BALTIC COASTAL ZONE
Journal of Ecology and Protection of the Coastline

Vol. 20 pp. 137-159 2016

ISSN 1643-0115

Original research paper

Received: **29/08/2016**
Accepted: **04/11/2016**

MUSCLE BIOCHEMISTRY IN RAINBOW TROUT *ONCORHYNCHUS MYKISS* FOLLOWING *YERSINIA RUCKERI* VACCINATION

Halyna Tkachenko[1], Joanna Grudniewska[2], Agnieszka Pękala[3]

[1]Department of Zoology and Animal Physiology,
Institute of Biology and Environmental Protection,
Pomeranian University in Słupsk,
ul. Arciszewskiego 22B, 76-200 Słupsk, Poland
e-mail: tkachenko@apsl.edu.pl, biology.apsl@gmail.com
[2]Department of Salmonid Research, Stanisław Sakowicz Inland Fisheries Institute,
83-330 Żukowo, Poland
e-mail: jgrudniewska@infish.com.pl
[3]Department of Fish Diseases, National Veterinary Research Institute,
Aleja Partyzantów 57, 24-100 Puławy, Poland

Abstract

Vaccination of rainbow trout against Yersiniosis confers a high degree of protection to the fish (Raida et al. 2011). On the other hand, vaccination could alter metabolitic reactions in organisms. Therefore, exploring the effects of vaccination against *Y. ruckeri* on health condition of trout in general, and oxidative stress biomarkers and biochemical alterations in different tissues specifically, would be valuable. This prompted us to investigate theeffects of vaccination against *Y. ruckeri* on muscle function, and the oxidative mechanism underlying those effects, by detecting relevant lipid peroxidation (2-thiobarbituric acid reactive substances, TBARS) and protein oxidation biomarkers (aldehydic derivatives and ketonic derivatives) as well as biochemical alterations (aminotransferases and lactate dehydrogenase activity, lactate and pyruvate levels) in rainbow trout *Oncorhynchus mykiss* following *Y. ruckeri* vaccination at first month after oral immunization. Concentrated vaccine with inactivated by formalin *Y. ruckeri* strains was enclosed by fish feed, and was administered three times every other day. Rainbow trout from each group were euthanized 30 days after the immunization, and then muscle tissue were sampled for analysis. 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. Pyruvate level was increased by 47% ($p = 0.013$) in vaccinated trout compared to values of untreated fish. Lactate level, aminotransferases and lactate dehydrogenase activities were non-significantly altered in vaccinated trout. Our results suggest that vaccination could promote the ac-

tivation of the gluconeogenic substrate-providing enzymes, as well as substrates for aerobic metabolism that might in turn contribute to increase of oxidatively modified proteins. The oxidative stress biomarkers, i.e. content of oxidative protein damage, as well as biochemical enzymes and substrates were sensitive to vaccination of trout against *Y. ruckeri* and may potentially be used as biomarkers in evaluating vaccine toxicity in rainbow trout. From a practical point of view, the results may be useful in relation to studies of infections and the development, administration and uptake of new vaccines applicable for large amounts of fish.

Key words: yersiniosis, rainbow trout *(Oncorhynchus mykiss)*, lipid peroxidation, oxidatively modified proteins, aminotransferases, lactate dehydrogenase, lactate, pyruvate

INTRODUCTION

Enteric redmouth (ERM) or Yersiniosisis an acute or chronic generalized bacterial septicaemia in fish caused by a Gram-negative enteric bacterium, *Yersinia ruckeri*. The early terminology, redmouth, redthroat or bacterial septicaemia, used to describe the disease was derived from the gross clinical signs which, in many cases, were caused by mixed infections of Gram-negative organisms, particularly *Aeromonas liquefaciens* (Barnes 2011). The disease gets its name from the subcutaneous hemorrhages, it can cause at the corners of the mouth and in gums and tongue. Other clinical signs include exophthalmia, darkening of the skin, splenomegaly and inflammation of the lower intestine with 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). The vent area may also become inflamed, both externally and internally, at the distal end of the intestine (Barnes 2011). Exophthalmia has been reported in some cases in the later stages of infection, with haemorrhaging of the ocular cavity and iris (Fuhrmann et al. 1983, 1984). Internally, there are congestion of the blood vessels throughout the peritoneum, and petechial haemorrhages, affecting liver, pancreas, swim-bladder, lateral muscles and adipose tissues associated with the pyloric caecae (Wobeser 1973). The kidney and spleen may be swollen and there may be fluid inboth the stomach and the intestine which has a yellowish, opaque, mucoid appearance (Busch 1982). The spleen may increase up to threefold in size and this is associated with influx of bacteria, which increase in number over the first 3 days of infection in trout before declining (Raida and Buchmann 2008, Barnes 2011).

Prevention of ERM outbreaks is achieved by avoiding the introduction of infected stock and ensuring that all imported eggs have been disinfected properly (Barnes 2011). If a serious outbreak does occur, antibiotics may need to be administered. ERM was one of the first diseases for which an effective commercial fish vaccinewas developed (Busch 1978, Cossarini-Dunier 1986) and vaccination was widely employed as an effective aid in its control. Most commercial vaccines are bacterins prepared from virulent isolates, some using whole bacterial cells and others exposing internal bacterial cell components by lysis at pH 9.8. Most commercial vaccines use the whole-cell serotype, serovar I (Hagerman strain), which is the cause of the majority of disease outbreaks. Some outbreaks have been attributed to serovar II (Cipriano et al. 1986). Commercial vaccines are administered by intraperitoneal (intracoelomic) injection or immersion. In the latter case, the major source of uptake is

the gill, where antigen-trapping cells have been detected (Torroba et al. 1993) and high levels of antibody-secreting cells are detected after immersion vaccination (Santos et al. 2001). Under field conditions, the effectiveness of ERM vaccines may vary. In situations where fish are highly stressed through inappropriate handling, poor water quality, overstocking or poor nutrition, vaccines rarely prevent disease completely as the immune system is also affected by these conditions (Pickering and Pottinger 1987, Barnes 2011). Interestingly, Raida and Buchmann (2007) have indicated that expression of both innate and specific adaptive immune-response genes are highly temperature-dependent in rainbow trout following vaccination with a bacterin of *Y. ruckeri*. The up-regulation of cytokine genes was generally faster and higher at high water temperature, with major expression at 25°C. The pro-inflammatory cytokine interleukin IL-1β and interferon IFN-γ were significantly up-regulated in all immunized groups, whereas the cytokine IL-10 was only up-regulated in fish kept at 15 and 25°C. The gene encoding the C5a (anaphylatoxin) receptor was expressed at a significantly increased level in both head kidney and spleen of immunized fish. The secreted immunoglobulin M (IgM)-encoding gene was significantly up-regulated in the head kidney of immunized trout reared at 25°C, and a positive correlation (r = 0.663) was found between gene expression of secreted IgM in the head kidney and *Y. ruckeri*-specific antibodies in plasma (Raida and Buchmann 2007). Humoral factors such as Ig and complement are less important in the protection induced by bath vaccination while cellular factors including cytotoxic T-cells could play a role in immunity against *Y. ruckeri* (Raida and Buchmann 2008). Gene expression in fish vaccinated at 15°C (the protected fish) 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. Passive immunization using transfer of plasma from vaccinated fish to naïve fish conferred no protection (Raida and Buchmann 2008).

The immune mechanisms responsible for protection may comprise both cellular and humoral elements (Raida et al. 2011). Fish are the most primitive gnathostomes (jawed vertebrates) possessing an adaptive immune system. A key hallmark of the vertebrate adaptive immune system is the generation of antigen-specific antibodies from B cells. ERM is traditionally associated with rainbow trout (Ormsby et al. 2016). ERM has been controlled successfully using immersion-applied bacterin vaccines for several decades (Welch and LaPatra 2016). Immersion-vaccines (bacterins) are routinely used for aquacultured rainbow trout to protect against *Y. ruckeri*. During immersion vaccination, rainbow trout take up and process the antigens, which induce protection (Korbut et al. 2016). Welch and LaPatra (2016) have speculated that the exceptionally high potency of *Y. ruckeri* lipopolysaccharide (LPS) accounts for the unusual success of this vaccine when delivered by immersion.

On the other hand, Ghosh and co-workers (2016) have revealed that oral vaccination of first-feeding Atlantic salmon confers greater protection against Yersiniosis than immersion vaccination. In their study, first-feeding Atlantic salmon fry (<0.5 g) were immunized either by oral administration of a microencapsulated *Y. ruckeri* vaccine formulation (0.38 g initial weight), or *via* immersion in bacterin suspension (0.26 g), with and without a booster immersion vaccination at 1g size. Protection in groups

receiving only immersion immunization did not differ significantly from untreated controls when challenged with *Y. ruckeri* at approximately 5 g size, while orally immunized fish were significantly better protected than untreated controls. A quantitative real-time PCR assay was used to successfully detect covertly infected fish among challenge survivors, indicating more than 50% of surviving fish in each group were infected with no significant differences between immunized fish and untreated controls (Ghosh et al. 2016).

