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EFFECTS OF *FLAVOBACTERIUM PSYCHROPHILUM*, *FLAVOBACTERIUM PSYCHROPHILUM* EXTRACELLULAR PRODUCTS, AND LIPOPOLYSACCHARIDE ANTIGENS ON THE ANTIOXIDANT ENZYME SYSTEM ACTIVITY OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) FRY

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The aim of this study was to investigate the response of different doses of lipopolysaccharide (LPS) and extracellular product (ECP) antigens (25, 50, 100, 250, and 500 μg) obtained from *Flavobacterium psychrophilum* to changes in oxidative stress parameters (catalase [CAT], superoxide dismutase [SOD], glutathione peroxidase [GPx], glucose 6-phosphate dehydrogenase [G6PD], glutathione reductase [GR], and malondialdehyde [MDA]) in juvenile rainbow trout. For this purpose, 6 different doses of *F. psychrophilum* (10^2–10^6) were applied to the fish, and the lethal dose 50 % (LD_{50}) value was determined as $1.33 \cdot 10^4$. Changes in MDA levels and antioxidant enzymes were determined by applying 25 % of the LD_{50} value to fish for 14 days. Accordingly, while *F. psychrophilum* increased GR, G6PD, GPx, and SOD activity, it decreased MDA levels and CAT activity. Initially, 25, 50, 100, 250, and 500 μg doses of ECP and LPS were applied to the fish; after 21 days, a 25 % dose of LD_{50} was applied to these fish. Fourteen days later, the fish were taken, and the changes in MDA levels and antioxidant enzymes were examined. As a result, it was determined that while ECP and LPS application decreased GR, G6PD, GPx, and SOD activity, there was an increase in MDA levels and CAT activity.

Keywords: *Flavobacterium psychrophilum*; fish; antioxidant enzymes; lipopolysaccharide; extracellular products

ЕФЕКТИ НА *FLAVOBACTERIUM PSYCHROPHILUM,* НА НАДВОРЕШНИ ПРОИЗВОДИ НА *FLAVOBACTERIUM PSYCHROPHILUM* И НА ЛИПОПОЛИСАХАРИДНИ АНТИГЕНИ ВРЗ АНТИОКСИДАЦИСКАТА СИСТЕМСКА АКТИВНОСТ КАЈ ПОДМЛАДОК НА КАЛИФОРНИСКА ПАСТРМКА (*ONCORHYNCHUS MYKISS*)

Целта на оваа студија е да се испита одговорот на различни дози на антигени (25, 50, 100, 250 и 500 μg) од липополисахариди (LPS) и екстрацелурани производи (ECP) добиени од *Flavobacterium psychrophilum* на промените на оксидативните параметри на стрес (каталаза [CAT], супероксидна дизмутаза [SOD], глутатион-пероксидаза [GPx], глукоза 6-фосфатна дехидрогеназа [G6PD], глутатион-редуктаза [GR] и малондиалдехид [MDA]) кај подмладокот на калифорниска пастрмка. За таа цел беа применети шест различни дози на *F. psychrophilum* (10^2–10^6) и беше определено дека вредноста на смртоносната доза од 50 % (LD_{50}) изнесува $1{,}33 \cdot 10^4$. Промените во нивоата на MDA и оксидациските ензими беа определени со примена на 25 % од вредноста на LD_{50} на рибите во времетраење од 14 дена. Следствено, додека *F. psychrophilum* ја зголеми активноста на GR, G6PD, GPx и на SOD, ги намали нивоата на MDA и активноста на CAT. Во

почетокот на рибите им беа дадени дози од 25, 50, 100, 250 и 500 μg од ECP и LPS; по 21 ден им беше дадена доза од 25 % од LD_{50}. По 14 дена рибите беа земени, им беа измерени промените во нивоата на MDA и беа испитани антиоксидациските ензими. Како резултат на тоа беше одредено дека примената на ECP и LPS ја намалува активноста на GR, G6PD, GPx и на SOD, додека имаше зголемување на нивоата на MDA и активноста на CAT.

