatives of oxidatively modified proteins in the liver of vaccinated trout after two months after immunization was caused by a high antioxidant capacity of the liver. Activation of proteolytic degradation of the modified amino acid residues may be one reason for the reduction of oxidatively modified derivatives. Increased total antioxidant capacity in the liver of individuals from control and immunized groups at second month after vaccination indicated the powerful adaptability of the liver, helping defend against oxidative stress induced by immunization (Tkachenko et al. 2015). The alterations in markers of oxidative stress and antioxidant defenses suggest that glutathione-dependent enzymes may contribute to balance of oxidative stress in the liver of trout vaccinated against *Y. ruckeri*. We did not find any alterations in the hepatic tissue after 60 days of immunization. This is likely a result of long-term adaptation to immunization (Tkachenko et al. 2016c).

The effects of vaccination against *Y. ruckeri* on muscle function, and the oxidative mechanism underlying those effects, by detecting relevant lipid peroxidation and protein oxidation biomarkers as well as biochemical alterations in rainbow trout following *Y. ruckeri* vaccination at first and second months after oral immunization (Tkachenko et al. 2016a, b). The TBARS level in the muscle tissue of vaccinated group was at same level compared to unhandled group. The ketonic derivatives of oxidatively modified proteins in the trout following *Y. ruckeri* vaccination at first month after immunization were significantly increased compared to the level in the controls, while the aldehydic derivatives of oxidatively modified proteins were non-significantly increased (Tkachenko et al. 2016b). All these culminated in a depletion

of GPx activity and low TAC level. Correlations between CAT activity and lipid peroxidation and TAC confirmed the pivotal role of CAT in antioxidant defense during immunization. From a broader perspective, it is suggested that immunization of fish with anty-*Yersinia* vaccine is associated with induced free radical formation and oxidative stress. Free radicals would therefore be at least partially responsible for the induction of both humoral and cellular elements of the immunity and increased protective immunity against *Y. ruckeri* infection (Tkachenko et al. 2016e). In our study, vaccination against *Y. ruckeri* at second month after oral immunization results to non-significant decrease of TBARS as lipid peroxidation level, aldehydic and ketonic derivatives of OMP level in the muscle tissue during the second month after immunization against *Y. ruckeri*, while significant changes occurred in GR activity (decreased by 46%, $p = 0.017$) and TAC (increased by 44%, $p = 0.045$). The alterations in markers of oxidative stress and antioxidant defenses suggest that glutathione-dependent enzymes may contribute to balance of oxidative stress in the muscle tissue of trout vaccinated against *Y. ruckeri* (Tkachenko et al. 2016a).

CONCLUSIONS

Vaccinated trout showed tissue-specific oxidative stress responses in gills, liver, and heart. The gill tissue was the most sensitive to oxidative damage among these specimens. Accumulation of oxidative stress biomarkers in the rainbow trout was tissue-specific as follows: gills > heart > liver. These results suggest that the trout expressed tissue-specific oxidative stress mechanisms due to anti-*Yersinia* vaccine treatment. The oxidative stress biomarkers, i.e. content of oxidative modified proteins in the muscle tissue, gills, and liver, and 2-thiobarbituric acid reactive substances level in the gills, and total antioxidant capacity in the liver, were sensitive to vaccination of trout against *Y. ruckeri* and may potentially be used as biomarkers in evaluating vaccine toxicity in rainbow trout.

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EFEKTY DOUSTNEGO SZCZEPIENIA PRZECIWKO *YERSINIA RUCKERI* NA STĘŻENIE MARKERÓW STRESU OKSYDACYJNEGO W SKRZELACH, WĄTROBIE I SERCU PSTRĄGA TĘCZOWEGO *(ONCORHYNCHUS MYKISS* WALBAUM)

Streszczenie

Celem pracy było zbadanie wpływu doustnego szczepienia przeciwko *Yersinia ruckeri* na poziom biomarkerów stresu oksydacyjnego w różnych tkankach pstrąga tęczowego (*Oncorhynchus mykiss* Walbaum). Szczepionka składała się z trzech szczepów *Y. ruckeri* (serotyp O1) pochodzących od pstrąga tęczowego z klinicznymi objawami jersiniozy. Inaktywowane antygeny *Y. Druckeri* wprowadzono do paszy; immunizacja ryb odbywała się trzykrotnie w odstępach jednodniowych. Po upływie pierwszego miesiąca po skończeniu szczepienia do badań biochemicznych pobrano próbki skrzeli, tkanki wątrobowej i sercowej ryb. Szczepione ryby wykazały specyficzne zmiany poziomu markerów stresu oksydacyjnego w skrzelach, wątrobie i sercu. Skrzela okazały się najbardziej wrażliwe na uszkodzenia oksydacyjne wśród badanych tkanek. Akumulacja biomarkerów stresu oksydacyjnego u pstrąga tęczowego charakteryzowała się następującą specyficznością: skrzela > serce > wątroba. Wyniki te sugerują, że mechanizmy stresu oksydacyjnego są specyficzne dla różnych tkanek pstrąga tęczowego w wyniku szczepienia przeciwko jersiniozie. Nie stwierdzono statystycznie istotnych zmian w aktywności przeciwutleniaczy oprócz dysmutazy ponadtlenkowej w tkance wątroby i serca oraz peroksydazy glutationowej w skrzelach szczepionych ryb. Analiza korelacyjna potwierdziła ważną rolę katalazy w obronie antyoksydacyjnej immunizowanych ryb. Biomarkery stresu oksydacyjnego, tj. zawartość zmodyfikowanych oksydacyjnie białek w skrzelach i wątrobie, poziom produktów reagujących z kwasem 2-tiobarbiturowym w skrzelach oraz całkowita zdolność antyoksydacyjna w wątrobie, są wrażliwe na wpływ szczepienia przeciwko *Y. ruckeri* i mogą potencjalnie być wykorzystane jako biomarkery w ocenie toksyczności szczepionek w akwakulturze pstrąga tęczowego.