In our previous study, we have assessed the influence of vaccination against ERM on oxidative stress biomarkers and antioxidant defense in the muscle tissue of rainbow trout vaccinated against *Y. ruckeri* in the first and second month after immunization. No significant difference was noted in lipid peroxidation level in either the first or second month after vaccination, while aldehydic and ketonic derivatives of oxidatively modified proteins (OMB) in the vaccinated group were significantly lower in the second month compared to those in the first month after vaccination ($p < 0.05$). The content of ketonic derivatives of OMB in muscles in the first month after immunization was higher compared to untreated control. All these culminated in a depletion of glutathione peroxidase activity and low level of total antioxidant capacity (TAC). Correlations between catalase activity and lipid peroxidation and TAC confirmed the pivotal role of catalase in antioxidant defense during immunization. From a broader perspective, it is suggested that immunization of fish with *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. 2016b).

The balance between prooxidant endogenous and exogenous factors and antioxidant defenses (enzymatic and nonenzymatic) in biological systems can be used to assess toxic effects under stressful conditions, especially oxidative damage induced by different classes of xenobiotics. The role of these antioxidant systems and their sensitivity can be of great importance in toxicology studies (Valavanidis et al. 2006, Lushchak 2011). Therefore, exploring the effects of vaccination against *Y. ruckeri* on health condition of trout in general, and oxidative stress biomarkers and biochemical alterations in different tissues specifically, would be valuable. This prompted us to investigate theeffects 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 (aminotransferases and lactate dehydrogenase activity, lactate and pyruvate levels) in rainbow trout following *Y. ruckeri* vaccination at first month after oral immunization.

MATERIALS AND METHODS

Experimental animals. Clinically healthy rainbow trout with a mean body mass of 107.9 ± 3.1 g were used in the experiments. The experiments were performed in water at 14.5 ± 0.5°C and pH 7.2-7.4. The dissolved oxygen level was about 9 ppm with additional oxygen supply, with a water flow of 25 L/min, and a photoperiod of

12 h per day. The same experimental conditions were used during the whole research. The water parameters were maintained under constant surveillance. The fish were held in square tanks (150 fish per tank) and fed commercial pelleted diet.

Experimental design. The fish were divided into two groups: untreated control (n = 15) and immunized against ERM (n = 15). The vaccine against ERM (Department of Fish Diseases, National Veterinary Research Institute, Pulawy, Poland) contained three inactivated *Y. ruckeri* strains originating from rainbow trout cultured at different farms, in which fish were exhibiting clinical signs of ERM. The bacteria isolates belonged to O1 serotype and showed some differences in their biochemical properties. Concentrated vaccine was enclosed by fish feed, and was administered three times every other day. Fifteen rainbow trout from each group were euthanized 30 days after the immunization, and then muscle tissue samples were collected.

Laboratory analyses. The samples were homogenized in ice-cold buffer (100 mMTris-HCl, pH 7.2) using a glass homogeniser immersed in ice water bath. Homogenates were centrifuged at 3000 g for 15 min at 4°C. After centrifugation, the supernatant was collected and frozen at -20°C until analyzed. Protein contents were determined with the method described by 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 adding the tissue supernatant.

Determination of 2-thiobarbituric acid reactive substances (TBARS). The level of lipid peroxidation was determined by quantifying the concentration of TBARS with the Kamyshnikov method (2004) for determining the MDA concentration. Briefly, 2.1 mL of sample homogenate (in tris-HCl buffer, 100 mM, pH 7.2) was added to 1 mL of 20% of 2-thiobarbituric acid (TCA), and 1 mL of 0.8% of trichloroacetic acid (TBA). The mixture was heated in a boiling water bath for 10 min. After cooling, the mixture was centrifuged at 3,000 g for 10 min. The absorbance of the supernatant was measured at 540 nm. The concentration of MDA (nmol/1 mg of protein) was calculated using $1.56\ 10^5\ mM^{-1}\ cm^{-1}$ as the extinction coefficient.

Measurement of carbonyl groups of oxidatively modified protein. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2,4-dinitrophenylhydrazine (DNPH) as described by Levine and co-workers (1990) and as modified by Dubinina and coworkers (1995). DNPH was used for determining carbonyl content in soluble and insoluble proteins. Briefly, 1 mL of 0.1M DNPH (dissolved in 2M HCl) was added to 0.1mL of the sample after denaturation of proteins by 20% TCA. After addition of the DNPH solution (or 2M HCl to the blanks), the tubes were incubated for 1 h at 37°C. The tubes were spun in a centrifuge for 20 min at 3,000 g. After centrifugation, the supernatant was decanted and 1 mL of ethanol–ethylacetate solution was added to each tube. After mechanical disruption of the pellet, the tubes were allowed to stand for 10 min and then spun again (20 min at 3,000 g). The supernatant was de-

canted, and the pellet was washed thrice with ethanol–ethylacetate solution. After the final wash, the protein was solubilised in 2.5 mL of 8 M urea solution. To speed up the solubilisation process, the samples were incubated at 90°C in a water bath for 10 to 15 min. The final solution was centrifuged to remove any insoluble material. The carbonyl content was calculated from the absorbance measurement at 370 nm and 430 nm, and an absorption coefficient of 22,000 M^{-1} cm^{-1}. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehydic derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}).

Assays of ALT (E.C. 2.6.1.2) and AST (E.C. 2.6.1.1) activities. ALT and AST activity was analyzed spectrophotometrically by standard enzymatic method (Reitman and Frankel 1957). The ketoacids produced by the enzyme action reacts with 2,4-dinitrophenylhydrazine producing hydrazone complex measured calorimetrically at 530 nm. ALT and AST activities were expressed as μmol pyruvate per h per mg of protein.

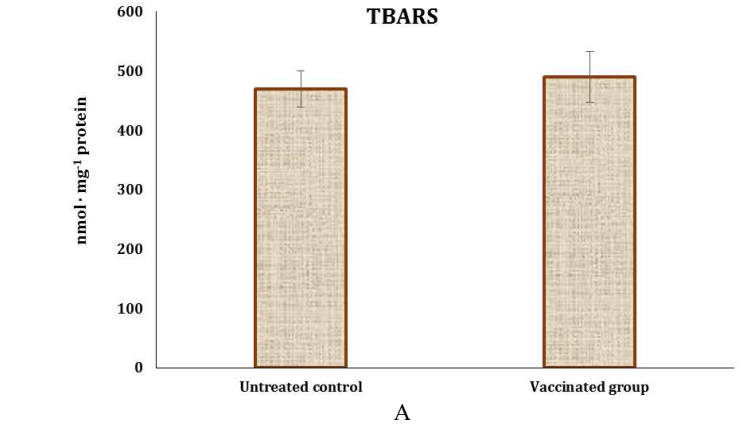
Assay of LDH (E.C. 1.1.1.27) activity. The colorimetric method of Sevela and Tovarek (1959) was used for the determination of LDH activity LDH activity was expressed as μmol pyruvate per h per mg of protein.

Assays of lactate and pyruvate concentrations. Lactate and pyruvate concentration was measured according to the procedure described by Herasimov and Plaksina (2000). Seven mL of tissue homogenate sample was added to 1 mL of metaphosphoric acid (10%). The mixture was centrifuged at 800 g for 5 min to separate the supernatant. 1 mL $CuSO_4$ (25%) and 0.5 g $Ca(OH)_2$ were added to the supernatant, which was then mixed for 30 min. The mixture was centrifuged at 1,000 g for 10 min. For lactate concentration assay the resulting supernatant was resuspended in 3 mL *p*-dimethylaminobenzaldehyde and 1 mL NaOH (25%). Solutions were heated in a water bath at 37°C for 45 min, which was then centrifuged at 1000 g for 10 min. The absorbance was measured at 420 nm. Solution with *p*-dimethylaminobenzaldehyde and NaOH (25%) was used as blank. For pyruvate concentration assay the resulting supernatant was resuspended in 0.1 mL $CuSO_4$ (10%), 4 mL H_2SO_4, and 0.1 mL hydroquinone, which was then heated in a water bath at 100°C for 15 min. The absorbance was measured at 430 nm. Calibration curve of lactate (0.1-5 mM) and pyruvate (0.1-5 mM) was used, and results were expressed in nmol per mg protein.

