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# Pre-probiotic effects of different bacterial species in aquaculture: behavioral, hematological and oxidative stress responses

by

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## Abstract

There is very limited aquaculture research on candidate probiotics and their effects on fish physiology. In this study, acute applications of four different molecularly identified bacterial species – *Brevibacillus brevis* FD-1 (A), *Pseudomonas fluorescens* FDG-37 (B), *Bacillus sphaericus* FD-48 (C), and *B. amyloliquefaciens* TV-17C (D), with potential in aquaculture, were tested in rainbow trout (*Oncorhynchus mykiss*) under static conditions. Physiological changes in blood tissue [hematological indices: erythrocyte count (RBC), leukocyte count (WBC), hemoglobin (Hb), hematocrit (Hct), platelet count (PLT), mean cell hemoglobin concentration (MCHC), mean cell hemoglobin (MCH), and mean cell volume (MCV)], oxidative stress responses in liver and gill tissues [malondialdehyde (MDA) level, antioxidant enzyme activities: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase ($GP_x$), glutathione reductase (GR), glutathione-S-transferase (GST), glucose-6-phosphate dehydrogenase (G6PD)] and acetylcholinesterase (AChE) activity in brain tissue (as neurotoxic biomarker) were investigated. Additionally, behavioral differences were recorded by measuring swimming performance to support neurotoxic findings in all treatment groups. The $LC_{50}$24 value of FDG-37 strain was determined through analysis as $1.0 \times 10^8$ CFU $ml^{-1}$. Inhibition of enzyme activity, increase in the MDA level, as well as significant differences in hematological indices and swimming performance were determined in rainbow trout treated with B compared to control and other bacterial groups in gills. The potential for using group FD-48 and TV-17C bacterial strains as probiotics in aquaculture is more pertinent when considering the research findings and water quality parameters.

**Key words:** fisheries, ecosystem management, probiotic, oxidative stress, $LC_{50}$

# 1. Introduction

There is a growing demand for probiotics, which are very common in therapeutic, prophylactic, and growth supplements in animal and human health (Pandiyan et al. 2013). Probiotics have recently gained popularity as candidates for beneficial microbes in cultured organisms to maintain the health and well-being of different aquatic organisms (Dawood et al. 2019). Of the many probiotics that have been discovered, *Bacillus* ssp. have proven to have superior probiotic properties due to their ability to produce pathogenic and non-toxic antimicrobial substances that are active against many microbes. It has also been documented that *Bacillus* ssp. increase digestive and antioxidant enzyme activity, increase the expression of immune and stress-related genes, and above all improve the resistance of fish to pathogenic microbes (Kuebutornye et al. 2019). However, due to the paucity of research on probiotics and the selection of unsuitable microorganisms, there are significant difficulties in understanding the mechanism of the effects of these microorganisms and determining the selection criteria for potential probiotics. Evaluation of blood parameters is an important marker and a commonly used method in assessing the effects of similar therapeutics on fish health. With this technique, it is possible to make reliable decisions in determining the physiological state and environmental conditions of an animal (Uçar et al. 2019; Alak et al. 2020a; Uçar et al. 2021). By studying the hematological parameters, effects of different social, physiological and environmental factors (such as social hierarchy, disease, nutritional deficiencies, toxic substances, changes in water quality, temperature, photoperiod, density, salinity, pH, oxygen, heavy metals, pesticides, detergents) on fish health and physical state can be determined (Uçar et al. 2019; Alak et al. 2020b). Analysis of hematological and biochemical parameters in fish contributes to the assessment of animal health and ecological conditions (Uçar et al. 2019; Alak et al. 2020b; Atamanalp et al. 2021; Uçar et al. 2021). Any disorder in the blood has a serious effect on physiological functions of the whole organism. The liver, which has many other functions in the body, including production of red blood cells, glycogen storage, plasma, and protein synthesis, hormone production, and detoxification, is also very effective in the production, storage and release of many enzymes (Alak et al. 2019). Some xenobiotics cause oxidative stress in these organs. In such a case, an increase in free radical production and a decrease in antioxidant defense are observed. For this reason, MDA (which is generally the end product of lipid peroxidation), antioxidant enzymes such as SOD, GPx, CAT, GST, GR, and 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, were measured to determine oxidative stress (Alak et al. 2019; Atamanalp et al. 2021). The main purpose of this study is to identify new probiotic candidates that can be used in aquaculture and to support the effects of these candidate probiotics on fish health using rapid and reliable biomarkers (hematological indices and enzyme activity), as well as behavioral changes using swimming performance tests. The second objective of the study is to understand the necessity of using probiotics as a sustainable alternative to regulate the general health status of the aquatic environment and their different functionality in aquaculture.

