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Antioxidant defense in the hepatic tissue of rainbow trout (*Oncorhynchus mykiss*) following first month after vaccination against *Yersinia ruckeri*

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Vaccination of rainbow trout against the enteric redmouth disease confers a high degree of protection to the fish. On the other hand, exploring the effects of vaccination against *Yersinia ruckeri* on health condition of trout in general, and oxidative stress and antioxidant defense biomarkers in different tissues specifically, would be valuable. Therefore, the aim of our study was to assess the effects of the oral vaccination against *Y. ruckeri* on the enzyme antioxidant defenses (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, total antioxidant capacity) in the hepatic tissue of rainbow trout (*Oncorhynchus mykiss*) at first month after immunization. Concentrated vaccine with *Y. ruckeri* strains inactivated by formalin 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 hepatic tissue was sampled. In our study, antioxidant defenses not differed from that of the control group. Results of correlative analysis indicated the main role of catalase that serve as a defense against oxidative stress. The results suggest that antioxidant responses may have potential as biomarkers for evaluating physiological effects of vaccination on rainbow trout.

Key words: immunization, rainbow trout (*Oncorhynchus mykiss*), hepatic tissue, superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, total antioxidant capacity.

Introduction

Yersinia ruckeri, a Gram-negative bacterium, is the etiological agent of enteric redmouth disease (ERM), a hemorrhagic septicemia in fish, leading to significant economic losses in salmonid aquaculture worldwide [Cascales et al., 2016]. Infection may result in a septicaemic condition with haemorrhages on the body surface and in the internal organs [Tobback et al., 2007]. The clinical signs of ERM include the subcutaneous hemorrhages, 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].

Conditions predisposing fish to clinical infection relate primarily to stress [Barnes, 2011]. Healthy laboratory populations can withstand exposure to high numbers of cells without succumbing to clinical disease [Ross et al., 1966]. Infection may occur where fish are obese through poor feeding regimes, but poor water quality is the prime cause. Common causes are high ammonia and low oxygen due to poor water flow or excessive stocking densities, high temperatures (which also reduce the oxygen-carrying capacity of the system), or the presence of a high level of suspended organic and siliceous matter [Bullock and Snieszko, 1975]. When these conditions are marginal, handling stress may trigger infections where the fish would have remained healthy if left untouched. The expectation of trouble, therefore, is in summer conditions where temperatures rise and water flows are reduced. The peak is considered to be 15–18 °C and monitoring oxygen and temperatures daily is an effective warning system where *Y. ruckeri* is endemic [Barnes, 2011].

Yersiniosis is successfully controlled with commercial vaccines and in fact represents one of the first diseases to be controlled by vaccination [Thomson, Adams, 2004]. An enteric redmouth bacterin was the first commercially-produced fish vaccine, and the formalin-killed whole-cell product continues to be highly effective whether administered by immersion, spray, injection, or oral routes [Stevenson, 1997]. Two of the most predominant groups of *Y. ruckeri* belong to serovar type 1 (Hagerman) which is more commonly isolated from rainbow trout, and serovar type II (O'Leary) first isolated from chinook salmon (*Oncorhynchus kisutch*) [Thomson, Adams, 2004]. Serovar 1 Hagerman strains are the basis for most commercial bacterins. Lipopolysaccharide (LPS) of serovar 1 *Y. ruckeri* elicits negligible or weak antibody responses in fish and low cell-proliferation memory responses compared with serovar 2 strains [Stevenson, 1997].

Salmonid fish are usually immunized with multivalent vaccines by intraperitoneal injection. In marine fish species vaccination is generally performed by immersion, but use of injection vaccination is increasing, particularly in the Mediterranean region. Only limited use of orally administered fish vaccines is reported [Håstein et al., 2005]. The major disadvantage with this route of administration is that lower levels of protection are achieved and the duration of protection elicited is shorter [Thomson, Adams, 2004]. 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 the injection and immersion administration and is suitable for vaccinating large numbers of fish of all sizes. It is also avoids the handling stressors experienced by the fish with the other methods of vaccination [Thomson, Adams, 2004].