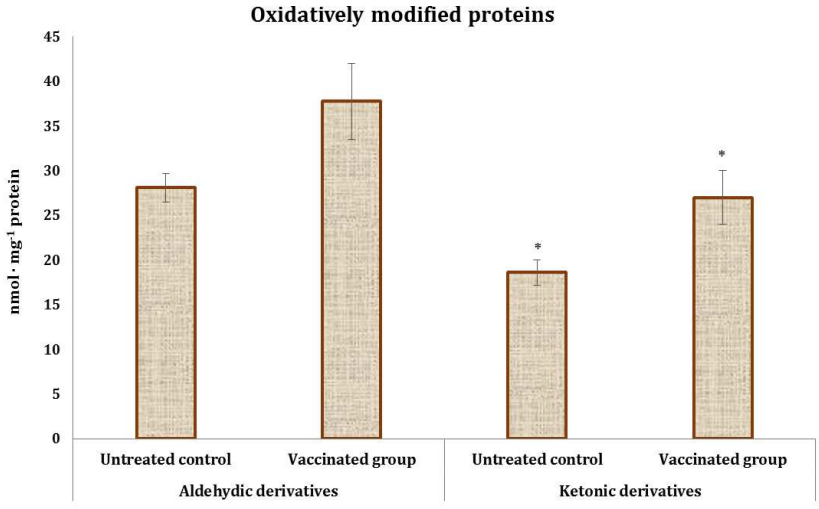
Statistical analysis. The mean ±S.E.M. values was calculated for each group to determine the significance of inter group difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test ($p > 0.05$). Significance of differences between the oxidative stress biomarkers level (significance level, $p < 0.05$) was examined using Mann-Whitney *U* test. Correlations between parameters at the set significance level were evaluated using Spearman's correlation analysis (Zar 1999). All statistical calculation was performed on separate data from each individual with STATISTICA 8.0.

RESULTS

Vaccination caused a non-significant changes of the TBARS level in the muscle tissue (490.15 ± 43.23 vs. 469.92 ± 30.71 nmol·mg^{-1} protein) (Fig. 1A). The ketonic derivatives of oxidatively modified proteins in the trout following *Y. ruckeri* vaccination at first month after immunization were significantly increased by 45% ($p = 0.037$) compared to the level in the controls (27 ± 3.01 vs. 18.61 ± 1.43 nmol·mg^{-1} protein) (Fig. 1B). The aldehydic derivatives of oxidatively modified proteins were non-significantly increased in vaccinated group compared to controls (37.72 ± 4.27 vs. 28.07 ± 1.57 nmol·mg^{-1} protein) (Fig. 1B).



A



B

Fig. 1. TBARS level (A), aldehydic and ketonic derivatives of oxidatively modified proteins (B) in the muscle tissue of the rainbow trout following *Y. ruckeri* vaccination at first month after immunization. Data are represented as mean ±S.E.M., n = 15
* – the significant difference was shown as $p < 0.05$ when compared vaccinated group and unhandled group values

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are found in the liver, heart, skeletal muscle, kidney, pancreas, spleen, erythrocyte, brain and gills (Banaee et al. 2011). When diseases or injuries affect these tissues, the cells are destroyed and these enzymes are released into plasma. ALT and AST activities according to Fig. 2A were non-significantly alteredin the muscle tissue of the rainbow trout following *Y. ruckeri* vaccination at first month after immunization as compared with control group (5.95 ± 1.17 vs. 3.57 ± 0.29 µmol·h^{-1}·mg^{-1} protein).

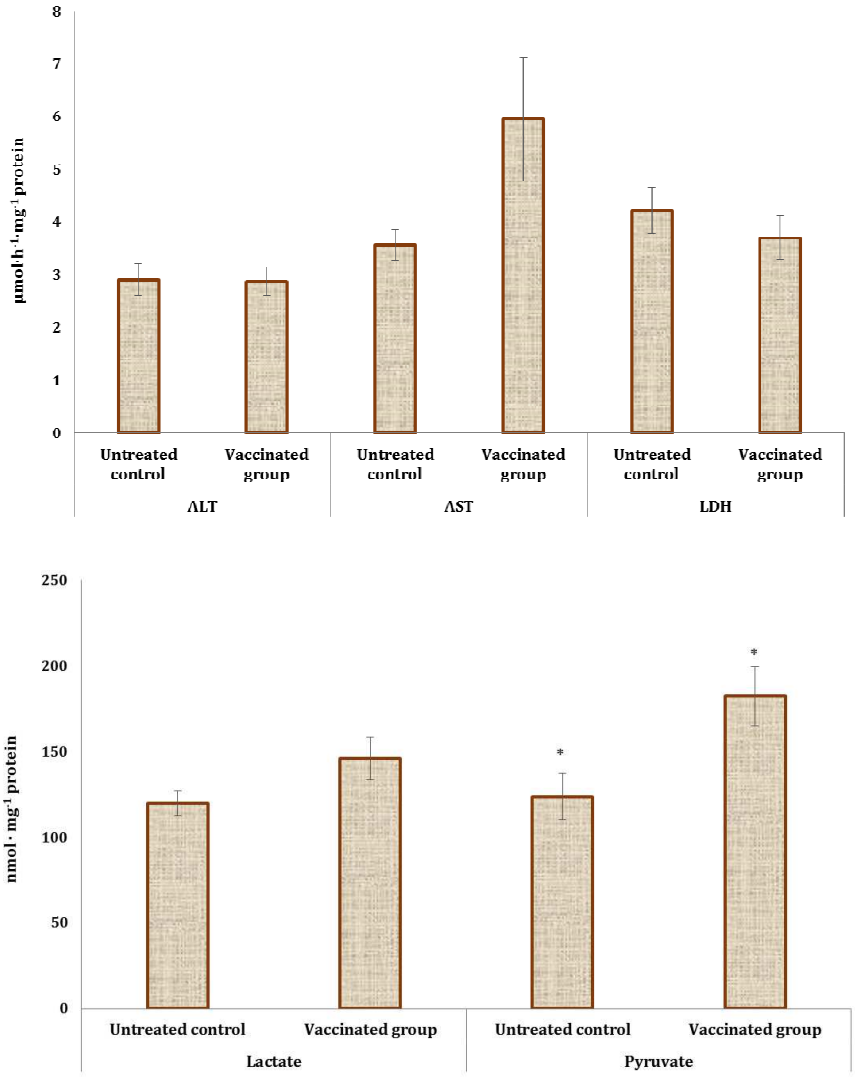
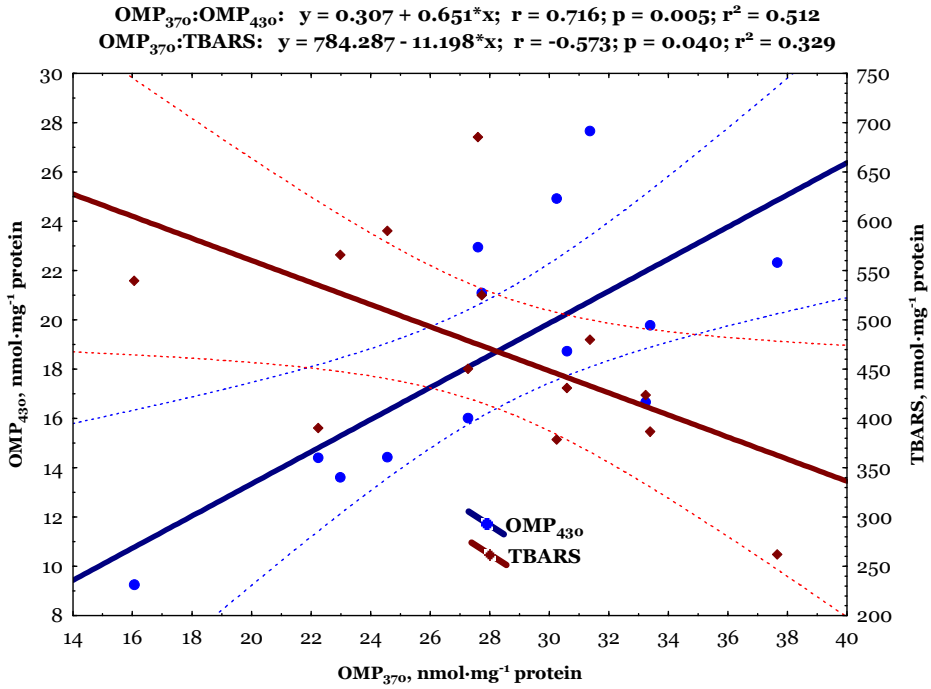