# 2. Materials and methods

## 2.1. Bacteria

The coding contents, bacterial strains, and Genbank numbers used in the study are presented in Table 1. *Bacillus* species can be found in nature in quite different substrates, including decaying organic material, dust, soil, green vegetables, water, and in some species, normal body flora (Kara et al. 2021). *Pseudomonas fluorescens*, on the other hand, has a versatile metabolism and can be found in soil and water.

**Table 1**

Results of sequences

| Treatment code | Strains | Identification results | Genbank Numbers |
|---|---|---|---|
| A | FD-1 | *Brevibacillus brevis* | MW847612 |
| B | FDG-37 | *Pseudomonas fluorescens* | MW740242 |
| C | FD-48 | *Bacillus sphaericus* | MW751888 |
| D | TV-17C | *Bacillus amyloliquefaciens* | MW751910 |

## 2.2. Fish

A total of 3700 rainbow trout (*Oncorhynchus mykiss*) with an average weight of 18–20 g (60 days after hatching according to the OECD 210 report) were used in the study, 25 fish in each group. From this pool of fish, 3200 individuals (4 bacteria × 8 concentrations × 4 replications × 25 fish) were used in the $LC_{50}$ analysis. The remaining 500 fish were placed in the experimental groups that were redesigned based on the determined $LC_{50}$ value: 400 (4 bacteria × 1 concentration × 4 replications × 25 fish) and 100 individuals (25 fish × 4 replicates).

### 2.3. Determination of bacterial 16S-23S rDNA gene sequences

Design of primers: The 16S-23S rDNA region was expressed using universal primers (Jiang et al. 2006). The universal primer pairs used for this are provided in Table 2. DNA isolation: Isolation was performed using the Qiagen Qiacube DNA Isolation Robot with the aid of the Qiamp DNA Mini Qiacube Kit. Colonies were homogenized in PBS solution for 2 min and then centrifuged at 5000 g for 10 min to obtain a bacterial pellet. After the isolation step of the tube containing the bacterial pellet was completed in the Qiacube device, 1.5 ml volume tubes collected from the L3 position and stored at –20°C until the PCR analysis following the relevant labeling process.

**Table 2**

Sequences of primers

| Primer | Primer Sequence (5'-3') |
|---|---|
| 27 Forward | AGAGTTTGATCCTGGCTCAG |
| 1492 Reverse | GGTTACCTTGTTACGACTT |

The PCR reaction mixture and the reaction cycle are presented in Table 3. PCR products were visualized on 1% agarose gel (1 g agarose, 100 ml TAE, 4 µl ethidium bromide, 10 mg $ml^{-1}$). PCR products were loaded onto the frozen gel and run at 90V for 50 min. Purification was performed with a commercially available PCR purification kit (Invitrogen). The purified sample was obtained using sequencing service from Refgen Biotechnology Company (Turkey, Ankara). The sequenced samples were analyzed using the Bioedit software.

**Table 3**

PCR mix content and cycle conditions

| PCR Mix | Amount | PCR Cycle | | | |
|---|---|---|---|---|---|
| $dH_2O$ | 37.2 µl | 1 | 95°C | 2 min | 1 cycle |
| 10X PZR tampon | 5 µl | 2 | 94°C | 1 min | |
| $MgCl_2$ | 3 µl | 3 | 53°C | 1 min | 35 cycle |
| dNTP mix | 0.7 µl | 4 | 72°C | 1.30 min | |
| Forward primer | 0.8 µl | 5 | 72°C | 10 min | 1 cycle |
| Reverse primer | 0.8 µl | 6 | 4°C | ∞ | |
| DNA | 2 µl | | | | |
| Taq Polymerase (250 U) | 0.5 µl | | | | |
| Total | 50 µl | | | | |

### 2.4. Bacterial growth

For bacterial growth, dilution tubes were prepared separately for each bacterium. With gentle stirring, 100 µl of the solution was transferred onto nutrient agar (NA) and then inoculation was carried out on six Petri dishes. The seeded Petri dishes were left to incubate at 30°C in aerobic conditions for 48 h. Purification was performed from samples in which individual colonies were observed on Petri dishes. They were stored at –86°C in stock media containing Loria Broth (LB) and 18% glycerol until diagnosis and characterization processes (Kara et al. 2021). Frozen bacterial cultures (*FD-1, FDG-37, FDG-48*, and *TV-17C*; Table 4) were inoculated into Petri dishes containing NA medium. They were left to incubate at 32°C, after which 24-hour fresh cultures were obtained. Nutrient Broth (NB) was collected from the growing fresh cultures and transferred to the NB medium. The bacteria were cultured in a horizontal shaker incubator at 32°C for 24 h (Kara et al. 2021). Then, research concentrations ($1.0 \times 10^1$, $10^2$, $10^3$, $10^4$, $10^5$ $10^6$, $10^7$, and $10^8$ CFU/ml) were adjusted by a turbidimeter according to the standard turbidity tube by mixing in the vortex device.

