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Effects of Dietary Supplementation with Probiotics and Prebiotics on Growth, Physiological Condition, and Resistance to Pathogens Challenge in Nile Tilapia (*Oreochromis niloticus*)

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Abstract: This study aimed to investigate the effects of the probiotic *Betaplus*® and prebiotic *Technomos*® as feed additives for Nile tilapia in terms of growth performance, health profiles, and resistance to infection with *Aeromonas hydrophila* and *Pseudomonas fluorescens*. A total of 960 healthy juvenile Nile tilapia (1.5 ± 0.01 g) were randomly divided into four experimental variants with three replicates for each variant. Fish were fed a commercial diet (control group, V_0), supplemented with *BetaPlus*® probiotics–1% × BW (V_1), *TechnoMos*® prebiotics–1% × BW (V_2), and with *BetaPlus*® probiotics and *TechnoMos*® prebiotics in a ratio of 1:1% × BW (V_3). Results on growth performance showed the best values in the probiotic variant, correlated with the health profile and the relative survival percentage after the challenge test with *A. hydrophila* and *P. fluorescens*. Similarly, the effects of dietary supplementation with probiotics and prebiotics on physiological conditions also recorded beneficial results in the synbiotic variant, where a high survival percentage was obtained after infection with the two pathogenic bacteria. In conclusion, the results of this study indicate that dietary supplementation with feed additives consisting of mentioned probiotics, prebiotic, and their combination as synbiotics has the potential to promote growth performance, improving tilapia immunity and increasing survival after the challenge test.

Keywords: biochemistry; growth; hematology; infection; prebiotic; probiotic

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1. Introduction

The Nile tilapia ranked second in the world in terms of importance as an aquatic animal due to its high demand, rapid growth, and reasonable price [1]. Lately, the main concern in aquaculture is the maintenance of the health and welfare of fish, which can be greatly influenced by administrated feed and environmental conditions. In recent years, disease prevention substantially increased, and special attention focused on the use of feed additives to the detriment of chemical additives and veterinary medicines. Probiotics, prebiotics, and phytogenic compounds are commonly used feed additives [2–6]. Probiotics are defined as "live microorganisms that, when administered in adequate amounts confer a health benefit on the host" [7,8].

Probiotics have been shown (living, dead, or cell components) to influence the host by stimulating the growth of one or more healthy bacteria, improving survival by reducing pathogenic bacteria and modifying gut microflora [6,9,10]. Some of the positive effects of using probiotics include increasing immune responses, competition adhesion to the substrate of nutrients, and the production of antibacterial substances, which together protect against diseases.

Prebiotics are usually substances of non-microbial origin that are not digested or absorbed in the gut but create favorable conditions for growth and healthy intestinal microbiota [11]. Mannan oligosaccharides (MOSs) are prebiotics that are not digested by mammalian and fish enzymes but are digested by microbial enzymes [12]. Probiotics contribute both by maximizing feed utilization efficiency (i.e., reducing FCR) and by reducing infestation rates with various pathogens by activating immune cells. Prebiotics can help by influencing the modulation of beneficial gut bacteria and stimulating the secretion of essential digestive enzymes, thus making nutrients more readily available to fish. At the same time, synbiotics improve survival rates and alter the microbial composition in the gastrointestinal tract, thus contributing to improved productions in aquaculture more effectively than the application of probiotics or prebiotics alone [13].

Synbiotic, suppose the simultaneous use of prebiotics and probiotics, which beneficially affects the host by improving the survival and activity of beneficial microorganisms in the gut. During the administration of synbiotics, the main feature of prebiotics is the selective stimulation of the growth of probiotic bacteria. Thus, the high number of probiotic bacteria colonizes mucous membranes and prevents the adhesion of pathogens by competing on substrates and places of penetration [14].

Outbreaks caused by the pathogen *Aeromonas hydrophila* show high mortality rates in fish farming due to the occurrence of septicemia with mobile *Aeromonas* in two ways: the induction of internal bleeding and general bacteremia in the acute form as well as the appearance of skin ulcers and the underlying necrosis of muscles in the chronic form of the disease [15]. Some authors, such as [16,17], reported a better immune response to fish feed with the probiotic bacterium *Lactobacillus plantarum* AH 78 and prebiotic immunogen (*β-glucan* and *MOS*), especially after being infected with *Aeromonas hydrophila*.

Several studies have proved the role of probiotics in the control of pathogenic *Pseudomonas* species. Some data available evidence that *Bacillus species* can be considered as potential probiotics to fight *Pseudomonas* infections. Thus, in an experiment by [18], dead cells of *Bacillus sp.* and *Bacillus amyloliquefaciens* effectively inhibited the growth of the pathogen *Pseudomonas fluorescens*. Similarly, synthesized bacteriocins of *Bacillus subtilis LR1* showed inhibitory activities against *Pseudomonas fluorescens* [19].

Various commercial products, which contain several mixtures of probiotic bacteria, have been applied in aquaculture feed diets. Among these products, the most used was *BioPlus 2B* (Novus, St. Charles, MO, USA), which is a mixture of *Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749, isolated from soybean puree and earth [20], but they have lately been evaluated in fish farming. For example, *BioPlus 2B* was tested on rainbow trout and Nile tilapia, showing benefits in growth performance, immune response, and resistance against pathogenic bacteria. According to [21], the authors showed that *BioPlus 2B* improved the immune response and survival ratio in Nile tilapia fry after a challenge test with *Yersinia ruckeri*. Similarly, [22–24] demonstrated that *BioPlus 2B* contributes to the growth of rainbow trout fry and larvae. Other authors [25] combined *BioPlus 2B* with *Enterococcus faecium* (Lactosan GmbH & Co. KG, Kapfengerg, Austria) and showed an increase in the growth and survival of rainbow trout juveniles. However, [26] showed that *BioPlus 2B* did not significantly improve the benefits in adult Nile tilapia on growth, survival, and immune response after the *Streptococcus iniae* challenge test.

To our knowledge, until now, the evaluation of the dietary supplementation with *BetaPlus*® probiotic and *TechnoMos*® prebiotic against infection with *Aeromonas hydrophila* and *Pseudomonas fluorescens* has not been evaluated for juveniles of Nile tilapia. Therefore, this study aimed to evaluate the effect of commercial probiotics *BetaPlus*® (1% BW with 1×10^{12} CFU/kg *Bacillus subtilis*, 1×10^{12} CFU/kg *Bacillus licheniformis*) and *TechnoMos*® prebiotic (1% BW with *Saccharomyces cerevisiae*, *MOS* and *β-1,3-glucans*), combined as synbiotics or not, in promoting growth, physiological conditions, and protection against infection with *Aeromonas hydrophila* and *Pseudomonas fluorescens* of Nile tilapia juveniles.

2. Materials and Methods

2.1. Experimental Conditions and Fish

The study was organized in two stages: Stage I—40 days of administrating probiotics, prebiotics, and synbiotics in feed; stage II—50 days with the same feeding conditions in order to evaluate the effects on growth, health condition, and the challenge test.

Fish (Nile tilapia) were obtained after the reproduction of the mature tilapia at the Department of Food Science, Food Engineering, Biotechnology, and Aquaculture. For the first experimental stage, 960 juvenile tilapias (240 fish/variant, age 3 months, individual weight of 1.52 ± 0.01 g) were used, while for the second experimental stage, 360 juvenile tilapia (90 fish/variant, age five months, individual weight of 73.96 ± 0.45 g) were used.

For carrying out the experimental activity, two recirculating systems belonging to the Department of Food Science, Food Engineering, Biotechnology, and Aquaculture, University "Dunărea de Jos" of Galati, Romania, and the Institute for Research and Development in Aquatic Ecology, Fishing, and Aquaculture from Galati, Romania, were used.

For the first stage, the recirculation aquaculture system has 12 rearing units (10 mm thick glass) with a volume of 0.132 m^3 each (36 × 37.5 × 98 cm). The RAS is equipped with a pressurized sand filter to remove residual solids and a biological filtration unit—trickling filter to control the concentration of nitrogen compounds. Sterilization and disinfection of the water on the principal supply circuit have been provided using the Tetra Quiet UV-C 35,000 (Tetra GmbH, Melle, Germany). Recirculation of the water has been ensured using three pump types, DAB A 80 180 XM (Dab Pomps, Mestrino, Italy). The dissolved oxygen requirement has been assured by a compressor, type Fiap Air Active 10,000 (FIAP GmbH, Jakob–Oswald, Germany). The system was previously described by [27]. The second recirculating aquaculture system was used to perform the challenge test. The system has four glass units, with a volume of 130 L/unit (40 × 50 × 100 cm). Water recirculation and filtration were assured by the Tetratec EX 400 (Tetra GmbH, Melle, Germany) filters. Water oxygenation was assured with an aeration-oxygenation unit, consisting of two *Hagen* compressors with a flow rate of 1.5 m^3/h.