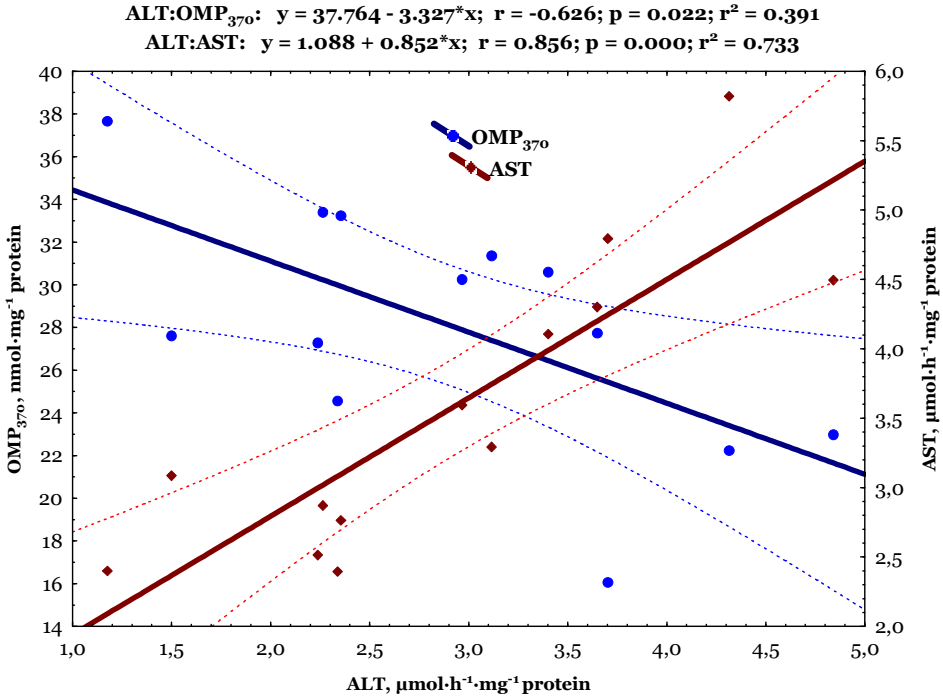
Fig. 2. Alanine and aspartate aminotransferases, lactate dehydrogenase activity (A), lactate and pyruvate level (B) in the muscle tissue of the rainbow trout following *Y. ruckeri* vaccination at first month after immunization. Data are represented as mean ±S.E.M., n = 15
* – the significant difference was shown as $p < 0.05$ when compared vaccinated group and unhandled group values

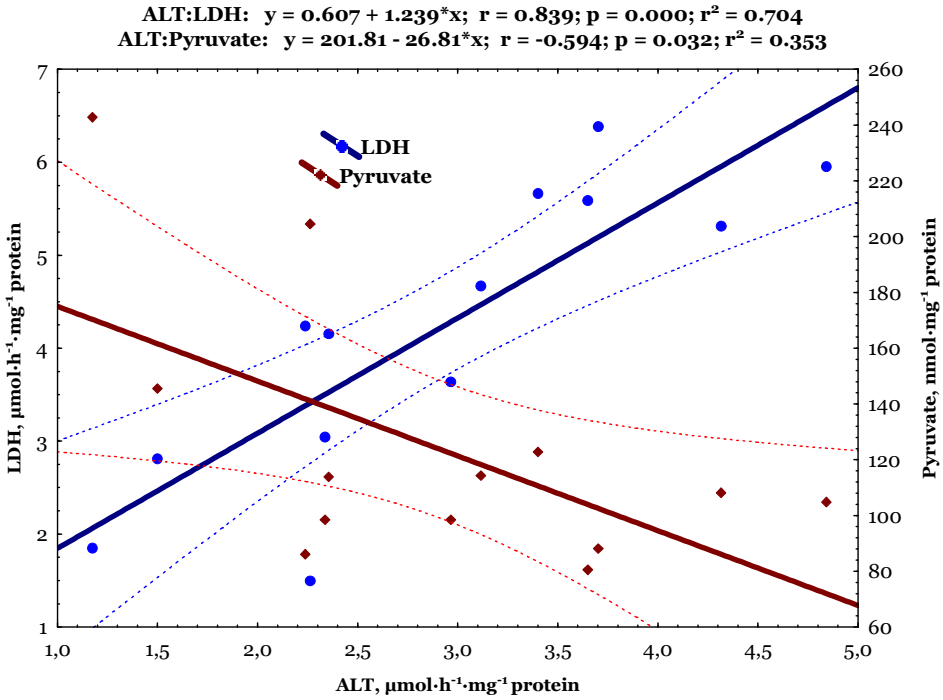
Lactate dehydrogenase (LDH) is an enzyme found in almost all body tissues, such as heart, kidneys, liver, skeletal muscle, brain, erythrocyte and gills. LDH measurement is used to detect tissue disorders and as an aid in the diagnosis of tissue damage (Banaee et al. 2011). The Fig. 2A also revealed that LDH activity in vaccinated trout was non-significantly altered compared to the controls (3.71 ± 0.41 vs. 4.22 ± 0.44 $\mu mol \cdot h^{-1} \cdot mg^{-1}$ protein).

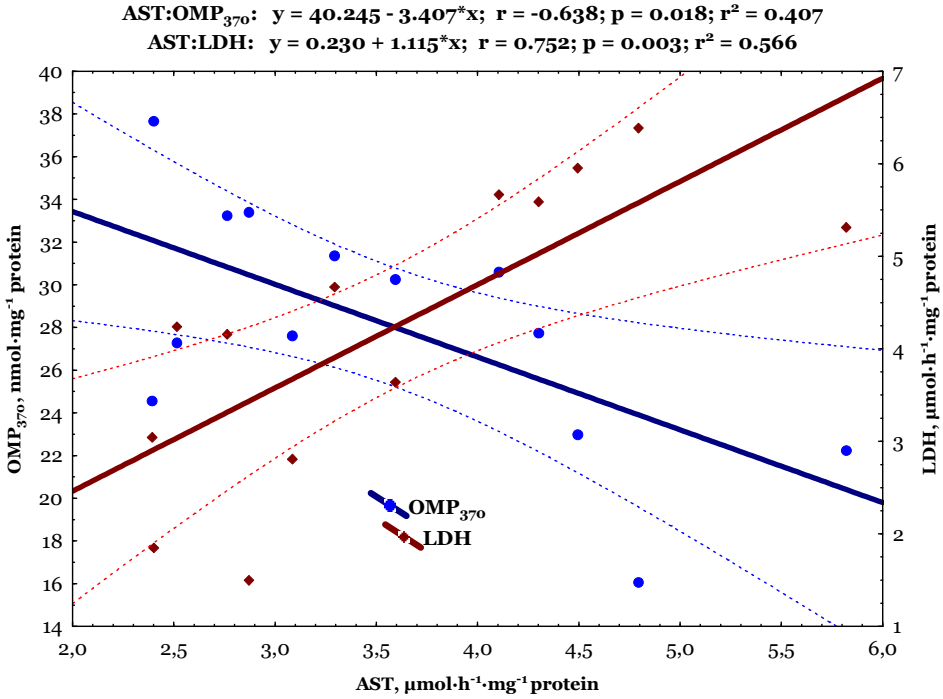
Fig. 2B represents the lactate and pyruvate concentrations in the muscle tissue of the rainbow trout following *Y. ruckeri* vaccination at first month after immunization. Regarding topyruvate, its level was increased by 47% ($p = 0.013$) in vaccinated trout compared to values of untreated fish (182.2 ± 17.44 vs. 123.73 ± 13.35 $nmol \cdot mg^{-1}$ protein). Lactate level was non-significantly altered in vaccinated trout compared to controls (146.09 ± 12.35 vs. 119.95 ± 119.95 $nmol \cdot mg^{-1}$ protein) (Fig. 2B).

Several correlations between checked parameters were found (Fig. 3). In unhandled group, aldehydic derivatives of OMP correlated positively with TBARS level ($r = 0.716$, $p = 0.005$) and inversely with ketonic derivatives of OMP ($r = -0.573$, $p = 0.040$), ALT activity correlated inversely with aldehydic derivatives of OMP ($r = -0.626$, $p = 0.022$) and positively with AST activity ($r = 0.856$, $p = 0.000$), ALT activity correlated positively with LDH activity ($r = 0.839$, $p = 0.000$) and inversely with pyruvate level ($r = -0.594$, $p = 0.032$). AST activity correlated positively with LDH activity ($r = 0.752$, $p = 0.003$) and inversely with aldehydic derivatives of OMP ($r = -0.638$, $p = 0.018$). Lactate level correlated positively with aldehydic derivatives of OMP ($r = 0.661$, $p = 0.013$) and inversely with TBARS level ($r = -0.712$, $p = 0.006$). Pyruvate level correlated positively with aldehydic derivatives of OMP ($r = 0.661$, $p = 0.013$) and inversely with LDH activity ($r = -0.747$, $p = 0.003$) (Fig. 3).




