та Chalmers (ATCC® 25922™), Escherichia coli (Migula) Castellani та Chalmers. (ATCC® 35218™), Pseudomonas aeruginosa (Schroeter) Migula (ATCC® 27853™) та грам-позитивних штамів, таких як Staphylococcus aureus subsp. aureus Rosenbach (ATCC 29213), Staphylococcus aureus subsp. aureus Rosenbach (ATCC® 25923™), метицилін-резистентного (MRSA), mecA-позитивного Staphylococcus aureus (NCTC® 12493), Enterococcus faecalis (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC® 51299™) (стійкий до ванкоміцину; чутливий до тейкопланіну) та Enterococcus faecalis (Andrewes and Horder) Schleifer и Kilpper-Balz (ATCC® 29212™) для оцінки можливого використання цієї олії для профілактики інфекцій, викликаних цими патогенами. Каєпутова ефірна олія була надана польськими виробниками ефірних олій (Bamer®, Влоцлавек, Польща). Антимікробну чутливість штамів визначали методом дискової дифузії. Наші дослідження показали, що каєпутова ефірна олія проявляє антибактеріальні властивості. Грам-позитивні бактерії були найбільш чутливими до дії каєпутової олії, що може вказувати на те, що біологічні активні речовини в цій олії (включаючи фенольні кислоти, дубильні речовини тощо) можуть бути потенційними речовинами щодо лікування та профілактики бактеріальних інфекцій. Серед грам-негативних бактерій, тільки штам Pseudomonas aeruginosa (Schroeter) Migula (ATCC® 27853™) був стійкий до каєпутової ефірної олії. У цьому випадку, ми не спостерігали статистично істотних змін у зоні затримки росту після застосування ефірної олії порівняно з контрольними зразками (96% етанол). Наше дослідження може свідчити про те, що використання каєпутової ефірної олії може бути корисним у терапії та профілактиці широкого спектру бактеріальних інфекцій у ветеринарії, аквакультурі, медицині та інших галузях.

Ключові слова: каєпутова ефірна олія, антибактерійні властивості, грам-негативні та грам-позитивні штами бактерій, диско-дифузійний метод Кірбі-Бауера.

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ORAL VACCINATION AGAINST *YERSINIA RUCKERI:* OXIDATIVE STRESS BIOMARKERS IN THE GILLS OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM)

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The aim of this study was to assess the effect of oral vaccination against Yersinia ruckeri based on oxidative stress biomarkers in the gills of rainbow trout (Oncorhynchus mykiss Walbaum). The vaccine consisted of three Y. ruckeri strains (O1 serotype) that originated from rainbow trout cultured on different farms, where fish ex-

hibited clinical signs of enteric redmouth disease. The concentrated vaccine was incorporated into the fish food; treatment was delivered three times at one-day intervals. Two months after immunization, gills were sampled. The vaccination against Y. ruckeri resulted in non-significant changes in TBARS level as lipid peroxidation marker, aldehydic and ketonic derivatives of oxidatively modified proteins level in the gills of trout at the second month after immunization against Y. ruckeri. A significant decrease in superoxide dismutase activity (by 36%, p = 0.002) compared to untreated controls occurred. The alterations in markers of oxidative stress suggest that antioxidant defenses may contribute to the balance between oxidative modification of proteins and antioxidant defenses in the gills of trout vaccinated against Y. ruckeri. We did not find any alterations in the gills after 60 days of immunization. This is likely a result of long-term adaptation to immunization. Understanding the role of oxidative stress in the tissues of vaccinated trout has important implications for the understanding of the complex physiological changes that occur in immunization and also for improving aquaculture practices to maximize tissue growth and health of vaccinated trout. The oxidative stress biomarkers in the gills were sensitive to vaccination 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 to large amounts of fish.