**Table 4**

Experimental design

| Treatment code | Strains |
|---|---|
| A | FD-1 |
| B | FDG-37 |
| C | FD-48 |
| D | TV-17C |
| E | No bacteria |

### 2.5. Design of trial groups and testing of bacteria in experimental equipment

In the experimental setup, pure bacteria to be tested were removed from the freezer and transferred into Petri dishes containing the NA medium, and then incubated for 24 h at 30°C to obtain fresh cultures. When transferring them to the nutrient medium containing 250 ml of NB, isolate numbers of bacteria were recorded on each of these cultures. The absorbance of these cultures, grown for 24 h in the horizontal shaker incubator, was adjusted to $1 \times 10^x$ CFU $ml^{-1}$ concentration using sterile distilled water in a biological turbidimeter. The experimental design was made based on static tests with four replications. Different doses were used in the experiment. First, limiting pretesting was performed by applying a control to cultured trout of $1.0 \times 10^1$, $10^2$, $10^3$, $10^4$, $10^5$ $10^6$, $10^7$, and $10^8$ CFU $ml^{-1}$ for each bacterial species. Then, considering these preliminary test results ($LC_{50}$ value), the dose to be applied in the main test ($1.0 \times 10^7$ CFU $ml^{-1}$) was determined and the experiment was

resumed. There were five groups in the tests, one of which was the control and four were the treatments. Bacterial cultures at a concentration of $1 \times 10^7$ CFU $ml^{-1}$ prepared in the experimental setup were applied to each aquarium (based on the 1/1000 inoculation culture ratio). In this application, taking into account the static test procedure, a solution containing 1 ml of bacteria was applied to each 100 ml, which was prepared separately for each bacterial species. Water quality parameters were measured for each application group at the beginning and at the end of the test (Table 5).

**Table 5**

Parameters of the trial water

| Water quality parameters | Beginning of the treatment (Hour 0) | | | | | End of the treatment (Hour 96) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | A | B | C | D | E |
| Temperature | 8.3 | 8.6 | 8 | 10 | 8.6 | 10.06 | 10.5 | 8.5 | 9.6 | 8.1 |
| Oxygen | 8.44 | 9.18 | 9.57 | 7.5 | 9.18 | 5.87 | 4.66 | 9.23 | 6.55 | 9.09 |
| EC | 0.220 | 0.222 | 0.215 | 0.217 | 0.222 | 0.237 | 0.237 | 0.220 | 0.241 | 0.205 |
| pH | 8.29 | 8.25 | 8.34 | 8.30 | 8.25 | 7.84 | 7.96 | 8.28 | 8.02 | 8.09 |

### 2.6. Determination of $LC_{50}$

The $LC_{50}$ value was calculated by Probit Analysis using Microsoft Excel software. For this purpose, rainbow trout were exposed to different bacteria. After 24 h, dead fish were detected and Probit analysis was performed. The data yielded an S-shaped curve, thus the LC value was calculated by converting doses into $\log_{10}$ to make this curve linear (Köktürk et al. 2021).

### 2.7. Swimming performance

Swimming performance was determined using a specific system. In this system, the temperature in the swimming tunnel was 10.0 ± 0.5°C and the oxygen level was 10.0 ± 0.5 ppm. The critical swimming speed (Ucrit) was calculated according to the equation given by Brett (1964). Swimming performance tests were carried out and the Ucrit was calculated using the said formula (Plaut 2001; Topal et al. 2015).

Ucrit = Uf + (tf/ti)Ui, where $U_f$ (cm $s^{-1}$) is the highest speed at which the fish was swimming for the entire time period and $U_i$ is the water velocity increment (proportionally to the body length); $t_i$ (30 min) is the prescribed period of swimming at a given speed and $t_f$ (min) is the time the fish were swimming at the final speed. All absolute $U_{crit}$ (cm $s^{-1}$) values were standardized for size by dividing the total length of fish to obtain a value in body lengths per second, which represents relative $U_{crit}$ (BL $s^{-1}$).

### 2.8. Determination of hematological indices

Erythrocyte count (RBC), leukocyte count (WBC), hemoglobin value (Hb), hematocrit ratio (Hct), platelet count (PLT), hemoglobin count per erythrocyte (MCHC), mean hemoglobin amount per erythrocyte (MCH) and mean erythrocyte volume (MCV) were determined in blood samples (Ucar and Atamanalp 2010; Parlak and Atamanalp 2017). Blood from fish was collected from the caudal vein. The cyanmethemoglobin method was used to determine hemoglobin and the microhematocrit method was used to determine hematocrit. For the determination of erythrocyte, leukocyte and thrombocyte levels, countings were made on areas determined under a microscope on a Thoma slide after staining with Dacie's solution. Values of other indices – mean erythrocyte volume (MCV), mean cell hemoglobin (MCH), and hemoglobin per erythrocyte (MCHC) – were calculated using the formulas provided by Parlak and Atamanalp (2017). To determine the erythrocyte sedimentation rate, anticoagulant blood samples were collected into hematocrit tubes (1.1 mm in diameter and 7 cm in length) and kept in an upright position (90°) for 1 h. The separated serum part was measured using a ruler and recorded in mm/h (Ucar and Atamanalp, 2010).