ALT:OMP370: y = 37.764 - 3.327*x; r = -0.626; p = 0.022; r² = 0.391
ALT:AST: y = 1.088 + 0.852*x; r = 0.856; p = 0.000; r² = 0.733
OMP370
AST
OMP370, nmol·mg-1 protein
AST, µmol·h-1·mg-1 protein
ALT, µmol·h-1·mg-1 protein


ALT:LDH: y = 0.607 + 1.239*x; r = 0.839; p = 0.000; r² = 0.704
ALT:Pyruvate: y = 201.81 - 26.81*x; r = -0.594; p = 0.032; r² = 0.353
LDH
Pyruvate
LDH, µmol·h-1·mg-1 protein
Pyruvate, nmol·mg-1 protein
ALT, µmol·h-1·mg-1 protein


AST:OMP370: y = 40.245 - 3.407*x; r = -0.638; p = 0.018; r² = 0.407
AST:LDH: y = 0.230 + 1.115*x; r = 0.752; p = 0.003; r² = 0.566
OMP370, nmol·mg-1 protein
LDH, µmol·h-1·mg-1 protein
AST, µmol·h-1·mg-1 protein
OMP370
LDH
40
38
36
34
32
30
28
26
24
22
20
18
16
14
7
6
5
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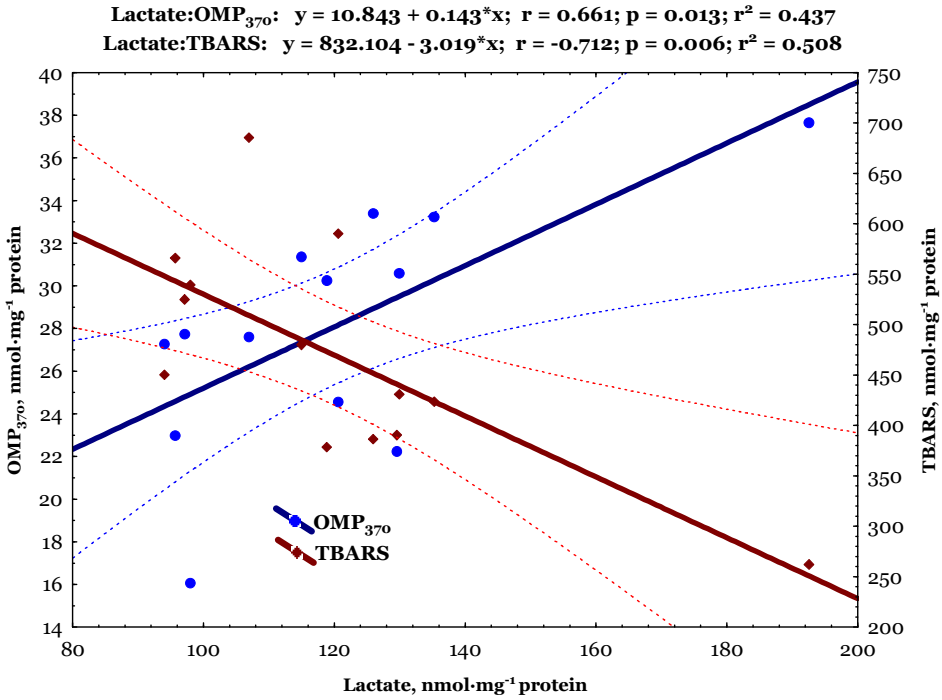
5,5
6,0


Lactate:OMP370: y = 10.843 + 0.143*x; r = 0.661; p = 0.013; r² = 0.437
Lactate:TBARS: y = 832.104 - 3.019*x; r = -0.712; p = 0.006; r² = 0.508
OMP370, nmol·mg-1 protein
TBARS, nmol·mg-1 protein
Lactate, nmol·mg-1 protein
OMP370
TBARS
40
38
36
34
32
30
28
26
24
22
20
18
16
14
750
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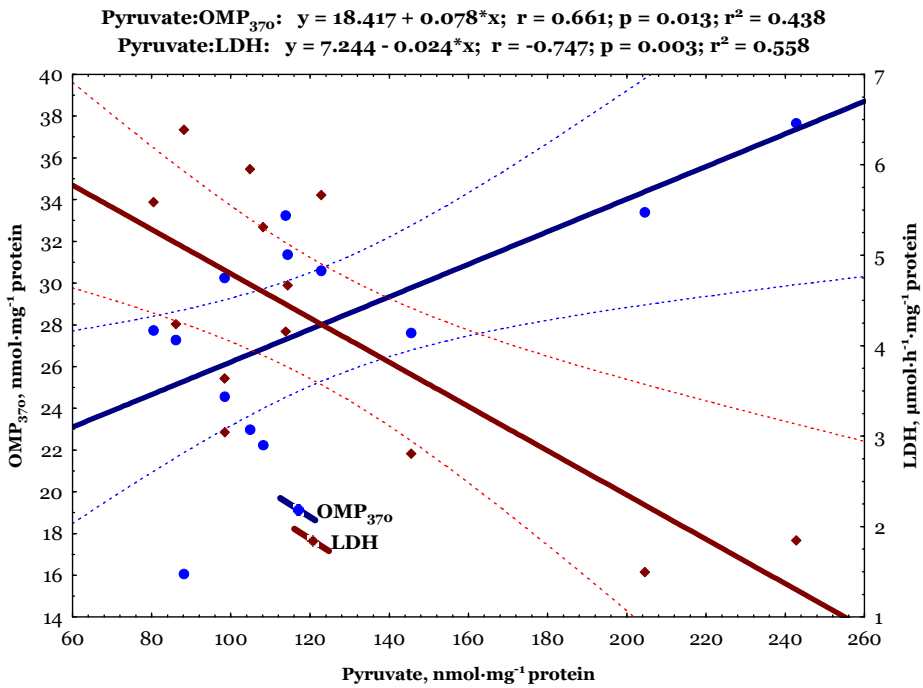
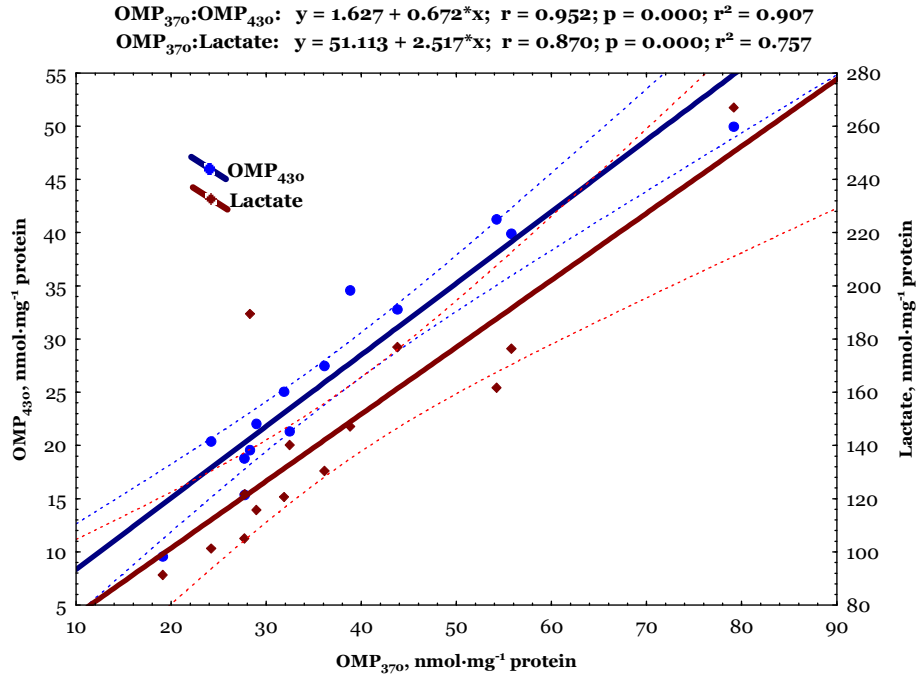


Fig. 3. Correlations between oxidative stress biomarkers and biochemical indices in the muscle tissue of the rainbow trout of unhandled group