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]. It should be interesting to study one-month alterations in antioxidant defenses in rainbow trout after oral vaccination against *Y. ruckeri*. Therefore, the present work aimed to assess the effects of oral vaccination against *Y. ruckeri* at first month after immunization in the rainbow trout (*Oncorhynchus mykiss*) in terms of enzyme antioxidant defenses (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, total antioxidant capacity) in specific organ such as liver.

Materials and methods

Experimental design. Clinically healthy rainbow trout 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.

The fish were divided into two groups: untreated control and immunized against ERM. The vaccine against ERM (Department of Fish Diseases, National Veterinary Research Insti-

tute, 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 31 days after the immunization, and then hepatic tissue samples were collected.

The samples were homogenized in ice-cold buffer (100 mM Tris-HCl, pH 7.2) using a glass homogenizer immersed in ice water bath. Homogenates were centrifuged at 3,000 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.

Superoxide dismutase activity assay. 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). Activity was expressed in units of SOD per mg of tissue protein.

Catalase activity assay. 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). 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.

Glutathione reductase activity assay. Glutathione reductase (GR, EC1.6.4.2) activity in the sample was measured according to the method described by Glatzle and co-workers (1974) with some modifications. The GR activity was expressed as μmol of $NADPH_2$ per min per mg of protein.

Glutathione peroxidase activity assay. Glutathione peroxidase (GPx, EC1.11.1.9) activity was determined by detecting the nonenzymatic utilisation of GSH (the reacting substrate) at an absorbance of 412 nm after incubation with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) according to the Moin (1986). Glutathione peroxidase activity was expressed as μmol GSH per min per mg of protein.

Total antioxidant capacity (TAC) assay. The TAC level in the sample was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm [Galaktionova et al., 1998]. The level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

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 antioxidant defense biomarkers (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 (StatSoft, Krakow, Poland).

Results

Antioxidant defense in the liver of the trout treated by vaccine against *Y. ruckeri* at first month after immunization are shown in Fig. 1. There were no statistically significant alterations in the activities of antioxidant enzymes in the hepatic tissue of the trout vaccinated against *Y. ruckeri* at first month after immunization (Fig. 1). The SOD and CAT activity was non-significantly decreased by 9% and 20% ($p>0.05$) after immunization, while GR and GPx was increased by 7.4% and 28% ($p>0.05$) compared to the controls (Fig. 1).

Non-significant decrease of TAC level in the liver of the trout treated by vaccine against *Y. ruckeri* at first month after immunization was found (Fig. 2).