Temperature and dissolved oxygen concentrations were measured with the *Hanna HI* 98,186 (HANNA Instruments, Cluj-Napoca, Romania), and the pH was measured with the pH meter *WTW*, 340 (Sigma-Aldrich, Darmstadt, Germany). The nitrogen compounds ($N\text{-}NH_4^+$, $N\text{-}NO_2^-$, and $N\text{-}NO_3^-$) concentrations were measured using the *Spectroquant NOVA 400* portable spectrophotometer (Merck general laboratory equipment, Enschede, Netherlands), using compatible kits from Merck (Merck general laboratory equipment, Enschede, Netherlands). The water's temperature was maintained at 27.82 ± 0.48 °C in the first experimental stage and 28.34 ± 0.44 °C in the second stage, with a pH of 7.77 ± 0.12 and 7.83 ± 0.15, respectively. The dissolved oxygen concentration was 7.28 ± 0.48 mg/L in the first experimental stage and 6.59 ± 0.38 mg/L in the second stage. The levels of ammonium ion, nitrates, and nitrites were 0.03 ± 0.01 mg/L, 20 ± 1.15 mg/L, and 0.06 ± 0.02 during the experimental period, respectively.

2.2. Preparation of Experimental Feed

During the first experimental stage, fish were fed with commercial extruded feed *ALLER FUTURA EX* (Aller Aqua Group, Christiansfeld, Denmark), with a protein content of 64% and 12% lipids. The daily ratio (8% of body weight BW) was divided into three equal meals per day at 8.00, 13.00, and 19.00.

In the second experimental stage (50 days), juvenile tilapia were fed with *ALLER SILVER* (Aller Aqua Group, Christiansfeld, Denmark), with a content of 45% crude protein and 20% lipids. The feeding intensity was 2% BW/day with a feeding frequency of twice/day.

The additives used are the *BetaPlus*® probiotic and *TechnoMos*® prebiotic. The probiotic *BetaPlus*® consists of *BioPlus*® *2B* and betaine (nitrogenous substance), the concentration being 1 × 10^{12} CFU/kg feed and betaine at 936,000 mg/kg. *BioPlus*® *2B* is a 1:1 mixture of *Bacillus licheniformis* (DSM 5749) and *Bacillus subtilis* (DSM 5750). The *TechnoMos*®

prebiotic is an extract from selected yeast, obtained from *Saccharomyces cerevisiae*, rich in mannan oligosaccharides and beta-glucans (β-1,3-glucans). These products were supplied by the company Biochem from Lohne, Germany, through the Romanian subsidiary Biochem Animal Health and Nutrition affiliated with the one from Lohne and located in Cluj-Napoca, Romania. The additives were used according to the manufacturer's recommendations.

The standard commercial pellets were mixed with the feed additives mentioned before by using gelatin as a binding material. The preparation of these experimental diets followed the same process: (a) dissolving the probiotic and prebiotic in distilled water; (b) stirring the solution for 10 minutes; (c) preparing a 2% gelatin solution in a water bath; (d) cooling the gelatine solution to 30 °C; (e) mixing probiotic, prebiotic, and gelatin solutions at a ratio of 2:1; (f) spraying the final solution on the surface of the feed granules by continuous stirring; (g) drying in an oven at T^0 = 20 °C, for 12 h. Feeding was performed every day and stored in polyethylene bags at 4 °C until use. The feed added with probiotics was adjusted to the daily needs of the fish. Four experimental variants were established (in triplicate) as follows:

I. Control variant (V_0)—commercial feed, without probiotics and prebiotics;
II. Probiotic variant (V_1)—commercial feed supplemented with *BetaPlus*® probiotics–1% × BW;
III. Prebiotic variant (V_2)—commercial feed supplemented with *TechnoMos*® prebiotics–1% × BW;
IV. Synbiotic variant (V_3)—commercial feed supplemented with *BetaPlus*® probiotics and *TechnoMos*® prebiotics–1:1% × BW.

2.3. Growth Measurements

All fish were measured and weighed at the beginning and end of each experimental stage. Growth performance and feed utilization parameters were calculated as follows:

Specific growth rate (SGR, % day^{-1}) = 100 × (lnWt − lnW0)/t (% BW/day),
where Wt denotes the final body weight (g), and W0 denotes the initial body weight (g).

Feed conversion ratio (FCR, g feed g $gain^{-1}$) = Dry feed consumed (g)/wet weight gain (g)

Protein efficiency ratio (PER, g gain g $protein^{-1}$) = Wet weight gain (g)/protein intake (g)

Survival (%) = 100 × [Final fish number/initial fish number]

2.4. Blood Samples, Hematological, Biochemical Parameters, and Oxidative Stress

2.4.1. Blood Sampling

Blood sampling has been carried out at the end of the two experimental stages and after the challenge test. Therefore, 15 fish were randomly sampled from each experimental variant to evaluate the hematological profile. To minimize the handling stress, the fish were anesthetized with 2-phenoxyethanol (0.7 mL/L) until deep anesthesia [28]. For the hematological analysis, blood was collected by caudal venous punctures using heparin as an anticoagulant, while fish blood was collected without an anticoagulant for the biochemical parameters.

2.4.2. Hematological, Biochemical Parameters, and Oxidative Stress

Blood analysis was performed by the method used in fish hematology. This analysis consisted of the determination of red blood cell count, RBCc ($\times 10^6$ cells/μL); hemoglobin, Hb (g/dL); and hematocrit, PVC (%). For the determination of erythrocyte numbers, we used the Neubauer hemocytometer, Potain pipette, and Vulpian diluting solution (prepared in the laboratory from sodium citrate, potassium iodide, and metallic iodine (Sigma-Aldrich, St. Louis, MO, USA)). The hematocrit (PVC %) was performed in duplicate using capillary tubes and centrifugated for 5 minutes at 12,000 rpm (13.709× *g*) in a Haematokrit 210 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). Hemoglobin

concentrations (Hb, g/dL) were measured spectrophotometrically with SPECORD 210 Analytikjena (Analytic Jena, Jena, Germany) at λ-540 nm using Drabkin reagent.

Using the standard formulas described by [29], we calculate the hematological indices: mean corpuscular volume MCV (fL), mean corpuscular hemoglobin MCH (pg), and mean corpuscular hemoglobin concentration MCHC (g/dL).

The relative and absolute numbers of leukocytes were determined by a microscopic examination of 200 leukocytes on blood smears (two per fish), using a *Zeizz Axio Imager* microscope (Zeiss International, Thornwood, NY, USA) with the immersion objective (10 oc. × 100 ob.). The absolute number of circulating blood leukocytes and platelets was determined in comparison with 1000 erythrocytes counted on a hemocytometer per blood volume unit. Blood smears were colored with the *May-Grünwald Giemsa* panoptic method (MGG), and the type of leukocytes was determined based on identification characters listed by [30].

Biochemical blood analyses consisted of the determination of glucose (mg/dL), total proteins (g/dL), and lysozyme activity (Units/mL). Due to the small weight of the fish from the first stage, these determinations were made only at the end of stage II and after the challenge test. For the determination of the glucose concentration (GLU mg/dL) and total protein (TP g/dL), we used the VetTest® Chemistry Analyzer and IDEXX VetTest kits (IDEXX Laboratories, Inc., Westbrook, ME, USA). Lysozyme activities were measured, from serum, based on a turbiometric assay, Enzymatic Activity of Lysozyme Protocol (Sigma, EC 3.2.1.17) (Sigma, EC 3.2.1.17, Sigma-Aldrich, St. Louis, MO, USA). For this test, *Micrococcus lysodeikticus* (Sigma, M3770, Sigma-Aldrich, St. Louis, MO, USA) was used as a substrate, Potassium Phosphate (with 6.24 pH at 25 °C) as a buffer, and white lysozyme was used as an enzyme-lyophilised powder of chicken egg (Sigma, L6876, Sigma-Aldrich, St. Louis, MO, USA). One unit of lysozyme activity was defined as a reduction in the absorbance of 0.001/min at a 450 nm wavelength, using an ELISA microplate reader (Tecan Sunrise, Tecan GmbH, Grödig, Austria).