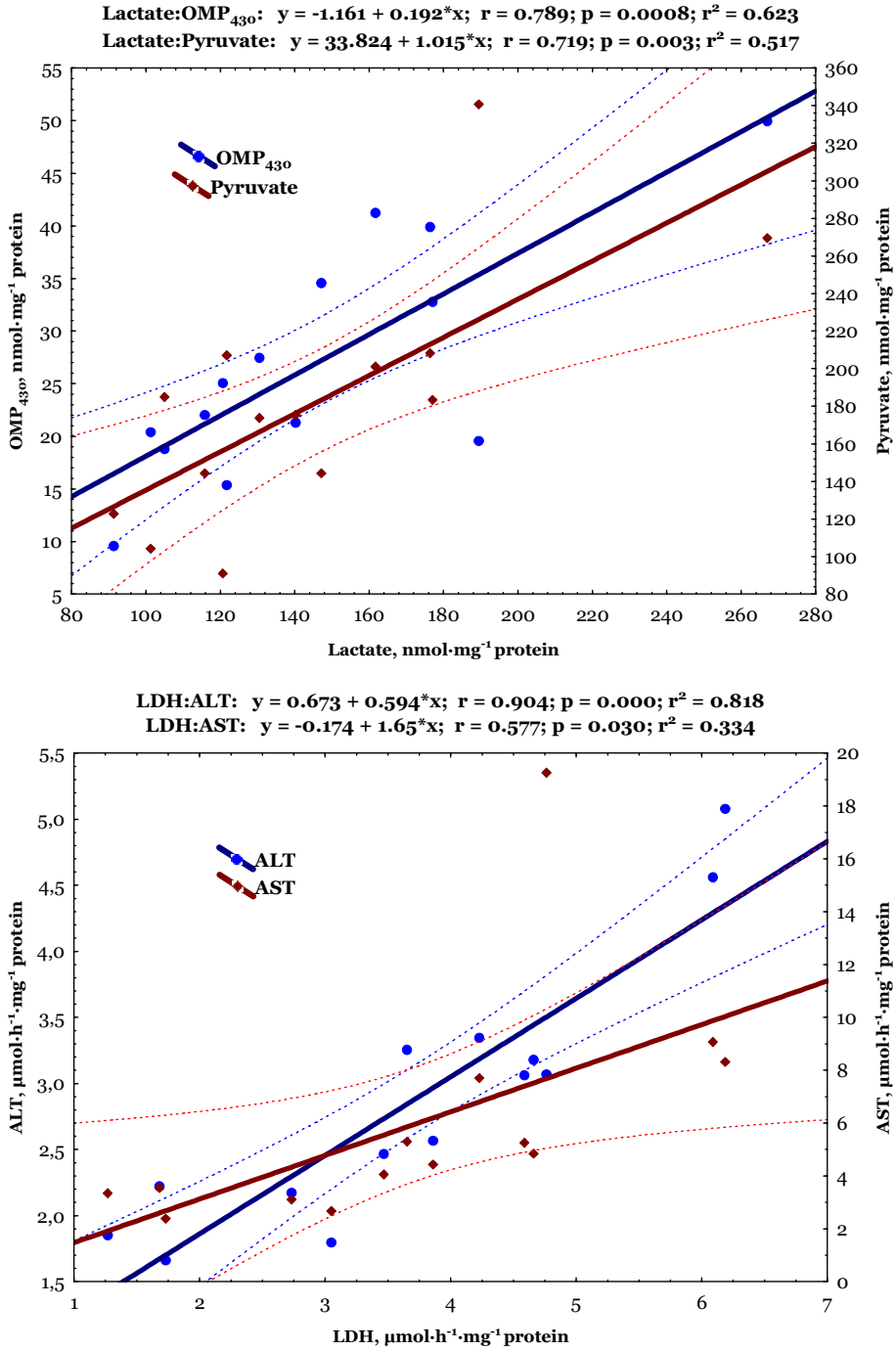


Fig. 4. Correlations between oxidative stress biomarkers and biochemical indices in the muscle tissue of the rainbow trout following *Y. ruckeri* vaccination at first month after immunization

In vaccinated group, aldehydic derivatives of OMP correlated positively with ketonic derivatives ($r = 0.952$, $p = 0.000$) and lactate level ($r = 0.870$, $p = 0.000$). The lactate level correlated positively with ketonic derivatives of OMP ($r = 0.789$, $p = 0.001$) and positively with pyruvate level ($r = 0.719$, $p = 0.003$). LDH activity correlated positively with ALT ($r = 0.904$, $p = 0.000$) and AST activity ($r = 0.577$, $p = 0.030$) (Fig. 4).

DISCUSSION

The major findings in the present study demonstrated that oral immunization by anti-*Yersinia* vaccine induces protein dysfunction in the muscle tissue by increasing protein damage due to the increased ketonic derivatives of OMP production (Fig. 1B). These findings suggest that vaccination against *Y. ruckeri* might contribute to increase of oxidatively modification of proteins.

Exposure to vaccination against *Y. ruckeri* might produce oxidatively consequences. Meanwhile, response of oxidative stress biomarkers in different tissues of fish is dependent of immune system activation and reactive oxygen species (ROS) generation due to respiratory burst in response to microbe recognition induced by vaccination. Paiva and Bozza (2014) described the mechanisms by which ROS directly kill microbes or interfere with the immune response, the role of ROS in pathogenic viral, bacterial, and protozoan infections (Paiva and Bozza 2014). Phagocytes recognize microbes through many molecular patterns displayed by them and try to engulf them. Once a microbe is phagocytosed, the nature of the molecules recognized on microbe's surface dictates the treatment enacted within the phagosome. Respiratory burst, a process by which NADPH oxidase generates ROS in response to microbe recognition, is a possible outcome of this process and helps to get rid of many microbes (Paiva and Bozza 2014). Once a pathogen is phagocytosed, it must subvert the respiratory burst, withstand its oxidative power, or escape the phagosome to survive (Paiva and Bozza 2014). 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 neutrophil extracellular traps (NETs); is actively involved in phagolysosomal formation and enzymatic degradation; autophagy; chemoattraction and inflammation; cell death of infection reservoirs; antigenic presentation, T-helper polarization, and lymphocyte proliferation; iron redistribution among tissues and cell compartment availability of iron (Paiva and Bozza 2014).

Indeed, non-specific and specific immune responses of fish against *Y. ruckeri* strains were activated in fish organisms. For example, Harun and co-workers (2011) have suggested that different types of adaptive responses are on-going within the vaccinated fish during infection with *Y. ruckeri*, potentially affected by the site and stage of infection. The bacterial burden in the spleen, the spleen index, and the expression profiles of pro- and anti-inflammatory cytokines and marker genes for T helper (Th) cells in the spleen and gills were analyzed, in comparison to the profiles in na-

ïve/challenged fish. As expected, the bacterial burden in the spleen of naïve fish increased over time and was correlated with the spleen index after *Y. ruckeri* challenge. The gene expression data showed that pro-inflammatory cytokines were up-regulated post-infection in the spleen of both naïve and vaccinated fish after *Y. ruckeri* challenge although the pro-inflammatory cytokine expression was much lower in vaccinated fish compared to the naïve fish. A correlated expression between pro-inflammatory cytokines and anti-inflammatory cytokines was only seen in spleen of ERM vaccinated fish, where a Th1-like response was indicated by the correlated gene expression of IFN-γ, T-β and IL-2. In contrast, in the gills, the inflammatory gene response was enhanced in vaccinated fish compared to naïve fish, but perhaps more importantly there was a strong up-regulation of IL-22 which was negatively correlated with IFN-γ gene expression at this site (Harun et al. 2011).

Recent discoveries suggest production of specific antibodies against *Y. ruckeri* may play a role in protection against disease. The significant increase in plasma antibody titers following immersion vaccination and significantly reduced mortality during *Y. ruckeri* challenge was demonstrated in study of Raida and co-workers (2011). Rainbow trout were immersion-vaccinated, using either a commercial ERM vaccine (AquaVac™ ERM vet) or an experimental *Y. ruckeri* bacterin. Half of the trout vaccinated with AquaVac™ ERM vet received an oral booster (AquaVac™ ERM Oral vet). Sub-groups of the fish from each group were subsequently exposed to 1 x 10^9 CFU *Y. ruckeri*/ml either eight or twenty-six weeks post vaccination. All vaccinated groups showed 0% mortality when challenged, which was highly significant compared to the non-vaccinated controls (40 and 28% mortality eight and twenty-six weeks post vaccination, respectively). Plasma samples from all groups of vaccinated fish were taken 0, 4, 8, 12, 16 and 26 weeks post vaccination and *Y. ruckeri* specific IgM antibody levels were measured with ELISA. A significant increase in titers was recorded in vaccinated fish, which also showed a reduced bacteremia during challenge. *In vitro* plasma studies showed a significantly increased bactericidal effect of fresh plasma from vaccinated fish indicating that plasma proteins may play a role in protection of vaccinated rainbow trout (Raida et al. 2011).