### 2.9. Biochemical analyses

#### Homogenate preparation and activity measurements

Tissue samples (brain, gill, and liver) collected from fish were homogenized in a homogenizer after adding the $KH_2PO_4$ buffer solution three times. The obtained homogenates were centrifuged at 4°C depending on the tissue type and the supernatants were used to measure enzyme activities (Alak et al. 2020a). The AChE activity and MDA content in brain homogenates, and antioxidant defense indicators (SOD, CAT, GPx, GR, GST, G6PD activity or MDA content) in gill and liver homogenates were determined. Protein concentration was determined spectrophotometrically at 595 nm

according to the Bradford method (using bovine serum albumin as standard; Bradford 1976).

#### Determination of acetylcholinesterase (AChE) activity

The enzyme activity was determined by absorbance readings at a wavelength of 412 nm according to the method of Ellman et al. (1961) as described by Alak et al. (2019).

#### Measurement of superoxide dismutase (SOD) enzyme activity

Xanthine produces a superoxide radical through the xanthine oxidase system. If there is no SOD in the environment, the superoxide radical reduces NBT (nitro-blue tetrazolium) and a blue color is produced. Removal of superoxide radicals from the environment and inhibition of NBT reduction causes a decrease in the intensity of the blue color depending on the amount of SOD in the environment. The produced superoxide radicals react with NBT, while SOD in the sample dismutates the produced radicals and slows down the NBT reduction reaction, and reduces the absorbance readings by forming absorbance formazan (Sun et al. 1988). Based on this principle, SOD activity was determined spectrophotometrically at 560 nm (Alak et al. 2020b).

#### Measurement of catalase (CAT) activity

The Aebi (1974) method was used to determine the catalase activity. It is based on the principle of measuring the decrease in absorbance at 240 nm when $H_2O_2$ in the activity measurement medium is converted to $H_2O$ via CAT. The reaction was determined by measurements made at 240 nm in a spectrophotometer and the rate of decrease in the amount of absorbance was associated with catalase activity.

#### Measurement of glutathione peroxidase (GPx) activity

GPx activity was determined according to Beutler (1984). It was calculated by measuring the difference in absorbance at 340 nm during the oxidation of NADPH consumed during the conversion of GSSG, which is formed as a result of the oxidation of GSH with $H_2O_2$ to GSH upon glutathione reductase (GSSG-Rd) catalysis, in a reaction catalyzed by GSH-Px (Atamanalp et al. 2021).

#### Measurement of glutathione S-transferase (GST) enzyme activity

The activity measurement is based on the absorbance at 340 nm of CDNB conjugated with glutathione at 37°C in a CDNB-containing medium. In all measurements, the amount of conjugate formed by the spontaneous reaction (nonenzymatic) was subtracted from the values obtained by the enzymatic reaction. In the absence of GSH, CDNB rapidly inactivates glutathione S-transferase. For this reason, the reaction was initiated by adding CDNB to the enzyme equilibrated with GSH at 37°C in reaction buffer (Habig et al. 1974).

#### Measurement of glucose-6-phosphate dehydrogenase (G6PD) enzyme activity

Enzyme activity is measured by detecting the difference in absorbance by NADPH formed during the reaction at a wavelength of 340 nm per unit time (Beutler 1975).

#### Measurement of the lipid peroxidation (MDA) level

MDA is an essential product of membrane lipid peroxidation and is a well-known indicator that reflects the degree of oxidative stress in cells (Chen et al. 2021). TCA was added to the extracted tissue samples and incubated at –20°C. The readings were made at 532 nm and the MDA level was calculated according to Atamanalp et al. (2021).

### 2.10. Statistical analysis

To ensure the correct interpretation of the results, including non-parametric and parametric tests obtained at the end of the study period, the normal distribution test was applied to the data using SPSS V25 software package. Data with normal distribution were subjected to the variance analysis and the significance level was set at 0.05.

## 3. Results

### 3.1. Diagnostic results of bacteria

Diagnostic results according to the 16S-23S rDNA gene sequences of the bacteria used in this research are presented in Table 1.