To quantify the lipid peroxidation (malondialdehyde-MDA nmol/mL) from the liver, kidneys, and muscle tissue, we used the method mentioned in [31], and the absorbance of the samples was read at an optical density of 532 nm.

2.5. Challenge Tests

At the end of the trial, 36 fishes from each treatment were randomly captured and subjected to bacterial challenge in order to create four experimental variants, in triplicate, as follows:

I. Positive control (C^+)—fish were injected with 0.5 ml of 0.85% saline solution;
II. *Aeromonas hydrophila* (Ah)—fish were injected with 0.5 ml solution of *Aeromonas hydrophila* with a concentration of 1.3×10^9 CFU/mL;
III. *Pseudomonas fluorescens* (Pf)—fish were injected with a 0.5 mL solution of *Pseudomonas fluorescens* with a concentration of 1.5×10^9 CFU/mL;
IV. Negative control (C^-)—fish were not injected.

Fish were injected intraperitoneally with the two bacterial strains of *Aeromonas hydrophila* and *Pseudomonas fluorescens*. These pathogenic bacteria were provided by the National Reference Laboratory for Fish Diseases within the Institute for Diagnosis and Animal Health, Bucharest, România.

The two strains of bacteria are as follows: (1) *Aeromonas hydrophila* with *RO AH ID, 10758/2009* which has cultural, morphological, and biochemical characteristics similar to the certified reference strain LMG 28,844 (this strain is being suspended in 0.85% saline solution with a concentration of 1.3×10^9 CFU/mL); (2) *Pseudomonas fluorescens* with *RO PF ID 14104/2008*, which has cultural, morphological, and biochemical characteristics similar to the VLA1219 certified reference strain (this strain is suspended in 0.85% saline solution with a concentration of 1.5×10^9 CFU/mL). These pathogenic strains were isolated from infected fish, diagnosed in the laboratory, and had a high virulence. Before infection, fish were anesthetized by bath for 2–5 min with 2-phenoxyethanol.

The challenge test lasted for 21-days. During this test, fish were starved and behavioral changes were observed. During that period, mortality and disease symptoms were observed closely in each group.

The cumulative mortality (%) and relative percent survival (RPS, %) were calculated according to [32].

Cumulative mortality (%) = (total mortality in each treatment after challenge/total number of fish challenged for same treatment) × 100;

Relative percent survival (RPS, %) = [1 − percent mortality in treatment/percent mortality in control group)] × 100.

2.6. Statistical Analysis

Statistical analyses were performed using SPSS software 21 for Windows (SPSS Inc., Chicago, IL, USA). Results regarding fish growth performance and hematological and biochemical parameters were expressed by means and standard error (Means ± SE) of the triplicates. One-way ANOVA and Duncan's multiple range tests were used to compare differences between all experimental groups. The level of significance was set at $p < 0.05$ for all analyses.

3. Results

3.1. Fish Growth Performance

In the first experimental stage, no significant differences ($p > 0.05$) were recorded between the FCR, SGR, and PER values. In the second stage, a *Duncan test* showed significant differences ($p < 0.05$) in FCR and PER values between V_1 and V_2 variants, indicating improved growths in the probiotic variant. (Table 1). In the first experimental stage, there were no significant differences ($p > 0.05$) between the FCR, SGR, and PER values between the treatments and control. In the second stage, the statistical analysis of the technological parameters (FCR and PER) showed a lower feed efficiency and protein conversion in the prebiotic variant compared to the control (Table 1).

Table 1. The main indicators of tilapia growth performance in the two experimental stages.

Experimental Variant	Experimental Stage I			Experimental Stage II		
	SGR	FCR	PER	SGR	FCR	PER
Control (V_0)	6.38 ± 0.01	0.92 ± 0.01	1.71 ± 0.01	1.99 ± 0.02	1.01 ± 0.02 ^{a}	2.21 ± 0.04 ^{a}
Probiotic (V_1)	6.72 ± 0.06	0.90 ± 0.03	1.73 ± 0.05	1.94 ± 0.04	0.97 ± 0.03 ^{a}	2.29 ± 0.07 ^{a}
Prebiotic (V_2)	6.74 ± 0.03	0.91 ± 0.01	1.71 ± 0.02	1.89 ± 0.03	1.12 ± 0.03 ^{b}	1.98 ± 0.06 ^{b}
Synbiotic (V_3)	6.85 ± 0.03	0.88 ± 0.01	1.77 ± 0.03	1.98 ± 0.01	1.00 ± 0.01 ^{a}	2.21 ± 0.02 ^{a}

SGR—specific growth rate; FCR—food conversion ratio; PER—protein efficiency ratio. Results are presented as triplicate means ± standard error. Values with a different superscript in a row differ significantly (ANOVA, $p < 0.05$).

3.2. Hematological and Biochemical Parameters

The results of the hematological analysis are summarised in Table 2. At the end of the first experimental stage, RBCc showed significant differences ($p < 0.05$), with values of the control variant (V_0) being higher than the other three variants (V_1, V_2, and V_3).

The hematocrit (PVC) at the end of the second experimental stage showed significant differences ($p < 0.05$). Thus, the Duncan test divided the values obtained into two groups, with values obtained for the control variant and V_1 being significantly lower ($p < 0.05$) than the V_2 and V_3 variants. At the end of the first experimental stage, hemoglobin concentrations registered significantly ($p < 0.05$) higher values in variant V_2. MCV showed significant differences ($p < 0.05$), with the values of the control variant being significantly lower than the other three variants both in the first and second experimental stages. MCH did not show significant differences ($p > 0.05$) between the four experimental variants, both at the end of the first stage and at the second stage. In the second stage, MCHC showed a significantly ($p < 0.05$) higher increase in the control variant.

Table 2. Hematological and biochemical parameters of tilapia in two experimental stages.

Hematological Parameters	Experimental Stage (I, II)	Control (V_0)	Probiotic (V_1)	Prebiotic (V_2)	Synbiotic (V_3)
RBCc ($\times 10^6$ cells/μL)	I	1.74 ± 0.05 ^{b}	1.59 ± 0.06 ^{a}	1.57 ± 0.03 ^{a}	1.50 ± 0.04 ^{a}
	II	1.82 ± 0.09	1.65 ± 0.09	1.69 ± 0.07	1.75 ± 0.05
PVC (%)	I	25.20 ± 1.11	25.80 ± 0.80	26.00 ± 0.74	27.07 ± 0.67
	II	22.00 ± 1.50 ^{a}	25.07 ± 1.09 ^{a}	27.27 ± 1.38 ^{b}	27.27 ± 1.22 ^{b}
Hb (g/dL)	I	7.88 ± 0.26 ^{a}	7.22 ± 0.24 ^{a}	8.05 ± 0.22 ^{b}	7.58 ± 0.19 ^{a}
	II	7.78 ± 0.14	7.68 ± 0.12	7.70 ± 0.22	8.00 ± 0.13
MCV (μm^3)	I	146.08 ± 6.57 ^{a}	165.12 ± 6.46 ^{b}	166.62 ± 6.36 ^{b}	183.04 ± 7.21 ^{b}
	II	123.89 ± 8.63 ^{a}	159.70 ± 12.05 ^{b}	168.83 ± 15.02 ^{b}	156.41 ± 6.44 ^{b}
MCH (pg)	I	46.35 ± 2.61	46.31 ± 2.00	51.35 ± 1.37	51.18 ± 1.85
	II	44.11 ± 1.90	48.88 ± 2.97	47.17 ± 3.00	46.33 ± 1.53
MCHC (g/dL)	I	32.01 ± 1.53	28.20 ± 0.92	31.43 ± 1.41	28.27 ± 1.04
	II	37.28 ± 2.12 ^{b}	31.40 ± 1.39 ^{a}	29.55 ± 1.92 ^{a}	30.34 ± 1.70 ^{a}
Glucose (mg/dL)	II	103.17 ± 1.46 ^{a}	111.93 ± 1.25 ^{b}	104.29 ± 1.95 ^{a}	102.83 ± 1.24 ^{a}
Total proteins (g/dL)	II	6.73 ± 0.23 ^{b}	6.04 ± 0.18 ^{a}	6.41 ± 0.28 ^{a}	5.76 ± 0.16 ^{a}
Lysozyme (U/mL)	II	11.79 ± 0.29 ^{a}	13.09 ± 0.3 ^{b}	12.29 ± 0.3 ^{a}	13.6 ± 0.52 ^{b}

RBCc—red blood cells count; PVC—hematocrit; Hb—hemoglobin; MCV—mean corpuscular volume; MCH—mean corpuscular hemoglobin; MCHC—mean corpuscular hemoglobin concentration. Results are presented as triplicate means ± standard error. Values with different superscript in a row differ significantly (ANOVA, $p < 0.05$).