Keywords: **rainbow trout *Oncorhynchus mykiss, Yersinia ruckeri,* immunization, oxidative stress, antioxidant defense, gills.**

Yersinia ruckeri is a ubiquitous pathogen of finfish capable of causing major mortalities in farmed fish stocks [13]. This bacterium is the aetiological agent of enteric redmouth (ERM) disease of farmed salmonids [29]. 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 [36] and defined as a new species, *Y. ruckeri*, in 1978 [10]. *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 [2]. It can be transmitted vertically from parent to progeny as well as horizontally in the water column from both clinically infected fish and asymptomatic carriers and is consequently capable of infecting fish at the early stages of development [13].

The disease gets its name from subcutaneous hemorrhages, which can cause at the corners of the mouth and in the gums and tongue. Other clinical signs include exophthalmia, darkening of the skin, splenomegaly, and inflammation of the lower intestine with an accumulation of thick yellow fluid. The bacterium enters the fish via the secondary gill lamellae and from there it spreads to the blood and internal organs (Kumar et al., 2015). Infected fish and asymptomatic carriers are the main sources of the infection, spreading bacteria with feces. Gills are regarded as the entry route of *Y. ruckeri* rods but the likelihood of the disease depends on the virulence of the given strain. Characteristic clinical signs of yersiniosis, such as hemorrhages around the oral cavity, are caused by extracellular products (ECPs) of *Y. ruckeri* [31].

Vaccination, or immunoprophylaxis, is based on the principle that when a foreign organism, such as a bacterium or virus, invades its hosts, the animal's immune response reacts against it in an attempt to remove it. If the fish is re-exposed to the same organism, the immune response is primed to respond against it. This is referred to as a memory response or adaptive immunity. Vaccination mimics the invasion of pathogens

and primes the animals' immune systems for a re-encounter with the pathogen without causing disease [39]. Yersiniosis is successfully controlled with commercial vaccines and in fact, represents one of the first diseases to be controlled by vaccination. Most vaccines are bacterin preparations using whole-cell preparations of serovar 1 (the Hagerman strain and the major cause of disease outbreaks). Bacteria are generally inactivated with formalin and sometimes pH lysed at pH 9.8 to expose internal cell components [39]. The success of the vaccine has been reported to be variable under field conditions and often does not completely prevent disease outbreaks when the level of infection is high, as seen when fish are stressed [18]. Clearly, a greater understanding of the fish response against *Y. ruckeri* and during vaccination against yersiniosis would help improve this situation.

Vaccination plays an important role in protecting salmonids against the bacterial pathogen *Y. ruckeri*. ERM is traditionally associated with rainbow trout [29]. Vaccination of rainbow trout against ERM by immersion in *Y. ruckeri* bacterin confers a high degree of protection to the fish [35]. ERM has been controlled successfully using immersion-applied bacterin vaccines for several decades [44]. 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 of this route of administration is that lower levels of protection are achieved and the duration of protection elicited is shorter [39].

Reactive oxygen species (ROS) production contributes to the elimination of pathogens and induces the activation of immune defense mechanisms [30]. However, excessive ROS formation can induce oxidative stress, leading to cell damage and cell death may follow [32]. 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) [32]. Lipid peroxidation of polyunsaturated lipids is a preferred method for marking oxidative stress levels [34]. The product of lipid peroxidation, malondialdehyde, is easily detected in blood/plasma and has been used as a measure of oxidative stress [34]. In addition, the unsaturated aldehydes produced from these reactions have been implicated in the modification of cellular proteins and other constituents [26]. The oxidative modification of proteins by ROS is implicated in the etiology or progression of diseases [24]. 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 the loss of catalytic and structural integrity or by interruption of regulatory pathways [38]. 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 [24]. 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 [5].

Therefore, exploring the effects of vaccination against *Y. ruckeri* on the 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 vaccination against *Y. ruckeri* on gill 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 experi-

ments. The study was carried out in the 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 an additional oxygen supply with a water flow of 25 L per min, and a photoperiod of 12 hours per day. The fish were fed with a commercial pelleted diet at an optimal level, using 12-hour belt feeders for fish. The daily dose of feed is calculated in accordance with the applicable table feed [11]. All enzymatic assays were carried out at the Department of Zoology and Animal Physiology, Institute of Biology and Earth Sciences, Pomeranian University in Słupsk (Poland).