The immunogenic antigens are taken up from the lumen of the gut in rainbow trout and presented to lymphocytes. The protective mechanism behind the immunity induced by oral and anal immunization is at present unknown, and the vaccine is likely taken up by the M-like cells in the lower intestine. It is therefore suggested that oral and anal vaccination induce a local immunity in the intestine due to activation of gut-associated lymphoid tissue (GALT) associated with the gastrointestinal tract in vaccinated trout (Villumsen et al. 2014). Fuglem and co-workers (2010) have showed that different intestinal segments from salmonid fish exposed to gold-BSA to identify antigen-sampling cells in the teleost intestine shared some morphological similarities with immature mammalian M cells and is believed to be an equivalent to or an early evolutionary precursor of GALT in fish. Lateral lines, dorsal fin, epidermis and gastro-intestinal tract mucosal tissue were the primary areas where bacterial uptake was demonstrated readily after exposure of *Y. ruckeri* in study of Khimmakthong and co-workers (2013). The central immune organs in rainbow trout are involved in the final antigen processing (Khimmakthong et al. 2013). An intestinal secretory immune system stimulated by oral administration of antigens is operative

in trout. The external mucosal layer of fish is the first physical barrier that comes into contact with the surrounding water containing various pathogenic microorganisms. This layer protects the fish against invasion of bacteria and is constantly sloughed off, which prevents bacteria from attaching and thereby invading the underlying epithelium (Georgopoulou and Vernier 1986). The mucous layer plays a crucial role in disease resistance which can be enhanced by vaccination (Davidson et al. 1993). These researchers have revealed that oral vaccination of rainbow trout with bacterin induced significant amounts of antibody-secreting B-lymphocytes in the intestinal epithelium eight weeks post vaccination, demonstrating an active role of the intestine in antibody secretion in rainbow trout (Davidson et al. 1993). The intestine is able to mount an immune response in a manner similar to that of other immunologically important tissue, i.e. spleen and head kidney (Villumsen et al. 2014).

In the study of Villumsen and co-workers (2014), the anally vaccinated group showed 100% survival relative to 44% survival in the un-vaccinated group. Full protection was obtained in both orally and anally vaccinated rainbow trout, but no increases in *Y. ruckeri* specific antibodies were detected in the plasma samples collected from these groups, compared to un-vaccinated control groups. Surprisingly, plasma proteins may play a role in protection of vaccinated rainbow trout (Raida et al. 2011). Chettri and co-workers (2012) have investigated the immune response of rainbow trout larvae and fry at an early stage of development against the bacterial pathogen *Y. ruckeri*. Gene expression studies showed an up-regulation of iNOS and IL-22 in infected larvae 24h post exposure but most of the investigated genes did not show any difference between infected and uninfected larvae. Immuno-histochemical studies demonstrated a high expression of IgT molecules in gills and CD8 positive cells in thymus of both infected and uninfected larvae. Infection of rainbow trout fry with *Y. ruckeri*, in contrast, induced a cumulative mortality of 74%. A high expression of cytokines (IL-1β, TNF-α, IL-22, IL-8 and IL-10), acute phase proteins (SAA, hepcidin, transferrin and precerebellin), complement factors (C3, C5 and factor B), antimicrobial peptide (cathelicidin-2) and iNOS was found in infected fry when compared to the uninfected control. IgT molecules and mannose binding lectins in gills of both infected and uninfected fry were detected by immunohistochemistry. The study indicated that early life stages (yolk-sac larvae), merely up-regulate a few genes and suggests a limited capacity of larvae to mount an immune response by gene regulation at the transcriptional level. It could be speculated that larvae may be covered by protective shield of different immune factors providing protection against broad range of pathogens. However, the increased susceptibility of older fry suggests that *Y. ruckeri* may utilize some of the immune elements to enter the naive fish. The up-regulation of iNOS and IL-22 in the infected larvae implicates an important role of these molecules in immune response at early developmental stages. A dense covering of surfaces of gill filaments by IgT antibody in the young fish suggest a role of this antibody as innate immune factor at early developmental stages (Chettri et al. 2012).

The findings of Evenhuis and Cleveland (2012) demonstrated that measurable alterations in immune gene expression occur in the intestine of rainbow trout following bath challenge with *Y. ruckeri*. The innate immune molecules, SAA, IL-8, INF-γ and TNF-α, as well as IgM, were up-regulated in intestinal tissue (Evenhuis and

Cleveland 2012). Wiens and Vallejo (2010) have demonstrated that rainbow trout had a strong innate response following challenge with BT2 *Y. ruckeri* strain YRNC10 indicating that flagellin expression is not required for production of a robust pro-inflammatory and acute-phase gene transcription response. TNFα1 and IL 1-β1 transcripts were increased by day 1 post-challenge, and on days 3, 5 and 7 maximal gene transcript up-regulation occurred at a threshold of approximately 64-256 CFU per mg spleen tissue following a primary challenge with biotype 2 *Y. ruckeri* strain YRNC10. Infection induced robust SAA gene up-regulation that was significantly correlated with increased gene expression of IL-1β1 and TNFα1. *Y. ruckeri* infection induced modest changes in INF-γ and Mx-1 gene transcript abundance at intermediate or high challenge doses and the expression patterns of both genes were positively correlated with pro-inflammatory gene and acute-phase gene transcription patterns. TNF superfamily 13b (BAFF) gene expression was significantly down-regulated in response to infection on days 3, 5 and 7 at the highest challenge doses. The spleen somatic index was significantly increased on days 3, 5 and 7 post-infection and positively correlated with spleen colony forming units and abundance of gene transcripts SAA, TNFα1, and IL1-β1 (Wiens and Vallejo 2010).

Fishes have to mobilize energy substrates to metabolically cope with oxidative stress (Iwama 1998). Muthappa and co-workers (2014) have noted that the production of glucose during stress assists the animal by providing energy substrates to tissues such as brain, gills, and muscles in order to cope with the increased energy demand. The extent, magnitude, and direction of biochemical/metabolic changes to compensate for increased energetic demand (glucose production) in response to low-dose stress decide how an animal will perform or perish in the hostile environment (Muthappa et al. 2014). The unique biochemical plasticity of fish, in terms of changes in activity of enzymes involved in anaerobic metabolism of carbohydrates (LDH), transaminases in amino acid metabolism providing gluconeogenic substrates (ALT and AST), lactate and pyruvate levels. As our results showed, after vaccination, protein catabolism increases to supply more glucose (energy) *via* up-regulation of AST and ALT, which play role in gluconeogenesis via a transamination reaction. So, we used these as markers along with several others. In our study, ALT and AST activities in the muscle tissue of trout following *Y. ruckeri* vaccination at first month after immunization were non-significantly altered compared to unhandled group. AST activity was non-significantly increased by 66% ($p > 0.05$).

Interestingly, the vaccination against yersiniosis in our study caused the decrease of oxidative modification of proteins in the muscle tissue of vaccinated fish as compared with the control group. In our study, the activities of aminotransferases were significantly determined by alterations of oxidative stress biomarkers. ALT ($r = 0.844$, $p = 0.000$) and AST activity ($r = 0.923$, $p = 0.000$), as well as LDH activity ($r = 0.899$, $p = 0.000$) and lactate level ($r = 0.681$, $p = 0.005$) correlated with TBARS level, while aldehydic derivatives of OMP correlated positively with ALT ($r = 0.924$, $p = 0.000$) and AST activity ($r = 0.978$, $p = 0.000$), as well as lactate ($r = 0.781$, $p = 0.001$) and pyruvate level ($r = 0.763$, $p = 0.001$), and ketonic derivatives of OMP correlated positively with ALT ($r = 0.932$, $p = 0.000$) and AST activity ($r = 0.986$, $p = 0.000$), as well as lactate ($r = 0.781$, $p = 0.001$) and pyruvate level ($r = 0.760$, $p = 0.001$) (Fig. 3). Correlative analysis confirmed that decrease of markers of protein damage

and lipid peroxidation may cause to inhibition of aminotransferases activity and pyruvate level (Fig. 3). Interestingly, the activities of aminotransferases in our study were significantly determined by alterations of oxidative stress biomarkers. In unhandled group, ALT ($r = -0.626$, $p = 0.022$) and AST activity ($r = -0.638$, $p = 0.018$), as well as lactate level ($r = 0.661$, $p = 0.013$) and pyruvate level ($r = 0.661$, $p = 0.013$) correlated with aldehydic derivatives of OMP (Fig. 3). In vaccinated group (Fig. 4), lactate level correlated positively with aldehydic ($r = 0.870$, $p = 0.000$) and ketonic derivatives of OMP ($r = 0.789$, $p = 0.001$). Correlative analysis confirmed that increase of protein damage markers may cause to inhibition of aminotransferases activity and to increase of lactate and pyruvate level (Figs 3 and 4). These correlations have revealed that aminotransferases as well as gluconeogenic substrates (lactate and pyruvate) in muscle tissue were differentially regulated by oxidatively modified proteins in vaccinated group.