Regarding glucose levels, the highest values were recorded in the fish fed with probiotics, while the total serum protein concentration registered a higher decrease in variants V_1, V_2, and V_3. Lysozyme activities were the highest in the fish fed with probiotics and synbiotics (Table 2).

Regarding the results obtained for malondialdehyde (MDA) from tissue, kidney, and liver, no significant differences were recorded between all experimental variants ($p > 0.05$) (Figure 1).

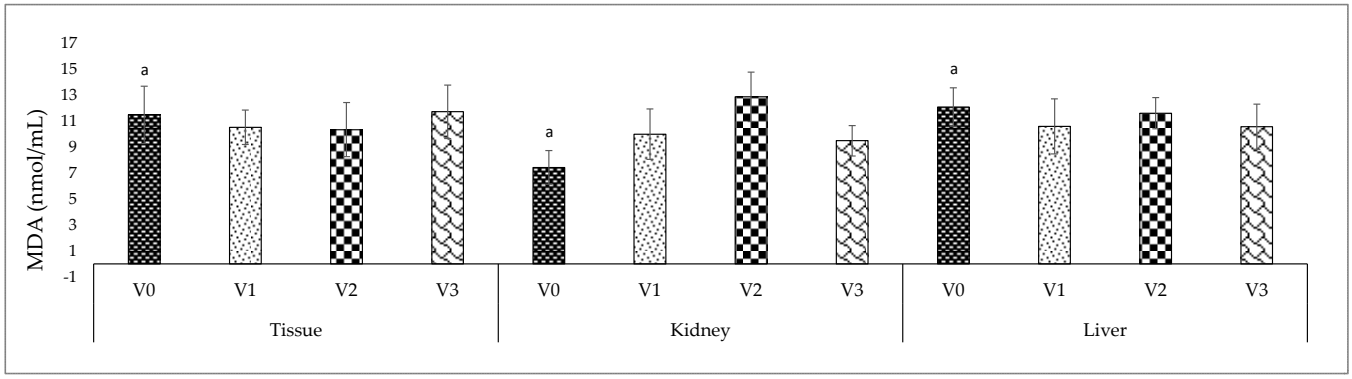

Figure 1. Variations of malondialdehyde in the tissue, kidney, and liver of the tilapia. Results are shown as mean values and standard errors. V_0—control variant; V_1—probiotic variant; V_2—prebiotic variant; V_3—synbiotic variant. The letter "a" means that there were no significant differences ($p > 0.05$).

At the end of the first experimental stage, the absolute number of leukocytes and small lymphocytes showed a significant decrease ($p < 0.05$) in variants V_2 and V_3, while in the second stage, the ANOVA test did not show significant differences ($p > 0.05$) between the experimental variants. Concerning the absolute number of large lymphocytes in the second

stage, a significant increase ($p < 0.05$) was observed in variants V_1, V_2, and V_3 compared to the control group. The absolute number of monocytes, neutrophil granulocytes, and platelets showed no significant ($p < 0.05$) differences both in the first and second stages. The results of the absolute number of leukocytes and platelet are summarized in Table 3.

Table 3. Variation of the absolute number of leukocytes and platelets in Nile tilapia in the two experimental stages.

Agranulocytes Granulocytes	Experimental Stage (I, II)	Control	Probiotic	Prebiotic	Synbiotic
		(V_0)	**(V_1)**	**(V_2)**	**(V_3)**
Leukocytes ($\times$1000 cell./mm^3)	I	65.25 ± 3.32 ^{b}	58.04 ± 6.72 ^{b}	46.25 ± 2.22 ^{a}	48.99 ± 3.67 ^{a}
	II	52.65 ± 3.94	51.64 ± 6.25	60.76 ± 6.36	56.31 ± 3.75
Lymphocytes small ($\times$1000 cell./mm^3)	I	65.58 ± 3.25 ^{b}	55.49 ± 6.52 ^{b}	43.70 ± 2.24 ^{a}	46.72 ± 3.50 ^{a}
	II	48.22 ± 3.77	47.05 ± 5.88	55.37 ± 4.03	51.00 ± 3.35
Lymphocytes large ($\times$1000 cell./mm^3)	I	1.11 ± 0.14	1.09 ± 0.22	0.94 ± 0.12	0.83 ± 0.08
	II	0.61 ± 0.08 ^{a}	1.12 ± 0.19 ^{b}	1.10 ± 0.17 ^{b}	1.14 ± 0.09 ^{b}
Monocytes ($\times$1000 cell./mm^3)	I	0.54 ± 0.11	0.55 ± 0.08	0.50 ± 0.06	0.36 ± 0.05
	II	0.65 ± 0.09	0.64 ± 0.11	0.53 ± 0.07	0.81 ± 0.17
Neutrophilic granulocytes ($\times$1000 cell./mm^3)	I	1.01 ± 0.19	0.89 ± 0.19	1.18 ± 0.11	1.09 ± 0.13
	II	3.16 ± 0.70	2.92 ± 0.38	2.63 ± 0.46	3.21 ± 0.58
Platelets ($\times$1000 cell./mm^3)	I	23.10 ± 4.01	20.72 ± 2.34	21.52 ± 2.06	15.84 ± 2.20
	II	32.82 ± 3.06	27.84 ± 3.15	25.57 ± 4.38	29.79 ± 3.79

Results are presented as triplicate means ± standard error. Values with different superscript in a row differ significantly (ANOVA, $p < 0.05$).

3.3. Challenge Tests

After 21 days, no mortalities were recorded in the positive (C^+) and negative control (C^-). In the groups injected with *A. hydrophila*, mortality started on day 2 (at fish provided from V_1 and V_2 variants) and reached maximum cumulative mortality (14.81%) on day 3 at fish from the control variant (V_0). Beginning with day 9, there were no mortalities registered in each treatment (Figure 2). Regarding the groups injected with *P. fluorescens*, the first mortalities started on the 16th day (in variants V_0 and V_2), reaching maximum cumulative mortality on the 17th day in the V_0 group (11.1%) (Figure 3).

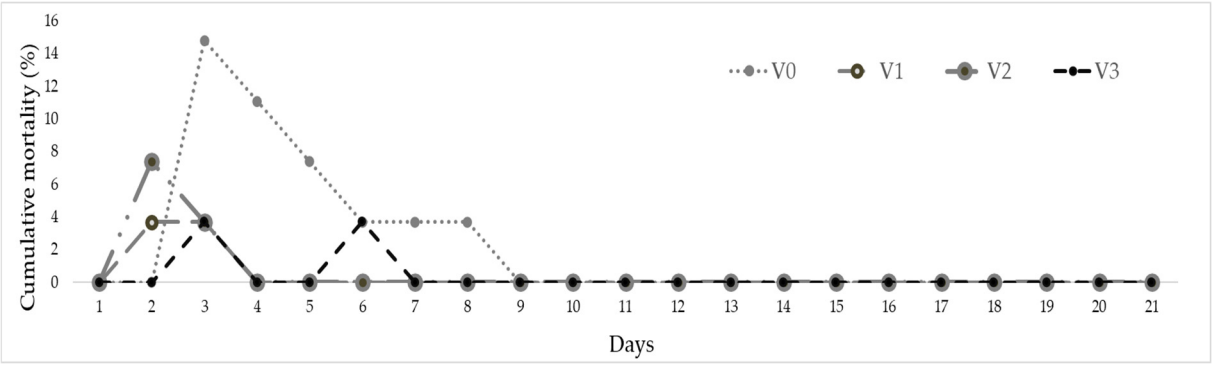

Figure 2. Daily cumulative mortality (%) of *O. niloticus* during the post-challenge test with *A. hydrophila*.