Experimental design. The fish were divided into two groups: I) control, and II) immunized by the 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 the Department of Fish Diseases, National Veterinary Research Institute in Puławy (Poland) according to the procedure covered by patent no. P.428259. The prepared vaccine at the concentration of $1 \cdot 10^9$ cells per mL was used to inoculate fish *per os*. Vaccine concentrate was added to fish feed and administered three times with one-day intervals between feedings. The fish were kept for 60 days after vaccination at a water temperature of 14.5 ± 0.5°C and a pH of 7.5. In our study, 15 rainbow trout from unhandled control and 15 vaccinated trout were used in the second month after immunization.

Sampling. The animals were captured and killed 61 days post-vaccination (n = 15 in each group). Gills 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 yield a homogenate in proportion 1:9 (weight/volume). The isolation buffer contained 100 mM tris-HCl; a pH of 7.2 was adjusted with HCl. Homogenates were centrifuged at 3,000 g for 15 min at 4°C. After centrifugation, the supernatant was collected and frozen at −25°C until analyzed. Protein contents were determined using the method of Bradford [3] 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 [19]. The absorbance of the supernatant was measured at 540 nm. TBARS values were reported as nmoles malonic dialdehyde (MDA) per mg protein.

Assay of carbonyl groups of the 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 the modification of Dubinina co-workers [8]. Samples were incubated at room temperature for 1 h with 10 mM 2,4-dinitrophenylhydrazine (DNTP) in 2M HCl. Blanks were run without DNTP. Afterward, proteins were precipitated with TCA and centrifuged for 20 min at 3,000 g. The protein pellet was washed three times with ethanol: ethyl acetate (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 [22]. The 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 [21]. One unit of CAT activity was defined as the amount of enzyme required for the 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 [14] 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 oxidizes $NADPH_2$, resulting in a decrease in the absorbance at 340 nm. 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 nonenzymatic utilization 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 [27]. 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 [12] 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, the Mann-Whitney *U* test was applied to the data [46]. 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 13.3 software (TIBCO Software Inc.).

Results. The level of lipid peroxidation in the gills of trout treated by vaccine did not significantly differ from that in the controls (Fig. 1). The content of ketonic derivatives of oxidatively modified proteins in the gills was non-significantly decreased in the group vaccinated against *Y. ruckeri* at second month compared to unhandled group (Fig. 1). The aldehydic derivatives of OMB in gill tissue of fish treated by the vaccine against *Y. ruckeri* at 61 days after immunization was non-significant higher compared to unhandled control (Fig. 1).