Chronic exposure of fish to stressors affects the energy requirements by changing anaerobic oxidation (Muthappa et al. 2014). In our study, vaccination against *Y. ruckeri* caused to elevation of pyruvate level in the muscle tissue (by 47%, $p = 0.013$), indicating a shift towards aerobic metabolism. Skeletal muscle has high energy demands, and to fulfill this demand, the activity of the gluconeogenic substrate-providing enzymes, as well as substrates for aerobic metabolism must have been increased. In this study, vaccination against *Y. ruckeri* had no significant effect on ALT and AST activity in the muscle tissue (controls compared). Thus influence on transaminases providing gluconeogenic substrates is the sum total of overall metabolic energetic demand and environment, and fish attempts to supply that by modulating enzymatic activities and substrates content. Correlative analysis confirmed our assumptions. Thus, in unhandled control, ALT activity correlated positively with AST activity ($r = 0.856$, $p = 0.000$), AST activity correlated positively with LDH activity ($r = 0.752$, $p = 0.003$), pyruvate level correlated inversely with LDH activity ($r = -0.747$, $p = 0.003$) (Fig. 3). In vaccinated group, pyruvate level correlated positively with lactate level ($r = 0.719$, $p = 0.003$), as well as LDH activity correlated positively with ALT ($r = 0.904$, $p = 0.000$) and AST activity ($r = 0.577$, $p = 0.030$) (Fig. 4).

In our previous study, 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 resulting from adaptation to the immunization. A high level of 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. The increased aspartate aminotransferases activity in the liver of individuals from control and immunized groups in the second month was noted. Activation of aminotrans-

ferases indicates on the metabolic transformations of proteins and carbohydrates. The significant decrease in the lactate concentration and LDH activity in hepatic tissue reflects the dynamic alterations in aerobic-anaerobic and aerobic metabolism as well as in the total energy supply. A significant decrease in the lactate level in hepatic tissue in the second month of the study both in the control and immunized groups indicates about the proper functioning of the mechanisms of metabolic activity in the long-term effects of the vaccination. The decrease of pyruvate and lactate levels in hepatic tissue both in control and immunized groups in the second month after vaccination confirms the high adaptive capacity of liver in compensation of metabolic alterations occurring as a result of immunization. Correlative dependence between levels of oxidative stress markers and metabolites in the liver of rainbow trout immunized with the vaccine against *Y. ruckeri* at first and second months after vaccination confirms the important role of metabolites and enzymes of energy transformation in the liver as the response to oxidative stress caused by immunization against *Y. ruckeri*. Our results confirm that vaccine against *Y. ruckeri* has no adverse effect on the condition and metabolism in the liver of the fish. Metabolic alterations recorded in our study is a proof that the vaccine against *Y. ruckeri* have no a negative effects (Tkachenko et al. 2015).

To determine the effects of vaccination against *Yersinia ruckeri* on health condition of rainbow trout in general, and oxidative stress biomarkers and metaboliteindices specifically, as well as to identify mechanisms that underpin the susceptibility of fish to vaccination, we also have compared the liver and heart function, and the oxidative mechanism underlying those effects, by detecting relevant lipid peroxidation and protein oxidation biomarkers, as well as aerobic-anaerobic metabolism in trout immunized against *Y. ruckeri* at 30 days post-vaccination and healthy individuals. In our previous study, hepatic aminotransferases activities were positively associated with the oxidative stress biomarkers in the trout vaccinated against *Y. ruckeri*. Moreover, similar associations were observed in the cardiac tissue of the immunized trout. Decreased aldehydic and ketonic derivatives of oxidatively modified proteins and the reduction of aminotransferases and lactate dehydrogenase activities were sensitive to vaccination of trout against *Y. ruckeri* and may potentially be used as biomarkers in evaluating vaccine effects in the liver of rainbow trout. Understanding the role of biochemical changes in the tissues of vaccinated trout has important implications for understanding of the complex physiological changes that occur in immunization but also for improving aquaculture practices to maximize tissues growth and health of vaccinated trout (Tkachenko et al. 2016a).

CONCLUSIONS

In summary, our results suggest that oral immunization by anti-*Yersinia* vaccine induces protein dysfunction in the muscle tissue by increasing protein damage due to the increased ketonic derivatives of OMP production. These findings suggest that vaccination against *Y. ruckeri* might contribute to increase of oxidatively modification of proteins. Taken together, these results suggest that vaccination could promote to 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. The oxidative stress biomarkers, i.e. content of oxidative protein damage, as well as biochemical enzymes and substrates were sensitive to vaccination of trout against *Y. ruckeri* and may potentially be used as biomarkers in evaluating vaccine toxicity in rainbow trout. From a practical point of view, the results may be useful in relation to studies of infections and the development, administration and uptake of new vaccines applicable for large amounts of fish.

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ZMIANY BIOCHEMICZNE W MIĘŚNIACH PSTRĄGA TĘCZOWEGO (*ONCORHYNCHUS MYKISS*) IMMUNIZOWANEGO PRZECIWKO *YERSINIA RUCKERI*

Streszczenie

Ciągłe ulepszanie i wprowadzanie nowych, cechujących się wysoką skutecznością, metod ochrony zdrowia ryb to podstawowe czynniki, decydujące o ekonomicznych efektach oraz postępie w chowie i hodowli ryb. Duże znaczenie mają metody ochrony zdrowia tarlaków, których kondycyja oraz stan zdrowia mają znaczący wpływ na uzyskanie od nich zdrowego potomstwa. Celem badań była analiza mechanizmów kształtowania się reakcji odpornościo-

wych ryb przez ocenę stężenia markerów stresu oksydacyjnego (produkty reagujące z kwasem 2-tiobarbiturowym, aldehydowe i ketonowe pochodne oksydacyjnej modyfikacji białek) i przemian metabolicznych (aktywność aminotransferaz alaninowej i asparaginianowej, dehydrogenazy mleczanowej, stężenie mleczanu i pirogronianu) w tkance mięśniowej młodocianych osobników pstrąga tęczowego, które to mechanizmy decydują o efektywności stosowania szczepionki przeciwko *Yersinia ruckeri* w pierwszym miesiącu po immunizacji doustnej. Ryby zostały podzielone na dwie grupy doświadczalne. Pierwszą z nich karmiono paszą bez dodatków i konserwantów (firma „Bestfeed"), drugą paszą z inaktywowanymi antygenami *Y. ruckeri* podawaną 3 razy co drugi dzień, a w pozostałe dni podawano paszę kontrolną. Po pierwszym miesiącu od zakończenia immunizacji szczepionką przeciwko *Y. ruckeri* z grup kontrolnej i doświadczalnej pobrano ryby do badań. Szczepionka zawierała trzy szczepy *Y. ruckeri* pochodzące z pstrągów tęczowych hodowanych na różnych farmach, gdzie ryby wykazywały kliniczne objawy jersiniozy. Wyizolowane bakterie należały do serotypu O1. Po określeniu opcjonalnych warunków szczepień (dawki antygenów, czasu ekspozycji ryb), młodociane osobniki pstrąga tęczowego karmiono paszą z inaktywowanymi antygenami *Y. ruckeri*. Zaobserwowano statystycznie istotne zwiększenie poziomu ketonowych pochodnych oksydacyjnie zmodyfikowanych białek między średnimi w grupach immunizowanej i kontrolnej po upływie pierwszego miesiąca po szczepieniu. Aktywacja proteolitycznej degradacji zmodyfikowanych reszt aminokwasowych może być jedną z przyczyn aktywacji metabolizmu pirogronianu w wyniku adaptacji do immunizacji. Korelacyjne zależności między stężeniem markerów stresu oksydacyjnego oraz metabolitami w tkance mięśniowej pstrąga tęczowego immunizowanego szczepionką przeciwko *Y. ruckeri* w pierwszym miesiącu po szczepieniu potwierdzają ważną rolę metabolitów i enzymów przemian energetycznych w przebiegu stresu oksydacyjnego spowodowanego immunizacją pstrąga tęczowego przeciwko *Y. ruckeri*. Wyniki badań wykazały, że szczepionka skierowana przeciwko jersiniozie nie wywiera negatywnego wpływu na kondycję i zdrowie ryb. W układach doświadczalnych nie zarejestrowano jakichkolwiek zmian o charakterze klinicznym u osobników poddanych wakcynacji, co jest dowodem na to, że szczepionka nie jest toksyczna.