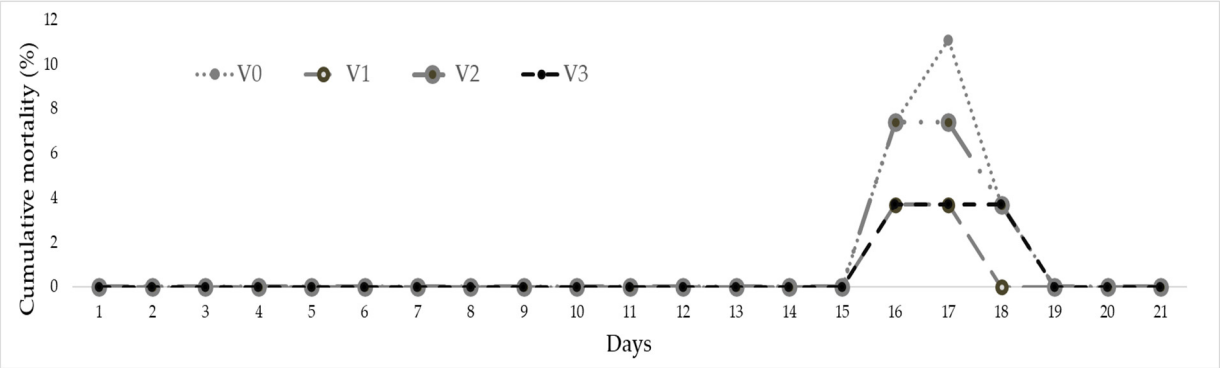

Figure 3. Daily cumulative mortality (%) of *O. niloticus* during the post-challenge test with *P. fluorescens*.

At the end of the challenge test, the cumulative mortality was higher in the control group, both in fish injected with *A. hydrophila* and *P. fluorescens*, followed by the variant V_2. The relative percent survival (RPS) was similar in the case of V_1 (81.33%) and V_3 (81.33%), followed by the V_2 variant (75%) for fish injected with *A. hydrophila*. For *P. fluorescens*, the RPS was the highest in V_1 (66.66%) and V_3 (50%) (Figure 4).

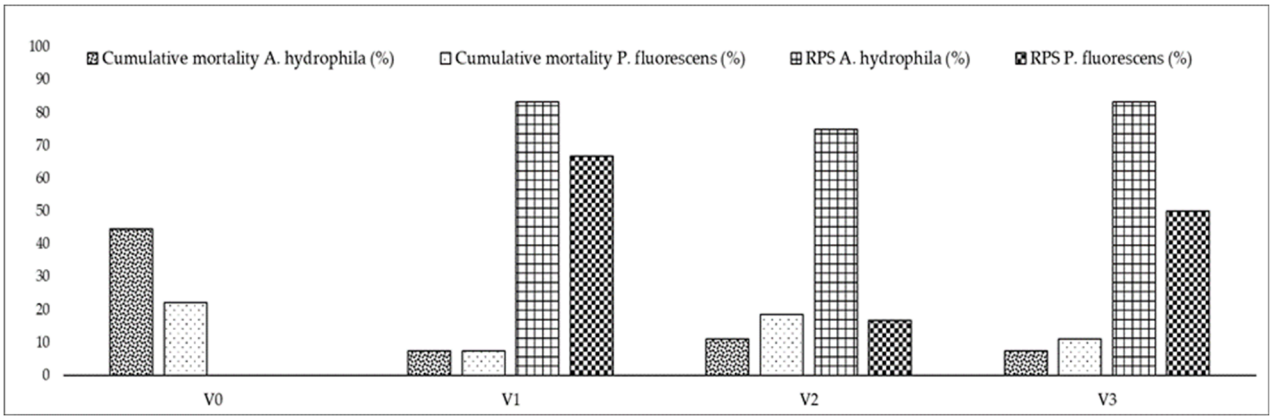

Figure 4. The cumulative mortality (%) and RPS (%) of *O. niloticus* after the post-challenge test.

The dead and moribund fish affected with *Aeromonas hydrophila* showed signs of external hemorrhaging at the base of the fins, around the anus, and at the skin. Internal symptoms were observed, such as the bloating of the abdomen, the hemorrhaging of the peritoneum, the presence of ascitic fluid, and an enlargement of internal organs, especially of the gallbladder (Figure 5).

Figure 5. Clinical signs after infection with *Aeromonas hydrophila*.

Hematological and Biochemical Parameters after the Challenge Test

To evaluate the influence of the challenge test with *Aeromonas hydrophila* and *Pseudomonas fluorescens* on the physiological condition of the Nile tilapia, an analysis of hematological and biochemical indicators was performed (Table 4). After challenge testing, RBCc showed significant differences ($p < 0.05$), with values in *A. hydrophila* and *P. fluorescens* variants being lower than those in positive and negative control variants. Hematocrit (PVC) showed significant differences ($p < 0.05$), with values obtained for the *A. hydrophila* and *P. fluorescens* variants being significantly lower ($p < 0.05$) than C^+ and C^- control variants. For these two hematological parameters, significant differences ($p < 0.05$) were recorded in batches that originated from the growth-period-specific control variant. After the challenge test, hemoglobin concentrations were significantly ($p < 0.05$) lower in the *A. hydrophila* variant from the growth control variant and also significantly ($p < 0.05$) lower in the *P. fluorescens* variant from the probiotic growth variant (V_1). MCV showed significant differences ($p < 0.05$), with the values of the *A. hydrophila* variant being significantly higher in the control growth variant than in other challenge test variants.

Table 4. Hematological parameters after challenge test with *Aeromonas hydrophila* and *Pseudomonas fluorescens*.

Hematological Parameters	Experimental Variant	Control Positive	*Aeromonas hydrophyla*	*Pseudomonas fluorescens*	Control Negative
		(C^+)	**(Ah)**	**(Pf)**	**(C^-)**
RBCc ($\times 10^6$ cells/µL)	V_0	1.15 ± 0.06 [a]	1.07 ± 0.02 [b]	1.01 ± 0.03 [b]	1.15 ± 0.06 [a]
	V_1	1.27 ± 0.08	1.21 ± 0.08	1.31 ± 0.04	1.27 ± 0.08
	V_2	1.30 ± 0.04	1.35 ± 0.03	1.29 ± 0.09	1.30 ± 0.04
	V_3	1.25 ± 0.02	1.14 ± 0.06	1.23 ± 0.03	1.25 ± 0.02
PVC (%)	V_0	21.33 ± 0.67 [a]	16.33 ± 1.20 [b]	15.00 ± 0.58 [b]	17.67 ± 1.67 [a]
	V_1	22.33 ± 1.33	22.00 ± 1.73	18.67 ± 0.67	15.33 ± 1.75
	V_2	19.33 ± 1.67	23.00 ± 1.00	19.67 ± 1.76	18.33 ± 1.86
	V_3	20.00 ± 1.73	23.33 ± 1.45	22.00 ± 1.15	21.00 ± 1.00
Hb (g/dL)	V_0	7.65 ± 0.64 [a]	6.58 ± 0.28 [b]	7.35 ± 0.31 [a]	7.36 ± 0.13 [a]
	V_1	7.62 ± 0.39 [a]	7.26 ± 0.31 [a]	5.67 ± 0.38 [b]	7.29 ± 0.30 [a]
	V_2	7.50 ± 0.22	8.03 ± 0.38	6.13 ± 0.51	7.15 ± 0.10
	V_3	7.11 ± 0.48	7.46 ± 0.18	7.50 ± 0.40	7.48 ± 0.07
MCV (µm^3)	V_0	186.49 ± 4.42 [a]	218.37 ± 5.93 [b]	149.28 ± 5.24 [a]	145.98 ± 14.57 [a]
	V_1	176.01 ± 9.71	170.66 ± 3.99	146.16 ± 2.85	126.99 ± 5.49
	V_2	148.56 ± 8.19	181.61 ± 3.99	150.30 ± 2.01	147.27 ± 8.09
	V_3	159.56 ± 13.62	144.93 ± 3.86	178.15 ± 5.30	164.88 ± 15.70
MCH (pg)	V_0	66.73 ± 4.23 [a]	61.83 ± 2.41 [a]	73.20 ± 4.01 [b]	60.77 ± 1.70 [a]
	V_1	59.99 ± 2.49	59.55 ± 1.70	47.45 ± 1.09	58.91 ± 2.05
	V_2	58.00 ± 3.15	60.24 ± 1.49[a]	43.34 ± 1.68	59.84 ± 3.56
	V_3	56.77 ± 3.82 [a]	66.07 ± 4.86 [a]	60.98 ± 4.37 [c]	58.59 ± 3.85 [a]
MCHC (g/dL)	V_0	35.79 ± 2.19 [a]	28.52 ± 2.67 [a]	49.01 ± 0.30 [b]	42.25 ± 3.17 [a]
	V_1	34.14 ± 0.52	34.90 ± 0.47	32.98 ± 3.27	40.21 ± 2.36
	V_2	39.53 ± 4.47	33.22 ± 1.31	29.01 ± 1.29	47.41 ± 2.14
	V_3	35.69 ± 0.78 [a]	46.04 ± 2.73 [b]	34.34 ± 2.88 [a]	35.77 ± 1.44 [b]
Glucose (mg/dL)	V_0	55.33 ± 1.17	57.01 ± 3.90	51.41 ± 0.50	62.44 ± 3.04
	V_1	60.55 ± 1.38	61.38 ± 4.39	59.52 ± 2.48	71.03 ± 0.64
	V_2	56.32 ± 2.36	60.94 ± 3.16	59.26 ± 1.19	76.56 ± 3.97
	V_3	60.42 ± 0.46	62.69 ± 1.72	57.46 ± 0.88	67.10 ± 4.81