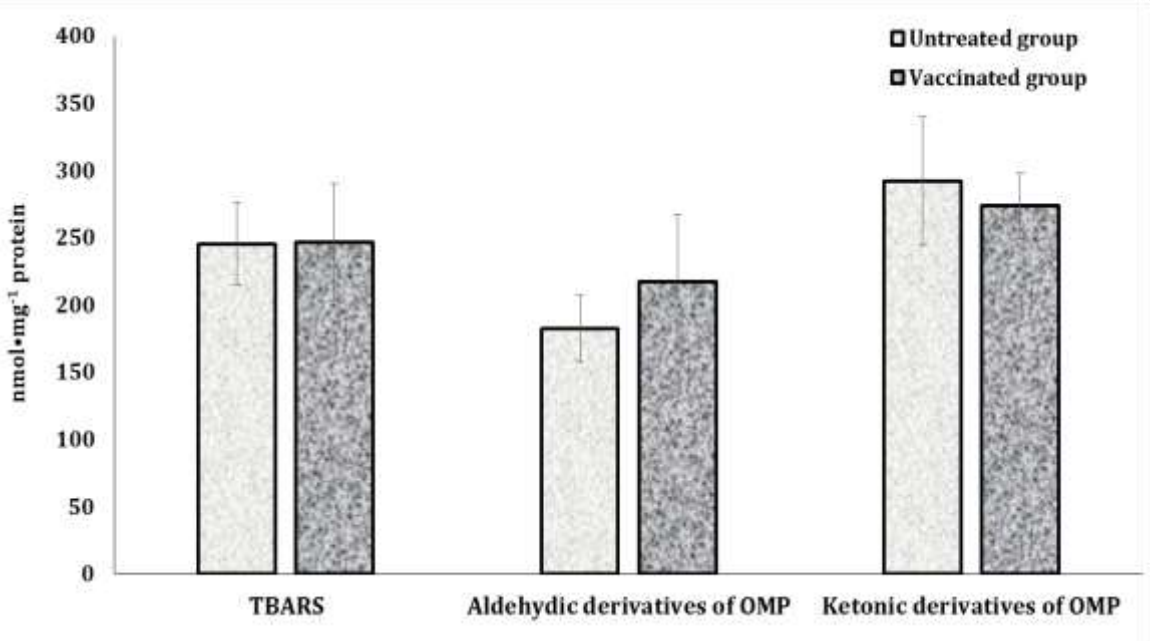

Fig. 1. The level of lipid peroxidation (nmol TBARS·mg^{-1} protein), aldehydic and ketonic derivatives of oxidatively modified proteins (nmol·mg^{-1} protein) in the gills of the trout treated by the vaccine against *Y. ruckeri* at second months after immunization. Data are represented as mean ± S.E.M. (n = 15).

Antioxidant defense in the gills of the trout treated by the vaccine against *Y. ruckeri* in the second months after immunization is shown in Table 1. No statistically significant alterations in the activities of antioxidant defenses instead SOD activity in the gill tissue of the trout treated with the vaccine against *Y. ruckeri* in the second months after immunization were not observed (Table 1). The SOD activity was decreased by 36% ($p = 0.002$) after immunization. A non-significant increase in TAC level in the gills of the trout treated with the vaccine against *Y. ruckeri* in the second month after immunization was found (Table 1).

Table 1

The activity of antioxidant enzymes and total antioxidant capacity (TAC) in the gills of the trout treated with the vaccine against *Y. ruckeri* in the second month after immunization

Activity of antioxidant enzymes	Untreated group	Group treated by the vaccine against *Y. ruckeri*
SOD, U·mg^{-1} protein	453.16 ± 47.97	290.85 ± 10.13*
Catalase, μmol H_2O_2·min^{-1}·mg^{-1} protein	128.67 ± 11.14	176.78 ± 19.80
GR, μmol $NADPH_2$·min^{-1}·mg^{-1} protein	67.99 ± 13.50	53.05 ± 7.11
GPx, μmol GSH·min^{-1}·mg^{-1} protein	153.90 ± 13.84	135.86 ± 4.72
TAC, %	4.68 ± 0.51	7.76 ± 1.03

Values expressed as mean ± S.E.M. (n = 15); * – see Fig. 1.

Several correlations between checked parameters were found (Fig. 2). In the vaccinated group, aldehydic derivatives of OMP correlated positively with catalase ac-

tivity (r = 0.828, p = 0.002). The ketonic derivatives of OMP also correlated positively with catalase activity (r = 0.684, p = 0.020) (Fig. 2).