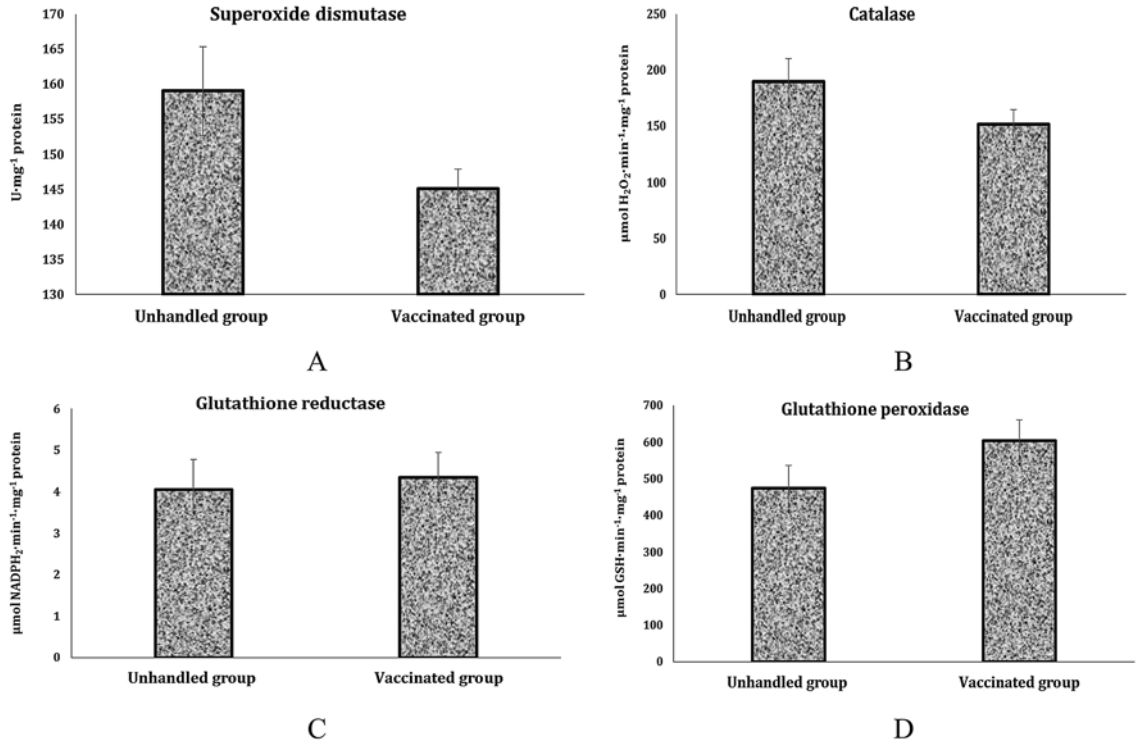

Fig. 1. Superoxide dismutase (A), catalase (B), glutathione reductase (C), and glutathione peroxidase (D) activities in the liver of the trout treated by vaccine against *Y. ruckeri* at first month after immunization. Data are represented as mean ± S.E.M. (n=15).

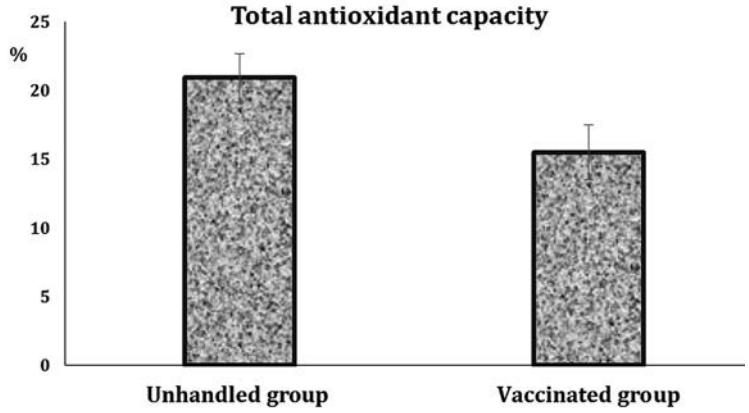

Fig. 2. The total antioxidant capacity in the liver of the trout treated by vaccine against *Y. ruckeri* at first month after immunization. Data are represented as mean ± S.E.M. (n=15).

Several correlations between checked parameters were found (Fig. 3). In vaccinated group, the CAT activity correlated positively with TBARS as biomarker of lipid peroxidation (r=0.666, ρ=0.009) (Fig. 3A) and with aldehydic derivatives of OMP (r=0.992, ρ=0.000) (Fig. 3B). On the other hand, aldehydic derivatives correlated positively both with TBARS (r=0.637, ρ=0.014) and ketonic derivatives of protein damage (r=0.837, ρ=0.000) (Fig. 3).

Discussion

Fish exposed to vaccination exhibit a variety of physiological responses, including oxidative metabolism imbalances [Tkachenko et al., 2015, 2016a-e]. In our previous study [Tkachenko et