Table 4. *Cont.*

Hematological Parameters	Experimental Variant	Control Positive (C^+)	*Aeromonas hydrophyla* (Ah)	*Pseudomonas fluorescens* (Pf)	Control Negative (C^-)
Total proteins (g/dL)	V_0	5.01 ± 0.29	4.86 ± 0.18	4.82 ± 0.11	5.64 ± 0.24
	V_1	5.33 ± 0.18	5.25 ± 0.16	5.10 ± 0.15	5.00 ± 0.17
	V_2	5.52 ± 0.19	5.47 ± 0.18	5.22 ± 0.43	5.32 ± 0.11
	V_3	5.03 ± 0.19	4.96 ± 0.92	5.00 ± 0.18	5.21 ± 0.25
Lysozyme (U/mL)	V_0	10.58 ± 0.39	10.62 ± 0.63	10.02 ± 0.33	11.38 ± 0.21
	V_1	11.99 ± 0.10	10.62 ± 0.36	11.37 ± 0.44	10.43 ± 0.54
	V_2	11.40 ± 0.36	10.81 ± 0.32	10.25 ± 0.67	11.10 ± 0.10
	V_3	11.89 ± 0.44	10.75 ± 0.29	10.89 ± 0.15	10.67 ± 0.67

RBCc—red blood cells count; PVC—hematocrit; Hb—hemoglobin; MCV—mean corpuscular volume; MCH—mean corpuscular hemoglobin; MCHC—mean corpuscular hemoglobin concentration. Results are presented as triplicate means ± standard error. Values with different superscripts in a row differ significantly (ANOVA, $p < 0.05$).

MCH showed significant differences ($p > 0.05$), and the values recorded in the *P. fluorescens* variant were higher in the control growth variant (V_0) and lower in the synbiotic growth variant (V_3) compared to the other variants after the challenge test. MCHC showed significantly ($p < 0.05$) higher growth in the *A. hydrophila* variant relative to the synbiotic growth variant (V_3) and the *P. fluorescens* variant relative to the control growth variant. There was also a significant decrease ($p < 0.05$) in the negative control variant after the challenge test to the synbiotic growth variant (V_3).

Regarding glucose levels, total serum protein concentration, and lysozyme activity after the challenge test, no significant changes ($p > 0.05$) were recorded between all experimental variants (Table 4). The results obtained for malondialdehyde (MDA) in tissues, kidneys, and liver showed no significant differences ($p > 0.05$) between experimental variants after the challenge test ($p > 0.05$) (Figure 6).

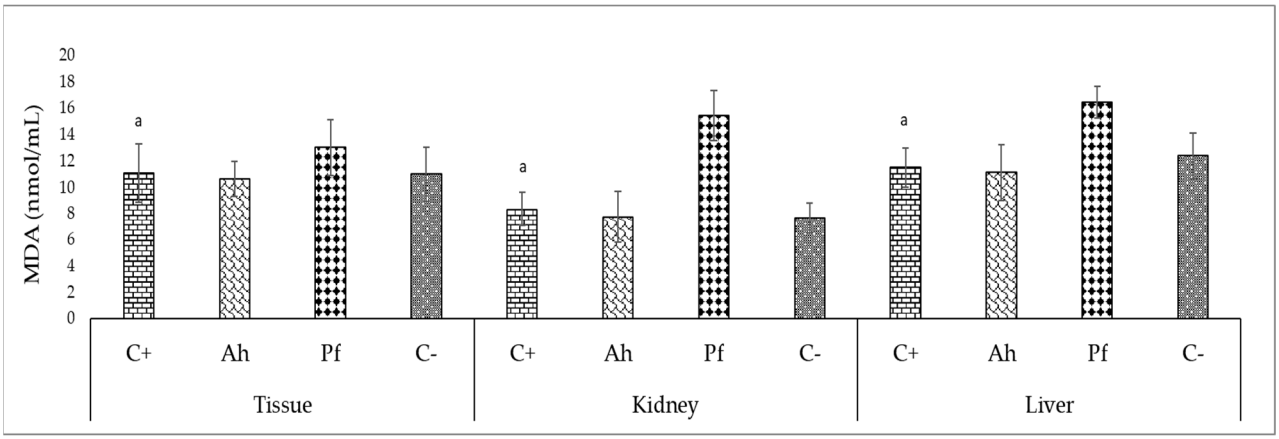

Figure 6. The level of oxidative stress biomarkers in the tissue, kidney, and liver of Nile tilapia, after a challenge test with *Aeromonas hydrophila* and *Pseudomonas fluorescens*. Results are shown as mean values and standard errors. (C^+)—positive control; (Ah)—*Aeromonas hydrophila*; (Pf)—*Pseudomonas fluorescens*; (C^-)—negative control. The letter "a" means that there were no significant differences ($p > 0.05$).

After the challenge test, significantly lower values of leukocytes, small lymphocytes, large lymphocytes, and neutrophilic granulocytes were observed in fish infected with *P. fluorescens* and *A. hydrophila* (Table 5).

Table 5. Variations in the absolute number of leukocytes in Nile tilapia after challenge tests with *Aeromonas hydrophila* and *Pseudomonas fluorescens*.

Experimental Challenge Test Variant	Experimental Growth Variant	Leukocytes	Lm	LM	M	GN	Platelets
		(×1000 cell./mm^3)					
Control positive (C^+)	V_0	53.63 ± 6.86 ^{a}	50.11 ± 14.12 ^{a}	0.90 ± 0.29 ^{a}	0.79 ± 0.23 ^{b}	1.58 ± 0.41 ^{a}	16.87 ± 0.66 ^{a}
	V_1	61.99 ± 5.53	59.78 ± 16.76	0.29 ± 0.08	0.29 ± 0.08	1.63 ± 0.43	16.72 ± 4.73
	V_2	84.02 ± 28.70 ^{b}	79.29 ± 20.96 ^{b}	1.54 ± 0.40 ^{b}	1.16 ± 0.38 ^{c}	2.03 ± 0.62 ^{b}	14.47 ± 4.56 ^{a}
	V_3	65.05 ± 8.16 ^{a}	62.82 ± 17.58 ^{a}	0.49 ± 0.14 ^{a}	0.63 ± 0.16 ^{b}	1.11 ± 0.29 ^{a}	23.05 ± 2.11 ^{b}
Aeromonas hydrophila (Ah)	V_0	48.34 ± 5.12 ^{a}	41.23 ± 12.56 ^{a}	1.54 ± 0.40 ^{a}	1.56 ± 0.42 ^{b}	4.00 ± 1.29 ^{b}	7.74 ± 2.09 ^{a}
	V_1	69.49 ± 6.31 ^{b}	62.59 ± 19.56 ^{b}	2.31 ± 0.83 ^{b}	1.06 ± 0.29 ^{a}	3.53 ± 1.08 ^{b}	14.59 ± 6.48 ^{c}
	V_2	74.28 ± 16,41 ^{b}	65.73 ± 17.08 ^{b}	3.40 ± 0.88 ^{b}	2.05 ± 0.53 ^{b}	3.10 ± 1.03 ^{b}	13.50 ± 8.99 ^{c}
	V_3	53.25 ± 5.43 ^{a}	50.02 ± 14.59 ^{a}	1.19 ± 0.35 ^{a}	0.84 ± 0.22 ^{a}	1.19 ± 0.35 ^{a}	10.64 ± 5.84 ^{b}
Pseudomonas fluorescens (Pf)	V_0	38.21 ± 0.01 ^{a}	35.18 ± 11.14 ^{a}	0.19 ± 0.06 ^{a}	1.23 ± 0.33 ^{a}	1.61 ± 0.51 ^{b}	16.03 ± 7.82 ^{b}
	V_1	41.65 ± 10.14 ^{b}	37.88 ± 9.97 ^{a}	0.94 ± 0.25 ^{a}	0.82 ± 0.21 ^{a}	2.00 ± 0.89 ^{b}	19.54 ± 4.96 ^{b}
	V_2	29.86 ± 2.73	27.99 ± 8.00	0.59 ± 0.19	0.74 ± 0.19	0.53 ± 0.14	3.52 ± 1.52
	V_3	52.90 ± 0.71 ^{b}	49.97 ± 15.55 ^{b}	1.01 ± 0.31 ^{b}	1.14 ± 0.52 ^{a}	0.76 ± 0.20 ^{a}	10.57 ± 8.59 ^{b}
Control negative (C^-)	V_0	77.14 ± 5.77 ^{a}	74.66 ± 22.35 ^{b}	0.98 ± 0.32 ^{a}	0.94 ± 0.24 ^{a}	0.57 ± 0.16 ^{a}	14.62 ± 6.66 ^{b}
	V_1	61.14 ± 6.90 ^{a}	58.47 ± 16.73 ^{a}	0.60 ± 0.17 ^{a}	0.43 ± 0.11 ^{a}	1.63 ± 0.52 ^{b}	10.82 ± 0.29 ^{a}
	V_2	69.09 ± 17.51 ^{a}	63.40 ± 16.39 ^{b}	1.45 ± 0.38 ^{b}	0.63 ± 0.16 ^{a}	1.56 ± 0.40 ^{b}	13.25 ± 2.42 ^{b}
	V_3	58.40 ± 2.05 ^{a}	55.73 ± 17.60 ^{a}	1.07 ± 0.33 ^{a}	0.53 ± 0.17 ^{a}	1. 07 ± 0.33 ^{a}	14.84 ± 4.86 ^{b}