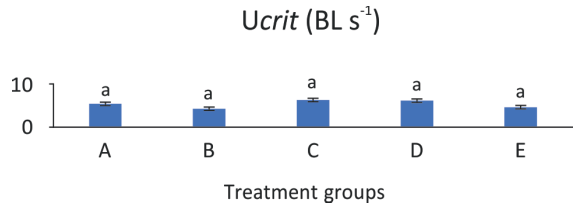
### 3.2. $LC_{50}$ value in rainbow trout

The $LC_{50}$ value for each of the four bacteria was tested in rainbow trout at eight different concentrations ($1.0 \times 10^1$, $10^2$, $10^3$, $10^4$, $10^5$ $10^6$, $10^7$, and

$10^8$ CFU $ml^{-1}$). In these applications, only the second bacteria (B) died at a concentration of $10^8$ CFU $ml^{-1}$ at the end of the 24th hour (72%). No death was observed in the three other bacterial groups. From the obtained results, the $LC_{50}$ 24 value for B was recorded as $10^8$ CFU $ml^{-1}$ (mortality rate of 72%), and for the other bacterial species it was > $10^8$ CFU $ml^{-1}$.

### 3.3. Swimming performance

The highest swimming performance was determined in group A (6.4 ± 0.14 BL $s^{-1}$), while the lowest in group B (6.4 ± 0.14 BL $s^{-1}$). Statistical analysis showed no significant differences in the application groups at the $p < 0.05$ level (Fig. 1).



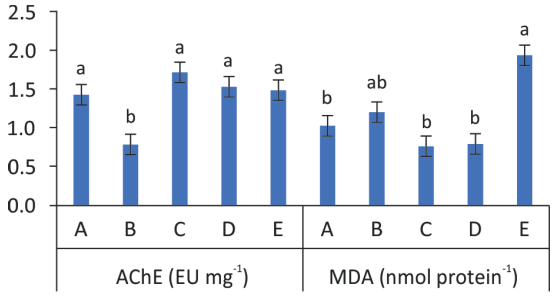

A – *Brevibacillus brevis*, B – *Pseudomonas fluorescens*, C – *Bacillus sphaericus*, D – *Bacillus amyloliquefaciens*, and E – control (n = 25, mean ± standard error). The same letters (a) indicate significant differences between the same columns within each experimental treatment group

**Figure 1**

Results of critical swimming performance

### 3.4. AChE activity

Changes in acetylcholinesterase activity were significant at the $p < 0.05$ level, and the highest inhibition was determined in group B (*Pseudomonas fluorescens*; Fig 2).




A – *Brevibacillus brevis*, B – *Pseudomonas fluorescens*, C – *Bacillus sphaericus*, D – *Bacillus amyloliquefaciens*, and E – control (n = 25, mean ± standard error). Different letters (a, b) indicate significant differences between the same columns within each experimental treatment group

**Figure 2**

Results of AChE enzyme activity in brain

### 3.5. Hematological indices

In terms of hematological indices, the difference between the groups was statistically significant ($p < 0.05$). No statistical difference was observed in RBC, ESR, and MCH between the treatment groups (Table 6).

### 3.6. Oxidative stress response

It was determined that different bacterial species (tested in this research) significantly increased antioxidant capacity in liver and gill tissues compared to the control group ($p < 0.05$, Table 7). Considering the lipid peroxidation level, it was determined that *Pseudomonas fluorescens* (group B) caused oxidative damage in the gill tissue, and the oxidative damage level in the liver tissue was much lower compared to the control group in four bacterial species ($p < 0.05$).

## 4. Discussion

The well-being and growth of organisms are directly dependent on their environment. Optimal properties and physicochemical status of water are important determinants in aquaculture. Exposure to different substances causes damage to the gills, difficulty in breathing, decreased oxygen uptake, and a decrease in critical swimming speed as a behavioral effect. The host gut microbiota affects the central nervous system by affecting local oxidative stress levels and the permeability of the gut, then subsequently the behavioral characteristics of the host (Chen et al. 2021). In this research, we observed that acute applications of bacterial strains significantly reduce the critical swimming speed, especially in group B. AChE inhibition is believed to be effective in this poor performance (Domingues et al. 2016). This reduction can be considered effective in possible changes in the gills. We believe that these behavioral changes are due to the association of deleterious effects between cerebral ROS and neurotransmission disorder. This is because its downregulation causes neuron depolarization and decreases neural activity, which has negative consequences on behavioral functions, including locomotor modification (Pereira et al. 2002). The non-specific response in fish is triggered by physical, chemical, and perceived stressors and enables fish to cope with the stressor. It is important to relate cellular responses to behavior, chemical stress, and higher levels of biological organization. Because behavior is the result of internal and external processes, changes in such parameters help to