Клучни зборови: *Flavobacterium psychrophilum*; риби; антиоксидациски ензими; липополисахарид; екстрацелуларни производи

1. INTRODUCTION

Flavobacterium psychrophilum, which causes bacterial coldwater disease (CWD) and/or rainbow trout fry syndrome (RTFS), is a Gram-negative bacterium that causes acute septicemia in salmonids and many other species. This pathogen often causes large economic losses associated with mortality and spinal deformities and is considered one of the most significant pathogens in freshwater salmonid aquaculture worldwide.[1]

The bacterial effect occurs at water temperatures below 16 ºC and most commonly acts at 10 ºC and below. Although fish of all ages are affected, small fish (fry and finger size) are particularly vulnerable to infections. CWD produces characteristic open lesions on the outer body surfaces of the fish. These lesions may appear as areas of rough skin or fin tip abrasions. As the infection continues, necrosis develops in the bacterial colonization areas, which are usually referred to as dorsal and adipose fin pathology. Lesion development has a preference for the caudal peduncle and caudal fin regions. Along with external pathology, systemic bacterial infections and extensive internal pathology will be present among many examples. As the disease form becomes more acute, external lesions will be less common and systemic infections and internal pathology will become predominant.[2]

Although CWD and RTFS are common all over the world, especially in freshwater salmonid cultivation, there is currently no commercial vaccine for this bacterium.[3] Vaccination is an important process to protect fish from infections. Inactivated bacteria applications are widely used as vaccines against bacterial fish diseases; however, the effect of antigens decreases during the inactivation of bacteria.[4] Although inactivated live or dead vaccines were developed against *F. psychrophilum*, it was found that the protection period was insufficient.[5–7]

In Gram-negative bacteria, the cell is surrounded by a double-layer membrane, and the outer membrane consists of phospholipids, lipopolysaccharides (LPS), lipoproteins, and integral outer membrane proteins, all synthesized in the cytoplasm.[8] LPS or lipoglycans are large molecules containing a covalently-bound lipid and a polysaccharide. They are found on the outer membrane of Gram-negative bacteria. They act as endotoxins that create a strong immune response in animals. LPS antigens are the main component of bacteria's outer membrane, and they protect bacteria from chemical attack. In addition, it provides a general stabilization by helping the cell membrane be negatively charged.[9] LPS consist of a lipid segment called lipid A and a polysaccharide chain; lipid A is responsible for its toxic effect. The polysaccharide group varies widely among different bacteria. Although the molecular weight of endotoxins is about 10 kDa, they can form clumps up to 1,000 kDa. After exposure to endotoxins, it creates antibodies against it in humans, but they do not provide protection against other endotoxins because the endotoxin is directed to the polysaccharide chain.[10] LPS found in bacteria are responsible for antigenic structures and have important effects on the development of the immune system and, therefore, constitute the basis for vaccine development studies.[11]

Extracellular products (ECPs) are metabolites released into the environment by pathogens during growth and reproduction. ECPs play an important role in the invasion of pathogens into host cells, absorption of nutrition, widespread dissemination, replication in the host, and action against the host immune system. Some of these proteins have good immunogenicity, can induce host immune response, and are effective protective antigens.[12] Although vaccines against bacterial diseases are needed in aquaculture, only a few commercial vaccines are available. In particular, typical components of commercial vaccines to protect fish against bacterial diseases are either killed bacterial whole cells or ECPs.[13]

The aim of this study was to: (I) examine the changes in the antioxidant enzyme (catalase [CAT], superoxide dismutase [SOD], glutathione peroxidase [GPx], glucose 6-phosphate dehydrogenase [G6PD], glutathione reductase [GR]) and malondialdehyde (MDA) levels of the juvenile trout treated with *F. psychrophilum* and (II) examine the changes in antioxidant enzymes of rainbow

trout with different doses of LPS and ECP antigens (25, 50, 100, 250, and 500 μg) obtained from *F. psychrophilum*.

2. EXPERIMENTAL

2.1. *Bacterial strain and growth conditions*

In this study, virulent bacteria isolates obtained after bacteriological screening in fish production and aquaculture facilities in the Eastern Anatolia Region in 2004 were used.[14] Using polymerase chain reaction (PCR), these isolates were determined to be *F. psychrophilum*.

For the production of *F. psychrophilum*, tryptone yeast extract salts (TYES) agar was prepared as reported by Holt *et al.*[15] The medium was heated to dissolve and autoclaved at 121 ºC for 15 minutes. After cooling, it was poured into petri dishes in 15–17 ml.

Before obtaining the antigenic structures of *F. psychrophilum*, some morphological and biochemical characteristics of the agent were redetermined using Holt *et al.*[16] methods, in order to determine whether the bacterium was exposed to any contamination. In the study, a strain obtained from abroad was used as a positive control. The bacteria were planted on TYES agar and incubated for 5–10 days at 15 ºC. Yellow-colored colonies were selected, subcultured on TYES agar, and incubated at 15 ºC for 5–10 days.