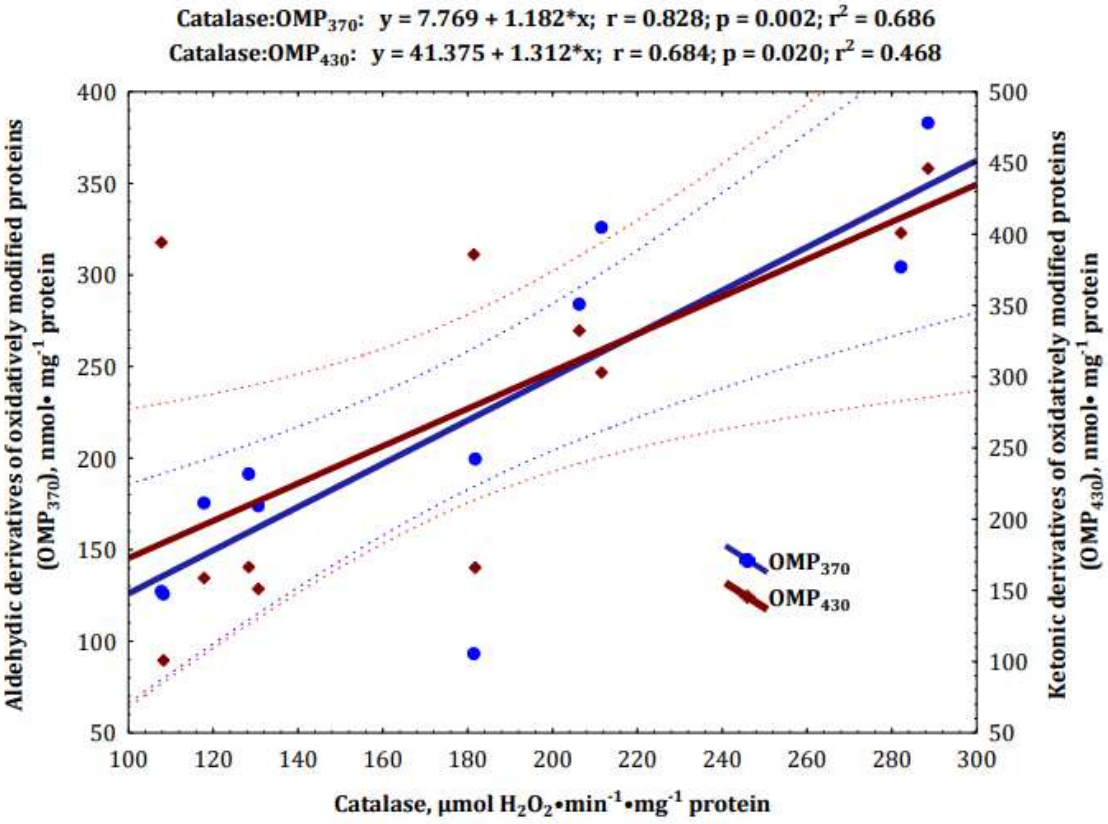

Fig. 2. Correlations between oxidative stress biomarkers in the liver of the trout treated by the vaccine against Y. ruckeri at second months after immunization.

In the present study, the most widely used and accepted markers were utilized to demonstrate the existence of oxidative stress in the gill tissue (TBARS as a marker of lipid peroxidation, aldehydic and ketonic derivatives of OMP, antioxidant defenses, and the total antioxidant capacity). In this study, our results clearly demonstrate that immunization with the anti-*Yersinia* vaccine does not alter the gills of rainbow trout. Oxidative stress parameters were examined in gills homogenate and lipid peroxidation as measured by the amount of TBARS, as well as aldehydic and ketonic derivatives of OMP, were non-significantly altered ($p > 0.05$) in the gills of vaccine-treated fish (Fig. 1). All these culminated in the decrease of SOD activity, while the total antioxidant capacity in the gills at second month after vaccination was increased (Table 1).

In summary, the findings described in the present study allow the conclusion that immunization by anti-*Yersinia* vaccine does not alter oxidative stress markers compared to unhandled control in the second month after immunization. We also have shown that antioxidant defense makes it possible to avoid the cellular lesions that cause the anti-*Yersinia* vaccine. Correlative analysis between protein oxidatively modification biomarkers and catalase activity confirm our conclusions (Fig. 2). From a broader perspective, it is suggested that immunization of fish by anti-*Yersinia* vaccine is associated with the induced free radical formation and oxidative stress. Free radicals would, therefore, be at least partially responsible for immunity with humoral and cellular elements and increased protective immunity against *Y. ruckeri* infection.

In our previous study, we assessed the influence of vaccination against enteric redmouth disease on oxidative stress biomarkers and antioxidant defense in the muscle tissue of rainbow trout vaccinated against *Y. ruckeri* in the first and second months 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 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 the untreated control. All these culminated in a depletion of GPx activity and a low level of 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 the *Yersinia* vaccine is associated with the 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 immunity and increased protective immunity against *Y. ruckeri* infection [40, 41].