understand the health and viability of natural populations exposed to pollutants. Stressors induce a nonspecific response in fish to adapt to or cope with the disturbance (Sharma 2018). Under stress conditions, along with the physiological effect of the primary response of aquatic organisms, secondary responses occur in a chain-like manner. Secondary responses can be determined by changes in histological, histopathological, biochemical, and hematological parameters. Homeostasis in fish after stress can be achieved through certain physiological changes that regulate hematological, hormonal, and energy metabolism (Uçar et al. 2021). Some studies have reported that probiotic supplementation can increase hematological indices in fish (Hassaan et al. 2021; Jahan et al. 2021). Previous studies have documented that *Bacillus* ssp. (added to water or feed) improve the aquatic environment with extracellular enzymes and antimicrobial peptides, and probiotic bacteria detoxify water and make it more suitable for culture organisms (Kuebutornye et al. 2019). According to the results of our research, the hematological index and oxidative stress responses were better for C and D groups compared to other groups. This shows that the studied bacterial species have the potential to improve water quality parameters. The erythrocyte count is an important parameter that determines the blood oxygen-carrying capacity and functions of erythropoietic tissues (Uçar et al. 2021). High RBC and Hb values are common responses to hypoxia or anoxia. In this study, we found that hemoglobin values were higher in group B than in the control group, and we concluded that this increase was related to the increase in the count of red blood cells. In this case, the fish attempted to eliminate this stress by increasing RBC and Hb in order to increase the oxygen-carrying capacity of the blood (Hedayati and Tarkhani 2014). The improvement in hematological parameters and the increase in Hb/Htc values can be explained by the increase in the release of some minerals (Ca, P, Fe, and Cu) by probiotics (Hassaan et al. 2021). The same researchers also stated that the increase in

**Table 6**

Hematological indices in the groups

| Treatment | WBC ($10^4$ mm$^3$) | RBC ($10^6$ mm$^3$) | PLT ($10^4$ mm$^3$) | ESR (mm h$^{-1}$) | Hb (g dl$^{-1}$) | Hct (%) | MCV (µm$^3$) | MCH (pg) | MCHC (g 100ml$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| A | 10.65 ± 2.03$^{c}$ | 3.07 ± 0.05$^{a}$ | 14.33 ± 2.16$^{b}$ | 0.18 ± 0.04$^{a}$ | 8.59 ± 0.8$^{ab}$ | 33.50 ± 5.57$^{b}$ | 117.07 ± 13.10$^{b}$ | 29.21 ± 3.91$^{a}$ | 27.30 ± 3.06$^{a}$ |
| B | 17.98 ± 2.03$^{a}$ | 3.42 ± 0.05$^{a}$ | 23.92 ± 2.16$^{a}$ | 0.13 ± 0.04$^{a}$ | 9.47 ± 0.8$^{a}$ | 47.58 ± 5.57$^{a}$ | 142.78 ± 13.10$^{ab}$ | 28.64 ± 3.91$^{a}$ | 20.58 ± 3.06$^{b}$ |
| C | 14.73 ± 2.03$^{b}$ | 3.33 ± 0.05$^{a}$ | 22.07 ± 2.16$^{a}$ | 0.16 ± 0.04$^{a}$ | 7.41 ± 0.8$^{b}$ | 44.25 ± 5.57$^{a}$ | 136.06 ± 13.10$^{ab}$ | 22.82 ± 3.91$^{a}$ | 17.11 ± 3.06$^{b}$ |
| D | 12.91 ± 2.03$^{bc}$ | 3.15 ± 0.05$^{a}$ | 16.00 ± 2.16$^{b}$ | 0.26 ± 0.04$^{a}$ | 8.07 ± 0.8$^{ab}$ | 52.56 ± 5.57$^{a}$ | 168.99 ± 13.10$^{a}$ | 26.44 ± 3.91$^{a}$ | 16.19 ± 3.06$^{b}$ |
| E | 15.00 ± 2.03$^{b}$ | 3.40 ± 0.05$^{a}$ | 22.92 ± 2.16$^{a}$ | 0.23 ± 0.04$^{a}$ | 8.10 ± 0.8$^{ab}$ | 47.50 ± 5.57$^{a}$ | 141.13 ± 13.10$^{ab}$ | 24.33 ± 3.91$^{a}$ | 17.15 ± 3.06$^{b}$ |

A – *Brevibacillus brevis*, B – *Pseudomonas fluorescens*, C – *Bacillus sphaericus*, D – *Bacillus amyloliquefaciens*, and E – control (n = 25, mean ± standard error). Different letters (a, b) indicate significant differences between the same columns within each experimental treatment group