2.2. *Fish*

The juvenile rainbow trout (*Oncorhynchus mykiss*) (approximately 1 g), which is the material of the study, was obtained from a commercial enterprise. The fish were brought to the Bingöl University Faculty of Agriculture Aquaculture Laboratory, placed in 60 l aquariums prepared previously, and adapted for 21 days. The average physicochemical properties of the water used in the study are as follows: temperature 13 ± 2 °C, dissolved oxygen 8.02 ± 0.5 mg/l, pH 7.3 ± 0.4, alkalinity 128 ± 7 mg/l, and total hardness 141 ± 23 mg/l. Fish were fed ad libitum with a commercial feed.

Before starting the study, bacteriological examinations were made from fish collected by random sampling, and it was determined whether the fish carried a pathogenic agent or not. Throughout the entire study, the fish were fed with a commercial trout feed twice a day, in the morning and evening, until they were full. The patient was fasted the day before vaccination studies and exposure with live bacteria.

2.3. *Lethal dose determination*

The lethal dose of *F. psychrophilum* bacteria used in the study was determined using the Reed and Muench method.[17] For this, 10^2–10^6 cells/ml live bacteria diluted with phosphate-buffered saline (PBS) was applied to 60 fish in each group (20 fish in each tank, 3 replicates) as a bath for 2 hours. Sterile PBS was added to the water of control fish. The deaths were recorded by checking fishes twice a day for 14 days. Colonies grown by planting on TYES agar were examined for *F. psychrophilum* from the dead fish. Samples isolated from internal organs of fish were considered to have originated from this bacteria. The lethal dose was calculated by Probit analysis with the SPSS 23.0 (Chicago, IL) statistical program.

2.4. *Preparation of ECPs*

In the preparation of ECPs, the cellophane film (gel drying, Sigma) technique reported by Liu was used.[18] A colony of *F. psychrophilum* bacteria, incubated at 15 ºC for 5–10 days in TYES agar, was transferred to tubes containing 5 ml of TYES broth. After the tubes were left to grow in the oven at 15 ºC for 5 days, 0.2 ml of this culture was taken and spread on TYES agar covered with sterile cellophane film with a Drigalski spatula. Petri dishes were incubated at 15 ºC for 5 days, and after incubation, bacterial cells were collected by washing with a small amount of cold PBS (pH 7.2). The cell suspension was centrifuged at 13,500 × G for 20 minutes at 4 ºC, then passed through a 0.45 μm membrane filter (Millipore) with the aid of a vacuum pump. Formalin was added to the obtained ECPs in a final concentration of 1 % and kept in the refrigerator at +4 ºC for one night; the samples taken from here were passed to TYES agar for sterility control. After the cultures were placed in an oven at 15 ºC and observed for 5–10 days, samples without any growth were stored in a refrigerator at 4 ºC until use.

2.5. *Preparation of LPS*

LPS antigen was made as reported by Swain *et al.*[19] Bacteria were incubated in TYES agar for 5–10 days at 15 ºC, and the bacteria weighing approximately 5 g were homogenized in 5 ml of distilled water then mixed in a water bath at 68 ºC. After adding an equal amount of 88 % phenol, preheated at 68 ºC, and mixing rapidly for 10 minutes, the mixture was cooled to 10 ºC. The supernatant was dialyzed against distilled water for 48 hours by centrifuging at 3,000 × G for 30 minutes. The obtained LPS molecule was adjusted to the appropriate con-

centration with polyethylene glycol (PEG-6000) and stored in a deep freezer at –20 ºC until use.

2.6. *Protein level of antigen preparations*

The protein level of ECP and LPS preparations were made as reported by Lowry *et al.*[20] After 1 ml of sample and 1 ml of alkaline copper reagent were placed in a glass test tube, the tubes were mixed thoroughly and kept for 10 minutes, then 4 ml of phenol reagent was added. After mixing thoroughly with vortex, the tubes were kept in a bain-marie at 55 ºC for 5 minutes. The tubes were cooled under tap water at the end of the incubation period, and the absorbance of the tubes at 650 nm was read against the blank.