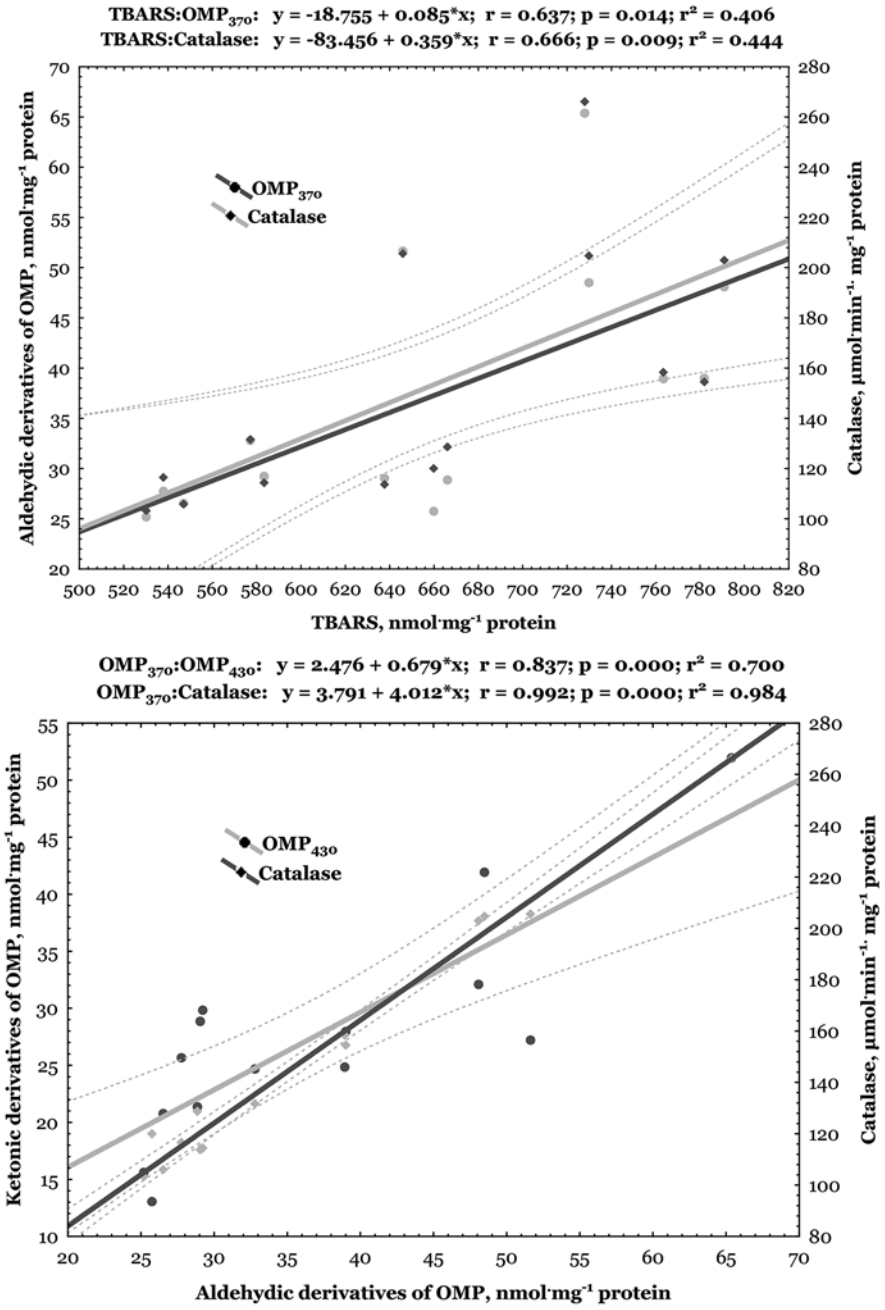

Fig. 3. Correlations between oxidative stress biomarkers and catalase activity in the liver of the trout treated by vaccine against *Y. ruckeri* at first month after immunization.

al., 2015], we have analyzed the level of oxidative stress biomarkers [2-thiobarbituric acid substances (TBARS), aldehydic and ketonic derivatives of oxidatively modified proteins (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 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].

In the present study SOD, CAT, GR, and GPx activity as well as TAC not differed from that of the control group (Figs 1 and 2). Results of correlative analysis indicated the main role of CAT that serve as a defense against oxidative stress along with elevated TBARS levels, and protein damage (Fig. 3). 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, 2016a-e] 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.