Lm—lymphocytes small; LM—lymphocytes large; M—monocytes; GN—neutrophilic granulocytes. Results are presented as triplicate means ± standard error. Values with different superscripts in a row differ significantly (ANOVA, $p < 0.05$). The statistical comparison was made between the V_0, V_1, V_2, and V_3 variants, within experimental groups C^+, Ah, Pf, and C^-.

4. Discussion

Commercially, Nile tilapia (*Oreochromis niloticus*) is considered to be an important species for freshwater aquaculture because of the quality of its meat, its market demand, and its well-established rearing protocol [33,34]. Environmental stressors and infectious diseases are among the most common main obstacles relative to the expansion of the aquaculture industry [35,36]. Over the past decades, the aquaculture industry has been heavily dependent on antibiotics and chemotherapeutics for controlling infectious diseases [37–39]. The aquaculture feed production industry is threatened by a glut of commercial products with probiotics, prebiotics, and synbiotics. The application of probiotics in RAS improved water quality, feed efficiency, and the growth of various culture organisms [40]. During our study related to the first growth stage in tilapia, it was found that there were no significant differences between the control variant and the probiotic-, prebiotic-, and synbiotic-tested variants, and similar results were also reported by other authors who reported positive weight-gain findings along with a lower feed conversion ratio (FCR) in the 1% probiotic supplemented group. Statistical analysis showed no differences in the administration of *Bacillus spp.* in the diet for improved growth parameters. Similar findings suggested that Nile tilapia (*O. niloticus*) fed with probiotics had insignificant effects on FCR improvement [41] and weight gain [42] during feeding trials. The results of the present study in the second stage showed significant differences in growth parameters FCR and PER between the probiotic variant (V_1), where the best feed conversion factor and protein efficiency was obtained, compared to the prebiotic variant (V_2) and control (V_0). These results can be correlated with the growth parameters of Nile tilapia (*Oreochromis niloticus*) fed with diets supplemented with feed additives (*Biogen*®) or (*Pronifer*®) for some period (90 days), which showed that the group of fish fed with supplemented diets had superior growth than the group fed with the control diet. In contrast to this study, [43,44] reported that Nile tilapia (*O. niloticus*) fry-fed diets supplemented with *Biogen* and yeast showed higher growth than those fed with the control diet. They also reported that the diet containing 30% protein supplemented with *Biogen* (synbiotic) at a level of 0.1% produced the best growth performance and feed efficiency. The authors concluded that *Biogen*® is a suitable growth promotion

additive in tilapia culture. Similarly, other studies have reported the efficacy of various *Bacillus sps.*, such as *B. subtilis, B. licheniformis, B. cereus, B. amyloliquefaciens, B. pumilus*, and *B. velezensis*, in promoting growth [45–47]. Our results on diet supplementation with the probiotic *BetaPlus*®, similarly to *Biogen* and formulated from commercially available probiotic mixtures supplemented in diets containing different levels of protein, produced improved growth performances in tilapia, supporting the observation that probiotics can improve tilapia growth [48,49]. The application of a commercial probiotic containing *B. subtilis* and *B. licheniformis* [50] and a mixture of *B. subtilis* and *B. licheniformis* and Chinese herbs [51] has been reported in tilapia. Other studies also report that there were no positive effects induced by some probiotic strains on the growth performance of Nile tilapia. After a 21-day growth study, the growth performance of tilapia in a diet group amended with the *B. subtilis* strain was similar to that of the control group [52]. The supplementation of probiotics in RAS improved the growth of fish, enhanced stress tolerances and immune responses of fish, and improved water quality and the feed utilization of farmed fish [53–55].

Regarding the assessment of the health status of animals, hematology is an appropriate study that can detect alterations caused by disease or physiological conditions. Erythrocyte counts and leukocyte profiles can be affected by intrinsic or extrinsic factors such as pathogen infections, water contaminants, and immunostimulant supply [56]. The RBCc results showed an increase of up to $1.74 \times 10^6 \pm 0.05$ cell/µL in the control variant compared to the synbiotic variant where $1.50 \times 10^6 \pm 0.04$ cell/µL was obtained at the end of the first experimental phase. No significant differences were recorded after the 90-day trial. The hematocrit (PVC) results showed a decrease in the control variant ($22 \pm 1.50\%$) compared to prebiotic and synbiotic variants ($27 \pm 1.22\%$) at the end of the second experimental stage. The results of the hemoglobin concentration showed an increase in the prebiotic variant (8.05 ± 0.22 g/dL) compared to the other variants at the end of the first experimental stage. The MCV results showed a significant increase in all treatment variants compared to the control over the entire experimental period. The MCHC results showed a significant increase in the control variant compared to the variants where treatments were applied at the end of the second experimental stage.

Hematology is an important factor that could be considered for the fish diet quality assessment. Ologhobo reported that one of the most common blood variables consistently influenced by diet is the hematocrit (PVC) and hemoglobin (Hb) levels [57]. Probiotics and prebiotics have been used alone and together in various animals, including the synbiotic in tilapia [58], which reported positive effects with respect to hematological parameters, and this was also confirmed by the results obtained in the present study. Most hematological and biochemical indices were assessed in different fish, crustaceans, and other invertebrate species to investigate the effects and potential usefulness of synbiotics in aquaculture. Some studies have reported that the application of synbiotics has positive effects on the hematological and biochemical parameters of fish, resulting in a significant increase in Hb, MCV, MCHC, RBC, and WBC compared to fish fed with non-synbiotic supplements [59–61].

Synbiotics can be used as an alternative to antibiotics to improve blood biochemical parameters and antioxidant activity [62]. Values of glucose contents offered improvements among the important signs of environmental stressors in fish [63]. The results on serum glucose content showed an increase of up to 111.93 ± 1.25 mg/dL in the probiotic variant compared to the control variant (103.17 ± 1.46 mg/dL). The results obtained for total serum protein showed a significant decrease in all treatment variants (5.76 ± 0.16 g/dL) compared to the control (6.73 ± 0.23 g/dL). The lysozyme's activity depends on the leucocyte counts that produce lysozymes that catalyze with the glycosidic bonds of pathogenic bacterial cell walls, resulting in an enhanced complement system and phagocytosis [64].