2.7. *Application and effect of ECP and LPS on fish*

ECP and LPS were applied to groups divided into 50 juvenile fish (50 × 3 repeats: 150 fish) at 25, 50, 100, 250, and 500 μg as an immersion bath for 2 hours. To measure the resistance of the fish against *F. psychrophilum*, a 25 % lethal dose 50 % (LD_{50}) dose of *F. psychrophilum* was applied to the fish 21 days after the first treatment. Fourteen days after the 25 % LD_{50} dose was administered, 20 fish were randomly taken from each group for the study.

2.8. *Preparation of the homogenate*

At the end of the experiment, the fish fry samples were anaesthetized in anesthetic matter (25 ppm, benzocaine). All fish fry samples were washed with physiological saline (0.9 % NaCl) and cut into small pieces using a scalpel. The fry samples were suspended in a 50 mM KH_2PO_4 buffer (pH 7.4) and were homogenized by liquid nitrogen. The homogenate was centrifuged at 27,000 × G for 60 min. The supernatant, existing in the upper section, was carefully taken with a dropper. Following this, the precipitated part was removed. The supernatant was used to determine enzyme activity.[21]

2.9. *Determination of lipid peroxidation and enzyme activities*

The levels of MDA, as indices of the LPO in all tissues, were measured using the thiobarbituric acid reactions according to the method of Placer *et al.*[22] G6PD activity was measured at 37 ºC and 340 nm, in accordance with the Beutler method.[21] This method is based on the fact that reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is formed as a result of reducing $NADP^+$. GR activity was measured at 25 ºC by the modified method of Carlberg and Mannervik.[23] SOD was assayed by the method of Sun *et al.*[24] at 560 nm and 20 ºC. CAT activity was determined by measuring the decomposition of hydrogen peroxide at 20 ºC and 240 nm, according to the method of Aebi.[25] GPx was determined according to the method of Beutler[26] by following the rate of NADPH oxidation at 340 nm.

2.10. *Statistical analysis*

Data were expressed as the mean ± standard error. The data were subjected to one-way Analysis of variance (ANOVA), and Duncan's test was used to determine the statistical significance between the control and experimental groups using the SPSS 23.0 (Chicago, IL) computer program. A p value less than 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

In aquatic animals, many chemicals and microorganisms can cause an imbalance between the production of reactive oxygen species and their removal, resulting in oxidative stress. Oxidative stress is one of the most important factors that exacerbate the damage caused by some drugs, pesticides, and other environmental chemicals. It has been suggested that toxicity biomarkers, such as MDA, reflect the oxidative status of the species. MDA is used as a marker for the oxidation of membrane phospholipids through lipid peroxidation.[27–34] The antioxidant defense system consists of antioxidant enzymes, such as SOD, CAT, GPx, and GR, and low molecular weight antioxidants, such as reduced glutathione, vitamin A, vitamin C, and vitamin E. Antioxidant levels can be used as an indicator of the antioxidant status of the organism and can act as biomarkers of oxidative stress. When antioxidant defenses are broken or exceeded, oxidative stress can cause DNA damage, enzymatic inactivation, and peroxidation of cell components, especially lipid peroxidation.[27–30]

The aim of this study was to: (I) examine the changes in the antioxidant enzymes of the juvenile trout treated with *F. psychrophilum* and (II) investigate different doses of LPS and ECP antigens (25, 50, 100, 250, and 500 μg) obtained from *F. psychrophilum* to examine the changes in antioxidant enzymes caused by rainbow trout. At the end of 14 days, the LD_{50} value of the bacteria was determined as $1.33 \cdot 10^4$. A 25 % ($3.33 \cdot 10^3$) LD_{50} dose was applied to the fish on the 21st day following the administration of the antigens. Fish were fol-

lowed for 14 days, and the study was conducted by taking 20 fish from each group at the end of the period. As a result of the study, it was observed that, in general, the bacteria applied to the juvenile fish disrupted the oxidant/antioxidant balance, but when the amount of LPS and ECPs applied before was increased, it started to rearrange the oxidant/antioxidant balance, which was damaged. Unlike these, it was observed that applying LPS and ECPs did not have a statistically ($p < 0.05$) significant effect on GPx and GR activity, respectively (Tables 1 and 2).