**Table 7**

Oxidative stress response of gill and liver tissues in the groups

| Tissues | Treatment | Oxidative stress response | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | CAT (EU mg$^{-1}$) | SOD (EU mg$^{-1}$) | GR (EU mg$^{-1}$) | GST (EU mg$^{-1}$) | G6PD (EU mg$^{-1}$) | GPx (EU mg$^{-1}$) | MDA (nmol protein$^{-1}$) |
| Gill | A | 0.98 ± 0.03$^{b}$ | 0.24 ± 0.03$^{ab}$ | 0.39 ± 0.08$^{ab}$ | 0.22 ± 0.04$^{a}$ | 0.26 ± 0.03$^{a}$ | 0.49 ± 0.02$^{b}$ | 3.30 ± 0.03$^{ab}$ |
| | B | 0.43 ± 0.03$^{b}$ | 0.20 ± 0.03$^{ab}$ | 0.38 ± 0.08$^{ab}$ | 0.19 ± 0.04$^{a}$ | 0.05 ± 0.03$^{b}$ | 0.26 ± 0.02$^{b}$ | 3.79 ± 0.03$^{a}$ |
| | C | 2.44 ± 0.03$^{a}$ | 0.29 ± 0.03$^{a}$ | 0.65 ± 0.08$^{a}$ | 0.30 ± 0.04$^{a}$ | 0.26 ± 0.03$^{a}$ | 1.45 ± 0.02$^{a}$ | 2.33 ± 0.03$^{b}$ |
| | D | 2.40 ± 0.03$^{a}$ | 0.28 ± 0.03$^{a}$ | 0.39 ± 0.08$^{ab}$ | 0.28 ± 0.04$^{a}$ | 0.38 ± 0.03$^{a}$ | 0.61 ± 0.02$^{ab}$ | 2.52 ± 0.03$^{ab}$ |
| | E | 0.55 ± 0.03$^{b}$ | 0.15 ± 0.03$^{b}$ | 0.34 ± 0.08$^{b}$ | 0.22 ± 0.04$^{a}$ | 0.05 ± 0.03$^{b}$ | 0.69 ± 0.02$^{ab}$ | 2.64 ± 0.03$^{ab}$ |
| Liver | A | 1.83 ± 0.02$^{ab}$ | 0.27 ± 0.04$^{bc}$ | 0.51 ± 0.02$^{a}$ | 0.22 ± 0.02$^{ab}$ | 0.17 ± 0.02$^{b}$ | 1.19 ± 0.01$^{ab}$ | 0.57 ± 0.03$^{ab}$ |
| | B | 0.39 ± 0.02$^{b}$ | 0.18 ± 0.04$^{cd}$ | 0.27 ± 0.02$^{a}$ | 0.16 ± 0.02$^{ab}$ | 0.14 ± 0.02$^{b}$ | 0.25 ± 0.01$^{b}$ | 0.76 ± 0.03$^{ab}$ |
| | C | 4.49 ± 0.02$^{a}$ | 0.43 ± 0.04$^{a}$ | 0.54 ± 0.02$^{a}$ | 0.33 ± 0.02$^{a}$ | 0.78 ± 0.02$^{a}$ | 2.03 ± 0.01$^{a}$ | 0.05 ± 0.03$^{b}$ |
| | D | 3.13 ± 0.02$^{ab}$ | 0.34 ± 0.04$^{ab}$ | 0.45 ± 0.02$^{a}$ | 0.24 ± 0.02$^{ab}$ | 0.40 ± 0.02$^{b}$ | 0.88 ± 0.01$^{b}$ | 0.24 ± 0.03$^{b}$ |
| | E | 0.25 ± 0.02$^{b}$ | 0.13 ± 0.04$^{d}$ | 0.18 ± 0.02$^{a}$ | 0.11 ± 0.02$^{b}$ | 0.09 ± 0.02$^{b}$ | 0.26 ± 0.01$^{b}$ | 1.16 ± 0.03$^{a}$ |

A – *Brevibacillus brevis*, B – *Pseudomonas fluorescens*, C – *Bacillus sphaericus*, D – *Bacillus amyloliquefaciens*, and E – control (n = 25, mean ± standard error). Different letters (a, b) indicate significant differences between the same columns within each experimental treatment group