Moreover, a statistically significant reduction in lipid peroxidation between the mean in groups immunized after the first and second months after vaccination indicated 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 the 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 in the 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 aminotransferases indicates the metabolic transformations of proteins and carbohydrates. The significant decrease in the lactate concentration and lactate dehydrogenase 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 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 the liver in compensation for 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* in the 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 the 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 proof that the vaccine against *Y. ruckeri* has no negative effects [43].

To determine the effects of vaccination against *Y. ruckeri* on health condition of rainbow trout in general, and oxidative stress biomarkers and metabolic 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 days post-vaccination and healthy individuals. In our 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 the 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 [42].

Non-specific and specific immune responses of fish against *Y. ruckeri* strains have been studied extensively [23]. A key hallmark of the vertebrate adaptive immune system is the generation of antigen-specific antibodies from B cells. Fish are the most primitive gnathostomes (jawed vertebrates) possessing an adaptive immune system. The immune mechanisms responsible for protection may comprise both cellular and humoral elements [35]. For example, both O-antigen and formalin-inactivated *Y. ruckeri* cells induced an immune response in rainbow trout, producing peak levels of antibody in the spleen at 14 days post-exposure and overall maximum titer at 28 days post-exposure. The neutrophil and macrophage responses that accompany inflammation in the peritoneal cavity of rainbow trout were studied by Afonso and co-workers [1]. Intraperitoneal injection of casein, Incomplete Freund's Adjuvant (IFA) and live or formalin-killed *Y. ruckeri* resulted in a rapid influx of neutrophils, peaking at 24 to 48 h post-injection and reaching values, in the case of live bacteria, 500x those in the resting, unstimulated peritoneal cavity. Peritoneal macrophages also increased, but the response was slower (peak at 5 d) and with more modest increases in number (7.5x). When *Y. ruckeri* was injected into resting peritoneal cavities, bacteria were ingested by the resident macrophages. When the bacteria were injected into cavities with high numbers of neutrophils (due to the previous injection of casein), more neutrophils than macrophages contained bacteria. Results show that the macrophages are the resident phagocytes of the peritoneal cavity of trout, while neutrophils are present in that body cavity in significant numbers only in situations of inflammation and only as long as the inflammation persists [1].

Recent discoveries suggest the 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 a study by Raida and co-workers [35]. 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 in 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 reduced bacteremia during the challenge. *In vitro* plas-

ma studies showed a significantly increased bactericidal effect of fresh plasma from vaccinated fish indicating that plasma proteins may play a role in the protection of vaccinated rainbow trout [35].

Harun and co-workers [16] have suggested that different types of adaptive responses are ongoing 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 naïve/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 the 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 [16].

Chettri and co-workers [4] 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 the thymus of both infected and uninfected larvae. Infection of rainbow trout fry with *Y. ruckeri*, in contrast, induced 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 the 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 a protective shield of different immune factors providing protection against a 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 suggests the role of this antibody as an innate immune factor at early developmental stages [4].

The findings of Evenhuis and Cleveland [9] demonstrated that measurable changes in immune gene expression occur in the intestine of rainbow trout following a 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 [9]. Wiens and Vallejo [45] 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 the production of a robust pro-inflammatory and acute-phase gene transcrip-

tion response. TNFα1 and IL1-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 [45].

The response of oxidative stress biomarkers in different tissues of fish is dependent on immune system activation and reactive oxygen species (ROS) generation due to respiratory burst in response to microbe recognition induced by vaccination. Paiva and Bozza [30] 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 [30]. 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 the 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 [30]. Once a pathogen is phagocytosed, it must subvert the respiratory burst, withstand its oxidative power, or escape the phagosome to survive [30]. 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 the 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 [30].