T a b l e 1

Changes in antioxidant parameters in rainbow trout fry treated with Flavobacterium psychrophilum and extracellular products (ECPs) antigens

	Control	**%25 LD_{50} *F. psychrophilum***	**%25 LD_{50} *F. psychrophilum* + 25 µg ECP**	**%25 LD_{50} *F. psychrophilum* + 50 µg ECP**	**%25 LD_{50} *F. psychrophilum* + 100 µg ECP**	**%25 LD_{50} *F. psychrophilum* + 250 µg ECP**	**%25 LD_{50} *F. psychrophilum* + 500 µg ECP**
MDA	346.21±5.23^{a}	251.73±5.01^{b}	250.61±4.46^{b}	254.07±4.77^{b}	261.26±3.97^{b}	297.37±5.71^{c}	321.96±4.53^{d}
GR	7.84±1.97^{a}	11.91±2.68^{b}	11.53±1.23^{b}	12.40±2.34^{b}	11.46±2.02^{b}	10.92±1.12^{b}	11.04±1.41^{b}
G6PD	3.36±0.96^{a}	8.64±1.07^{b}	6.23±1.89^{c}	5.53±2.01^{c}	6.01±1.38^{c}	5.19±0.83^{c}	5.21±1.01^{c}
GPx	121±4.56^{a}	176.05±5.14^{b}	163.93±6.82^{b}	170.10±5.41^{b}	149.74±4.72^{c}	145.28±4.98^{c}	140.71±5.28^{c}
SOD	222.73±13.26^{a}	271.35±16.66^{b}	278.09±11.89^{b}	269.21±11.76^{b}	255.46±12.38^{c}	224.01±11.94^{a}	249.49±11.42^{c}
CAT	16.43±2.55^{a}	12.38±2.96^{b}	12.51±3.85^{b}	11.58±2.01^{b}	14.64±1.99^{c}	15.99±2.50^{a}	14.11±2.33^{c}

$^{a, b, c, d}$ in the same row indicate significant differences in the same group ($p < 0.05$).
Each value is the mean ± standard error ($n = 50$).
MDA: malondialdehyde (nmol/mg protein); GR: glutathione reductase (EU/mg protein); G6PD: glucose 6-phosphate dehydrogenase; GPx: glutathione peroxidase activity (EU/mg protein); SOD: superoxide dismutase (EU/mg protein); CAT: catalase activity (EU/mg protein). (EU: Enzyme unit)

T a b l e 2

Changes in antioxidant parameters in rainbow trout fry treated with Flavobacterium psychrophilum and lipopolysaccharides (LPS) antigen

	Control	**%25 LD_{50} *F. psychrophilum***	**%25 LD_{50} *F. psychrophilum* + 25 µg LPS**	**%25 LD_{50} *F. psychrophilum* + 50 µg LPS**	**%25 LD_{50} *F. psychrophilum* + 100 µg LPS**	**%25 LD_{50} *F. psychrophilum* + 250 µg LPS**	**%25 LD_{50} *F. psychrophilum* + 500 µg LPS**
MDA	203.87±22.64^{a}	156.74±14.63^{b}	150.93±11.03^{b}	174.17±11.98^{c}	187.59±13.87^{c}	185.40±15.77^{c}	193.21±14.23^{a,c}
GR	10.03±2.63^{a}	14.11±3.83^{b}	14.63±3.79^{b}	15.64±3.14^{b}	13.56±3.84^{b,c}	12.67±3.29^{a,c}	11.16±2.99^{a,c}
G6PD	1.01±0.04^{a}	5.16±1.01^{b}	5.12±1.12^{b}	4.64±1.23^{b}	3.71±1.13^{b,c}	3.07±1.13^{c}	2.92±0.74^{c}
GPx	214.84±12.09^{a}	247.29±13.93^{b}	256.37±15.74^{b}	250.27±14.11^{b}	255.90±13.92^{b}	253.22±14.68^{b}	249.44±15.08^{b}
SOD	176.29±8.11^{a}	195.27±11.43^{b}	197.98±11.90^{b}	196.75±10.66^{b}	189.74±10.18^{b,c}	186.72±10.04^{c}	181.55±9.46^{c}
CAT	23.80±3.59^{a}	16.84±2.03^{b}	17.01±2.82^{b}	18.03±2.12^{b,c}	18.25±2.93^{b,c}	19.53±3.07^{c}	20.91±3.26^{c}

$^{a, b, c}$ In the same row indicate significant differences in the same group ($p < 0.05$).
Each value is the mean ± standard error ($n = 50$).
MDA: malondialdehyde (nmol/mg protein); GR: glutathione reductase (EU/mg protein);
G6PD: glucose 6-phosphate dehydrogenase; GPx: glutathione peroxidase activity (EU/mg protein);
SOD: superoxide dismutase (EU/mg protein); CAT: catalase activity (EU/mg protein). (EU: Enzyme unit)