To determine the effects of vaccination against *Y. ruckeri* on health condition of rainbow trout in general, and oxidative stress biomarkers and metabolitic parameters specifically, as well as to identify mechanisms that underpin the susceptibility of fish to vaccination, we 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 and 60 days post-vaccination and healthy individuals [Tkachenko et al., 2016a, d]. Decreased aldehydic and ketonic derivatives of OMP 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 [Tkachenko et al., 2016d]. The level of lipid peroxidation in the liver and heart on the 61st day after immunization of rainbow trout does not significantly differ from that in the control [Tkachenko et al., 2016a]. Vaccination caused a slight decrease of the aldehydic and ketonic derivatives level in the heart and liver against the backdrop of a significant decrease of total antioxidant activity in the cardiac tissue of the trout treated by the vaccine against *Y. ruckeri* on the 61st day after immunization. This is possibly a result of a long-term adaptation to immunization [Tkachenko et al., 2016a].

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., 2016b, c]. 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., 2016c]. 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., 2016b].

No significant difference was noted in lipid peroxidation level in the muscle tissue of rainbow trout in either the first or second months 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 ($P<0.05$) [Tkachenko et al., 2016e]. The content of ketonic derivatives of OMB in muscles in the first month after immunization was higher compared to untreated con-

trol. 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].

Glutathione-dependent antioxidant defenses can effectively regulate reactive oxygen species formation during the exposure period. This compensatory response accompanied by the induction of other antioxidants (SOD, CAT) may help to prevent accumulation of free radicals and their products in stressed organisms [Stara et al., 2013]. In our study, one-month alterations of antioxidant defenses in immunized fish resulted to the increase of GR and GPx activity (Fig. 1). Enzymatic antioxidants are essential to maintain the redox status of hepatic tissue and serve as an important defense against oxidative stress [Stara et al., 2013].

Conclusions

Oral immunization of rainbow trout by vaccine against *Y. ruckeri* was shown to alter non-significantly the antioxidant status of hepatic tissue. Non-significant changes in enzyme activity (SOD, CAT, GR, GPx) in the hepatic tissue of trout were seen with immunization at first month, with no observed oxidative damage to the tissue. Correlative analysis proved main role of catalase that serve as a defense against oxidative stress. This study provided important results for the evaluation of one-month effects of immunization against *Y. ruckeri*. Antioxidant responses could provide useful parameters for evaluating physiological effects of vaccination on rainbow trout, but the application of these findings will need more detailed laboratory study before they can be established as biomarkers for monitoring of effects of vaccination.

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Антиоксидантная защита в печени радужной форели (*Oncorhynchus mykiss*) после первого месяца вакцинации против *Yersinia ruckeri*

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Вакцинация радужной форели против иерсиниоза проявляет высокую степень защиты. С другой стороны, было бы ценным изучение эффектов вакцинации против *Yersinia ruckeri* на состояние здоровья форели в целом и содержание маркеров окислительного стресса и антиоксидантной защиты в различных тканях рыбы в частности. Изучение влияния пероральной вакцинации против *Yersinia ruckeri* на активность ферментативной антиоксидантной защиты (супероксиддисмутаза, каталаза, глутатионредуктаза, глутатионпероксидаза, общая антиоксидантная активность) в печеночной ткани радужной форели (*Oncorhynchus mykiss*) в первый месяц после иммунизации было целью нашего исследования. Инактивированные штаммы *Y. ruckeri* инкорпорировали в корм для рыб, используя его для иммунизации три раза с суточным интервалом между аппликациями. Радужную форель из контрольной и вакцинированной групп рыб через 30 дней после иммунизации использовали в исследованиях. Антиоксидантная защита вакцинированной группы рыб в настоящем исследовании не отличалась от контрольной группы. Результаты корреляционного анализа показали важную роль каталазы, которая служит защитой в условиях окислительного стресса, индуцированного процессом иммунизации. Полученные результаты свидетельствуют о том, что антиоксидантные маркеры могут быть индикаторами для оценки физиологического воздействия вакцинации на радужную форель.

Ключевые слова: иммунизация, радужная форель (*Oncorhynchus mykiss*), печеночная ткань, супероксиддисмутаза, каталаза, глутатионредуктаза, глутатионпероксидаза, общая антиоксидантная активность