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 are directly fed by arteriovenous-like anastomoses. It is likely that gill interlamellar and mammalian lymphatics are physiologically equivalent [28]. 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 [37]. IgM+ cells (B-cells, plasma cells, and IgM-bearing macrophages) are very abundant in the stratified epithelium of the gill arch and filaments [15]. The study of dos Santos and co-workers (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 [17, 20]. Therefore, as the first-line encounter with 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 [17].

Immunization against *Y. ruckeri* reported here might play an important role in vaccination-induced oxidative stress in the gills of trout due to phagocyte respiratory burst. ROS are used by the immune system as a weapon 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 that 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).

Conclusions. In the current study, vaccination against *Y. ruckeri* results in non-significant changes of TBARS level as lipid peroxidation marker, aldehydic and ketonic derivatives of oxidatively modified proteins level in the gills of trout at the second month after immunization against *Y. ruckeri*, while a significant decrease in superoxide dismutase activity (by 36%, $p = 0.002$) compared to untreated control was occurred. The alterations in markers of oxidative stress and antioxidant defenses suggest that these enzymes may contribute to the balance between oxidative modification of proteins and antioxidant defenses in the gills of trout vaccinated against *Y. ruckeri*. We did not find any alterations in the gills after 60 days of immunization. This is likely a result of long-term adaptation to immunization.

Understanding the role of oxidative stress in the tissues of vaccinated trout has important implications for the understanding of the complex physiological changes that occur in immunization and also for improving aquaculture practices to maximize tissue growth and health of vaccinated trout. The oxidative stress biomarkers in the gills were sensitive for trout to vaccination 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 to large amounts of fish.

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ПЕРОРАЛЬНА ВАКЦИНАЦІЯ ЩОДО YERSINIA RUCKERI: БІОМАРКЕРИ ОКИСНЮВАЛЬНОГО СТРЕСУ В ЗЯБРАХ РАДУЖНОЇ ФОРЕЛІ (ONCORHYNCHUS MYKISS WALBAUM)

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Метою даного дослідження була оцінка ефекту пероральної вакцинації щодо Yersinia ruckeri на основі біомаркерів окиснювального стресу в зябрах райдужної форелі (Oncorhynchus mykiss Walbaum). Вакцина складалася з трьох штамів Y. ruckeri (серотип O1), що походять від райдужної форелі, яка вирощується в різних фермерських господарствах, де у риб виявлялися клінічні ознаки ієрсинеозу. Концентровану вакцину вводили корм для риб; годування цим препаратом проводилося тричі з інтервалом в один день. Через два місяці після імунізації брали зразки зябер. Вакцинація щодо Y. ruckeri призводила до недостовірних змін рівня ТБК-активних продуктів як маркерів перекисного окиснення ліпідів, рівня альдегідних та кетонових похідних окиснювально-модифікованих білків у зябрах форелі на другий місяць після імунізації щодо Y. ruckeri. Ми спостерігали достовірне зниження активності супероксиддисмутази (на 36%, p = 0,002) порівняно з необробленим контролем. Зміни маркерів окиснювального стресу дозволяють припустити, що антиоксидантний захист може сприяти балансу між окиснювальною модифікацією білків та антиоксидантним захистом у зябрах форелі, вакцинованої щодо Y. ruckeri. Жодних змін у зябрах через 60 днів імунізації ми не виявили. Ймовірно, це є результатом тривалої адаптації до імунізації. Розуміння ролі октснювального стресу в тканинах вакцинованої форелі має важливе значення для розуміння складних фізіологічних змін, що відбуваються під час імунізації, а також для покращення практики аквакультури для максимального росту тканин та здоров'я вакцинованих риб. Біомаркери окиснювального стресу в зябрах були чутливі до вакцинації щодо Y. ruckeri і потенційно можуть використовуватися як біомаркери при оцінці токсичності вакцини у райдужної форелі. З практичної точки зору результати можуть бути корисні щодо вивчення інфекцій та розробки, введення та використання нових вакцин, що застосовуються до великої кількості риб.

Ключові слова: райдужна форель Oncorhynchus mykiss, Yersinia ruckeri, імунізація, окиснювальний стрес, антиоксидантний захист, зябра.