Fish exposed to vaccination exhibit a variety of physiological responses, including oxidative metabolism imbalances. Changes in oxidative stress biomarkers may indicate a compensatory response of the fish to vaccination. The differences observed in oxidative stress biomarkers obtained in this study may reflect differences in the antioxidant mechanisms of the vaccinated fish, exposure time, and the tested vaccine. Previous studies have shown that oxidative stress indices in fish can vary depending on the immunization time and the evaluated tissue. For example, Tkachenko *et al.*[32] used a vaccine produced from *Aeromonas salmonicida* and *Aeromonas hydrophila* bacteria, which are inactivated against furunculosis disease in rainbow trout. In this study, they investigated the effects of vaccination against furunculosis in trout muscle, gill, liver, and brain tissues on MDA levels and antioxidant defense systems (SOD, CAT, GR, and GPx). They applied the vaccination process be-

tween 60 and 120 seconds. As a result of the study, they found that the exposure of trout to the vaccine against furunculosis showed positive changes (increase or decrease) in the MDA level and antioxidant enzyme activities. It was determined that the inoculated trout showed a higher rate of change in antioxidant enzyme activities in the liver compared to other tissues. In addition, it was determined that vaccination against furunculosis triggers lipid peroxidation in gill, brain, and liver tissues.

In another study conducted with *Yersinia ruckeri* bacteria, changes in the antioxidant defense system created by the vaccine against this bacterium during 30 and 60 days in the muscle tissue of trout were examined. In the study, there was no significant difference in lipid peroxidation levels at the end of the first and second month after vaccination, while a significant decrease was found in GPx, GR, and CAT activity. In addition, it was determined that there was a significant increase in SOD activity after 30 days but a decrease after 60 days. As a result, it has been found that the *Yersinia* vaccine is partially responsible for the induction of increased protective immunity against *Y. ruckeri* infection[33] Tkachenko *et al.*[34] administered a vaccine against *Y. ruckeri* for trout in their study and examined the changes in lipid peroxidation level and SOD, CAT, GR, and GPx activities in fish gill, heart, and liver tissues four weeks after the administration of the vaccine. As a result of the study, they determined that each tissue shows its own specific oxidative stress response. They determined that the gill tissue was the tissue most susceptible to oxidative damage. Lipid peroxidation changes in heart and liver tissue were not found to be significant when compared with the control group, while the increase in gill tissue was statistically significant ($p < 0.05$). However, the increase in GPx activity in gill tissue, the decrease in SOD activity in liver tissue, and the increase in SOD activity in heart tissue were found to be statistically significant ($p < 0.05$).

4. CONCLUSION

Although it has been observed that ECPs and LPS have a positive effect on MDA levels and antioxidant enzyme activities, this may vary depending on time and dose. The changes caused by the study in fish metabolism should be examined in more detail in many metabolic parameters in future studies. In addition, it should be investigated whether the vaccine to be obtained based on ECPs and LPS can be used as a commercial vaccine against *F. psychrophilum* bacteria.

REFERENCES

(1) Ozdemir, N. S.; Ispir, U.; Kirici, M.; Ozcan, M., Changes of Fatty Acids Content in Rainbow Trout Infected with *Flavobacterium psychrophilum*. *Appl. Sci. Rep.* **2015**, *11*, 11–15. http://dx.doi.org/10.15192/PSCP.ASR.2015.11.1.1115

(2) Starliper, C. E., Bacterial Coldwater Disease of Fishes Caused by *Flavobacterium psychrophilum*. *J. Adv. Res.* **2011**, *2*, 97–108. https://doi.org/10.1016/j.jare.2010.04.001

(3) Schubiger, C. B.; Orfe, L. H.; Sudheesh, P. S.; Cain, K. D.; Shah, D. H.; Call, D. R., Entericidin is Required for a Probiotic Treatment (Enterobacter sp. strain C6-6) to Protect Trout from Cold-Water Disease Challenge. *Appl. Environ. Microbiol.* **2015**, 81, 658–665. https://doi.org/10.1128/AEM.02965-14

(4) Sommerset, I.; Krossøy, B.; Biering, E.; Frost, P., Vaccines for Fish in Aquaculture. *Expert Rev. Vaccines* **2005**, *4*, 89–101. https://doi.org/10.1586/14760584.4.1.89