hematological indices may increase the oxygen-carrying capacity of the blood when using applications of bacterial origin. The increase in Hb and RBC values in the blood by various types of probiotics may be caused by the presence of vitamin B complex (which plays an important role in the production of blood cells) and other hematinic substances (Jahan et al. 2021). It is believed that catecholamine-induced spleen contractions may be effective in sudden increases in the number of erythrocytes due to stress and the release of new erythrocytes into the bloodstream. Similarly, an increase in the number of erythrocytes may cause deformations in the gill structure and an increase in the oxygen demand of tissues, either as a result of hypoxic conditions originating from the gill covered with mucus or by the stimulating effect of bacteria on erythrocyte formation. The concentration of pollutants in hematopoietic tissues and exposure time are known to cause changes in the erythrocyte cells of fish (Atamanalp et al. 2021). An increase or decrease in the ESR value depending on the number of erythrocytes indicates physiological dysfunction in fish (Jagtap and Mali 2012). Atamanalp et al. (2021) and Uçar et al. (2021) stated that hematological parameters show different sensitivity levels to different environmental factors/chemicals. In conjunction with the present study, several studies have reported that dietary probiotics may play a role as an immunostimulating feed additive in fish (Jahan et al. 2021). According to the results obtained in this study, there are changes in leukocyte values resulting from bacterial applications. The number of leukocyte cells is affected by physiological and environmental factors. The change in WBC and differential leukocyte counts observed in fish exposed to a pollutant indicates immunomodulation by pollutants. It is known that xenobiotics have an immunosuppressive effect in aquatic organisms (Heyedati and Tarkani 2014). In fish, the immunological function and WBCs increase as a protective response to stress. Similarly, it is known that WBC levels increase in gill injuries (Atamanalp et al. 2021). The results of our research showed that high levels of WBC triggered hematopoietic stimulation. In the present study, it was observed that the increase in MCV resulted from macrocytosis due to swelling of erythrocytes, indicating anemia. The increase in MCV is also due to swelling of RBCs as a result of a hypoxic increase or impaired water balance (osmotic stress) or macrocytic anemia in stressed fish. This situation increases the affinity for oxygen in the blood (Harikrishnan et al. 2009). This study showed that the bacterial application caused the red cells to shrink (increased MCHC), and MCH increased significantly. It had been reported that high MCHC and MCH values indicate the presence of large-sized RBCs with lower hemoglobin content (Ucar et al. 2019; Alak et al. 2020b; Ucar et al. 2021). In our study, we believe that the anemic condition is effective in increasing MCV and MCH levels in fish exposed to different bacterial strains. Similarly, high MCV levels were reported in some studies on rainbow trout as a result of the macrocytic type of anemia. The stress response in fish effectively disrupts the osmotic balance and regulatory systems of ion metabolism, causing a decrease in blood pH, an increase in erythrocyte volume and a consequent increase in the percentage of hematocrit (Saravanan et al. 2011). The obtained hematocrit results are higher than the control group and support the above. Compared to the control group, the platelet count increased in group B applications. Under stress conditions, the fish blood coagulation system becomes more active and therefore the platelet count can increase significantly. The best known physiological role of platelets is to initiate blood coagulation in the process of hemostasis (Engelmann and Massberg 2013). In fish, platelet cells form protective walls, have phagocytic properties and participate in the defense mechanism. These cells represent the link between innate immunity and expressing intracellular/extracellular molecules, including immune functions (Uçar et al. 2019; 2021). Under stress conditions, the blood coagulation system becomes more active and can therefore cause an increase in platelet count. Thrombocytopenia may have an adverse effect on fish, because these cells are not only responsible for blood coagulation, but also play a role in the control of superficial wounds and blood flow. Most of the enzyme responses, which are physiological and biochemical markers, are secondary stress responses and occur with cellular damage. The antioxidant defense systems can be classified as enzymatic (glutathione peroxidase, catalase, superoxide dismutase, glutathione reductase) and non-enzymatic defense systems (vitamin E, vitamin A, vitamin C, glutathione) (Atamanalp et al. 2021). In general, SOD, CAT, GSH-Px, and MDA are considered important indicators for testing the antioxidant capacity of aquatic animals. In this study, we can conclude that the acute bacterial application effectively removes excess free radicals and then increases the antioxidant capacity of fish (Kong et al. 2021). According to our results, in addition to the increased SOD, CAT, GPx, GST, GR, and G6PD activity in the groups containing $1.0 \times 10^7$ CFU/ml A, C, and D bacteria, MDA was also at a very low level compared to the control and B groups in liver. These results are consistent with those obtained by Kong et al. (2021) and Chen et al. (2021).

## 5. Conclusion

The data obtained in this study for the four bacterial species showed that they support the antioxidant defense system, have positive effects on hematological indices, and positively affect the swimming performance of rainbow trout, except for group B. The best results were obtained from *B. sphaericus* FD-48 and *B. amyloliquefaciens* TV-17C strains in groups C and D., respectively. These results are also supported by water quality parameters. Despite a considerable number of studies on the use of probiotics, the molecular mechanisms are still unresolved. In addition, the immune effect of each probiotic strain is different. Therefore, it is necessary to reveal the interaction mechanisms by examining each strain specifically. To be able to make more effective interpretations, data on adherence and survival in the host should be available for the bacterial species used in this study. In future studies, it is necessary to investigate the growth performance and immunological data of bacteria in question as potential probiotics by using feeding regimes. It is believed, however, that it would be useful to investigate the said bacterial strains in different aquatic organisms in terms of hematological indices. Further studies are needed to demonstrate a positive relationship between individual supplementation of probiotics, organic acids, or salts on hematological parameters. Three different potential probiotic candidates *B. brevis* FD-1 (A), *B. sphaericus* FD-48 (C) and *B. amyloliquefaciens* TV-17C strains (D) show a significant free radical scavenging potential in liver and gill tissues, which can be attributed to their ability to regulate oxidative damage.

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