The results of lysozyme activity after 90 days of feeding probiotics, prebiotics, and synbiotics showed a significant increase in the probiotic (13.09 ± 0.3 U/mL) and synbiotic (13.6 ± 0.52 U/mL) variants compared to the control. These results may correlate with the fact that synbiotics also contain mannan-oligosaccharide, which could attach to some Gram-negative bacteria, thereby preventing infection, which subsequently increases fish

immunity [65]. The β-glucan content of synbiotics can increase lysozyme and nitric oxide production, promoting the immune system of fish, as stated by [66,67]. Engstad [68] reported that high levels of lysozymes in fish blood are correlated with the increased production of phagocytes or lysozymes. β-Glucan is well-known for its ability to activate phagocytic cells in producing antimicrobial substances such as lysosomal enzymes, the complement system, and the production of reactive oxygen metabolites [69].

MDA is an indicator of cell damage and lipid peroxidation and is antagonistic with SOD for antioxidant activities [70]. Our results on malondialdehyde (MDA) in tissue, kidney, and liver showed no difference in the treatment trial and control variants. Some authors have reported that fish physiological processes and immunity are highly associated with the antioxidant defense system maintained by enzymes and antioxidant statuses [71].

Studies have shown that the administration of *Bacillus* species stimulates the immune system (specific and non-specific) of fish. Interactions between *Bacillus* species and the phagocytic activity of fish have also been reported [72]. Components of the innate and adaptive immune system play crucial roles in host defense against infectious agents [73,74]; thus, the enhancement of these components by *Bacillus* species suggests that it helps fish fight infectious agents by increasing fish immunity. The results obtained after 40 days of feeding with probiotics, prebiotics, and synbiotics showed an increase in the absolute number of leukocytes in the probiotic compared to the synbiotic variant. After 50 days of feeding with these additives, the absolute number of lymphocytes showed an increase in V_1, V_2, and V_3 variants compared to the control. These results correlated with other studies that state that probiotic applications with *Bacillus* increases the number of immune cells, such as leukocytes, lymphocytes, monocytes, goblet cells, and erythrocytes, and interacts with immune cells such as neutrophils, macrophages, monocytes, and natural killer (NK) cells to induce and enhance innate immune responses [75,76]. Another study indicates that a mixture of *B. subtilis* and *B. licheniformis* demonstrated significant benefits in aquaculture, including improved growth performances, immunomodulation, and survival rates [51]. Recent studies have shown that a diet supplemented with yeast and probiotics caused a significant modulation in the gut microbiota of juvenile barramundi after 42 days of feeding [77].

Accordingly, the interaction between probiotics with the host immune system depends on a few aspects, including source, type, strain, and species of probiotics. Therefore, there is a probability that when a probiotic strain is supplemented singularly to a particular host, it may not positively affect the host's immune system. On the contrary, the combination of different species and genera of probiotics can work synergistically and enhance the host's immune response [78]. Similar effects have also been shown in several fish species that demonstrated improved immunity after feeding with probiotics, prebiotics, and synbiotics [68,79–88].

In aquaculture, most pathogenic diseases are often associated with the genus *Aeromonas, Vibrio, Streptococcus, Yersinia, Acinetobacter, Lactococcus, Pseudomonas,* and *Clostridium* [89,90]. *Aeromonas* includes pathogens that cause disease in fish and other cold-blooded species and is equally well regarded as etiological agents for a variety of infectious complications in both immunocompromised and immunocompetent individuals [38,91]. The genus *Pseudomonas* causes a disease that frequently generates bacterial infections in fish and is mostly stress-related and occurs in freshwater, brackish, and marine farmed fish [92,93]. Although some *Pseudomonas* have been used as probiotics [94,95], few have been reported to cause disease in fish. *P. fluorescens* and *P. aeruginosa* are considered opportunistic pathogenic microbes in aquaculture [96].

The effect of *A. hydrophila* and *P. fluorescens* over 21 days on cumulative mortality was 7.41% in the probiotic-infected variant and followed by the prebiotic-infected variant (18.52%) infected with *P. fluorescens*, compared to the control (44.44%) infected with *A. hydrophila*. These results are consistent with those studies that have elucidated the role of probiotics in combating pathogenic *Pseudomonas* species. There are data available demonstrating that the *Bacillus* species can be considered as potential probiotics in combating

Pseudomonas infections. In this regard, in an experiment conducted by Nandi [18], dead cells of *Bacillus sp.* and *B. amyloliquefaciens* effectively inhibited the growth of *P. fluorescens*. Similarly, bacteriocins synthesized from *B. subtilis* LR1 showed inhibitory activities against it [19].

Our results show that in the *A. hydrophila* group, the relative survival percentage (RPS) was higher in the probiotic and synbiotic variant (81.33%), followed by the *P. fluorescens* group, which was 66.66% in V_1 and 50% in V_3, indicating that the latter group showed more pathogenicity. In this regard, studies show that a significant survival of tilapia has been recorded after feeding *B. pumilus, B. firmus,* and *C. freundi* against an *A. hydrophila* challenge, and it has been reported that a mixture of *Bacillus spp.* and *L. acidophilus* works better in defending tilapia against *A. hydrophila* and P. *fluorescens* but not against *Streptococcus iniae* [97]. Moreover, a mixture of *Bacillus spp.* alone and with herbs was effective in increasing tilapia survival against *S. agalactiae* [51,52]. Although probiotic use in single-species forms, as indicated in previous reports [98,99], is useful in increasing disease resistance and survival in tilapia, recent studies recommend the use of multispecies probiotics due to the advantage of synergistic effects of individual species [51,52]. Similar results were also obtained in studies indicating a higher survival rate after *P. aeruginosa* infection was observed in the synbiotics group due to the positive effect of *S. cerevisiae*, *mannan-oligosaccharide*, and *β-glucan* on the immune response, leading to increased bacterial resistances, as shown in [66,100], and the efficacy of the antimicrobial agent against the pathogen. Regarding the effect of synbiotics, several studies are showing that supplementation with synbiotics has already proven the modulation of disease resistance against pathogenic bacteria in rainbow trout, *O. mykiss* [101]; rockfish, *Sebastes schlegeli* [102]; and Nile tilapia, *Oreochromis niloticus* [63]. Synbiotics demonstrably elevated disease resistance capabilities against various pathogenic bacteria [103–105].

After the challenge test with *A. hydrophila* and *P. fluorescens*, hematological and biochemical parameters of tilapia showed an increase in RBCc, hemoglobin, and hematocrit in fish-fed probiotic, prebiotic, and synbiotic compared to the control in this study. Studies have reported that *Bacillus subtilis* probiotic candidates stimulated both local and systemic immune responses in tilapia [106] and effectively improved the growth performance and disease resistance of Nile tilapia [33]. In accordance with Newaj-Fyzul [107], *Bacillus subtilis* indispensably lowers the amount of motile *Aeromonas*, *Pseudomonas*, and total coliforms in fishes. Some studies indicate that the oral administration of *MOS* and *B. subtilis* synbiotics to *C. mrigala* (Ham.) had a positive effect on heterotrophic bacteria and *Bacillus sp.* [108]. Presumably, the probiotic bacteria tested by these authors fermented MOS and FOS carbohydrates and consequently produced biological compounds that improved the growth of commensal microorganisms and host health [109].

In the present study, no significant differences in serum biochemical parameters were observed in the group receiving the control and experimental diets (probiotics, prebiotics, and synbiotics). However, the combined use or not of pre/probiotics with *S. cerevisiae*, *B. subtilis,* and *B. licheniformis* had a stimulatory effect and increased the survival rate after the challenge test with *A. hydrophila* and *P. fluorescens*.

5. Conclusions

In conclusion, the results of this study indicate that dietary supplementation with *S. cerevisiae, B. subtilis,* and *B. licheniformis* has the potential to promote growth performance and improve tilapia immunity. Of the three experimental groups, the *BetaPlus*®–1% × BW probiotic diet containing *B. subtilis* and *B. licheniformis* is the most promising, followed by the synbiotic group with *BetaPlus*® probiotics, and the *TechnoMos*®–1:1% × BW prebiotics diet containing *Saccharomyces cerevisiae*, *MOS*, and *β-1,3-glucans* can reduce the negative impact of *A. hydrophila* and *P. fluorescens* infection and has beneficial effects on tilapia. Consequently, the two *Bacillus* species are strained with the potential to be used as probiotics as well, in combination with *S. cerevisiae*, which exerts a synbiotic effect on the host's immune system.

This motivates further investigations on other single or multiple probiotics and prebiotics methods for applications in the intensive aquaculture industry.

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Data Availability Statement: All the data are available from the first author and can be delivered if required.

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