(5) Högfors, E.; Pullinen, K. R.; Madetoja, J.; Wiklund, T., Immunization of Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), with a Low Molecular Mass Fraction Isolated from *Flavobacterium psychrophilum*. *J. Fish Dis.* **2008**, *31*, 899–911. https://doi.org/10.1111/j.1365-2761.2008.00956.x

(6) Plant, K. P.; Lapatra, S. E.; Cain, K. D., Vaccination of Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), with Recombinant and DNA Vaccines Produced to *Flavobacterium psychrophilum* Heat Shock Proteins 60 and 70. *J. Fish Dis.* **2009**, *32*, 521–534. https://doi.org/10.1111/j.1365-2761.2009.01008.x

(7) Gliniewicz, K.; Plant, K. P.; Lapatra, S. E.; Lafrentz, B. R.; Cain, K.; Snekvik, K. R.; Call, D. R., Comparative Proteomic Analysis of Virulent and Rifampicin-Attenuated *Flavobacterium psychrophilum*. *J. Fish Dis*. **2012**, *35*, 529–539. https://doi.org/10.1111/j.1365-2761.2012.01378.x

(8) Bos, M. P.; Tomassen, J., Biogenesis of the Gram-Negative Bacterial Outer Membrane. *Curr. Opin. Microbiol.* **2004**, *7*, 610–616. https://doi.org/10.1016/j.mib.2004.10.011

(9) Anwar, M. A.; Choi, S., Gram-Negative Marine Bacteria: Structural Features of Lipopolysaccharides and Their Relevance for Economically Important Diseases. *Mar. Drugs.* **2014**, *12*, 2485–2514. https://doi.org/10.3390/md12052485

(10) Raetz, C. R. H.; Whitfield, C., Lipopolysaccharide Endotoxins. *Annu. Rev. Biochem.* **2002**, *71*, 635–700. https://doi.org/10.1146/annurev.biochem.71.110601.135414

(11) Sakai, M., Current Research Status of Fish Immunostimulants. *Aquaculture* **1999**, *172*, 63–92. https://doi.org/10.1016/s0044-8486(98)00436-0

(12) Zhang, D.; Pridgeon, J. W.; Klesius, P. H., Vaccination of Channel Catfish with Extracellular Products of *Aeromonas hydrophila* Provides Protection against Infection by the Pathogen. *Fish Shellfish Immunol.* **2014**, *36*, 270–275. https://doi.org/10.1016/j.fsi.2013.11.015

(13) Ispir, U.; Dorucu, M. Effect of Immersion Booster Vaccination with *Yersinia ruckeri* Extracellular Products (ECP) on Rainbow Trout *Oncorhynchus mykiss*. *Int. Aquat. Res.* **2010**, *2*, 127–130.

(14) İspir, Ü.; Şeker, E.; Sağlam, N.; Dörücü, M., A Study of *Flavobacterium psychrophilum* Infection in Some Cultured Rainbow Trout (*Oncorhynchus mykiss*) in Eastern Anatolia. *Science and Engineering Journal of Fırat University* **2004**, 16, 718–724. (in Turkish with English abstract)

(15) Holt, R. A.; Rohovec, J. S.; Fryer, J. L., Bacterial Coldwater Disease. In: *Bacterial diseases of Fish,* Blackwell Scientific Publications, 1993; pp 3–23.

(16) Holt, R. A., *Cytophaga psychrophila, the Causative Agent of Bacterial Cold-Water Disease in Salmonid Fish.* PhD thesis, Oregon State University, Department of Microbiology, Corvallis, USA, 1987.

(17) Reed, L. J.; Muench, H., A Simple Methods of Estimating Fifty Percent Endpoints. *Am. J. Epidemiol.* **1983**, *27*, 493–497. https://doi.org/10.1093/oxfordjournals.aje.a118408

(18) Liu, P.V., Survey of Hemolysin Production among Species of Pseudomonads. *J. Bacteriol.* **1957**, *74*, 718–727. https://doi.org/10.1128/JB.74.6.718-727.1957

(19) Swain, P.; Nayak, S. K.; Sahu, A.; Meher, P. K.; Mishra, B. K., High Antigenic Cross-Reaction among the Bacterial Species Responsible for Diseases of Cultured Freshwater Fishes and Strategies to Overcome It for Specific Serodiagnosis. *Comp. Immunol. Microbiol. Infect. Dis.* **2003**, *26*, 199–211. https://doi.org/10.1016/S0147-9571(02)00059-0

(20) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J., Protein Measurement with Folin Phenol Reagent. *J. Biol. Chem.* **1951**, *193*, 265–275. https://doi.org/10.1016/S0021-9258(19)52451-6

(21) *Red Cell Metabolism Manual of Biochemical Methods*, Beutler, E., Eds., Academic Press: London, 1971.

(22) Placer, Z. A.; Cushman, L.; Johnson, B. C., Estimation of Products of Lipid Peroxidation (Malonyldialdehyde) in Biological Fluids. *Anal. Biochem.* **1966**, *16*, 359–364. https://doi.org/10.1016/0003-2697(66)90167-9

(23) Carlberg, I.; Mannervik, B., Purification and Characterization of the Flavoenzyme Glutathione Reductase from Rat Liver. *J. Biol. Chem.* **1975**, *250*, 5475–5480. https://doi.org/10.1016/S0021-9258(19)41206-4

(24) Sun, Y.; Oberley, L. W.; Li, Y., A Simple Method for Clinical Assay of Superoxide Dismutase. *Clin. Chem.* **1988**, *34*, 497–500. https://doi.org/10.1093/clinchem/34.3.497

(25) Aebi, H., Catalase. In: *Methods in Enzymatic Analysis*. Academic Press, 1983; pp. 673–680. https://doi.org/10.1016/B978-0-12-091302-2.50032-3

(26) Beutler, E., Red Cell Metabolism. In: *A Manual of Biochemical Methods.* Grune Strottan, 1975; pp 112–114.

(27) Yonar, S. M.; Sakin, F.; Yonar, M. E.; Ispir, U.; Kirici, M., Oxidative Stress Biomarkers of Exposure of Deltamethrin in Rainbow Trout Fry (*Oncorhynchus mykiss*). *Fresenius Environ. Bull.* **2011**, *20*, 1931–1935.

(28) Yonar, M. E.; Ispir, U.; Yonar, S. M.; Kirici, M., Effect of Copper Sulphate on the Antioxidant Parameters in the Rainbow Trout Fry, *Oncorhynchus mykiss*. *Cell. Mol. Biol.* **2016**, *4*, 55–58. https://doi.org/10.14715/cmb/2016.62.6.10

(29) Ispir, U.; Kirici, M.; Yonar, M. E.; Yonar, S. M., Response of Antioxidant System to Formalin in the Whole Body of Rainbow Trout, *Oncorhynchus mykiss*. *Cell. Mol. Biol.* **2017**, *63*, 13–16. https://doi.org/10.14715/cmb/2017.63.1.3

(30) Kirici, M.; Turk, C.; Çağlayan, C.; Kirici, M., Toxic Effects of Copper Sulphate Pentahydrate on Antioxidant Enzyme Activities and Lipid Peroxidation of Freshwater Fish *Capoeta umbla* Tissue. *Appl. Ecol. Environ. Res.* **2017**, *5*, 1685–1696. http://dx.doi.org/10.15666/aeer/1503_16851696

(31) Taysi, M. R.; Kirici, M.; Kirici, M.; Sögüt, B.; Bozdayi, M. A.; Taysi, S., Cadmium Toxicity in Rainbow Trout (*Oncorhynchus mykiss*): A Study on Heart and Muscle Tissue. *Turkish Journal of Agricultural and Natural Sciences,* **2020**, *7*, 983–987. https://doi.org/10.30910/turkjans.802746

(32) Tkachenko, H.; Kurhaluk, N.; Grudniewska, J.; Andriichuk, A., Tissue-Specific Responses of Oxidative Stress Biomarkers and Antioxidant Defenses in Rainbow Trout *Oncorhynchus mykiss* during a Vaccination against Furunculosis. *Fish Physiol. Biochem.* **2014**, *40*, 1289–1300. https://doi.org/10.1007/s10695-014-9924-9

(33) Tkachenko, H.; Grudniewska, J.; Pękala, A.; Terech-Majewska, E., Oxidative Stress and Antioxidant Defence Markers in Muscle Tissue of Rainbow Trout (*Oncorhynchus mykiss*) after Vaccination Against *Yersinia ruckeri*. *J. Vet. Res.* **2016**, *60*, 25–33. https://doi.org/10.1515/jvetres-2016-0005

(34) Tkachenko, H.; Grudniewska, J.; Pękala, A., Effects of Oral Vaccinaton against *Yersinia ruckeri* on Oxidative Stress Biomarkers in Gills, Liver and Heart of Rainbow Trout (*Oncorhynchus mykiss* Walbaum). *Balt. Coast. Zone* **2017**, *21*, 109–128.