



## Article

# Exopolysaccharides-Mediated ZnO Nanoparticles for the Treatment of Aquatic Diseases in Freshwater Fish *Oreochromis mossambicus*

Muthukumar Abinaya <sup>1</sup>, Sathappan Shanthi <sup>1</sup>, Jesudasan Palmy <sup>2</sup> , Khalid A. Al-Ghanim <sup>3</sup>, Marimuthu Govindarajan <sup>4,5</sup>  and Baskaralingam Vaseeharan <sup>1,\*</sup>

- <sup>1</sup> Biomaterials and Biotechnology in Animal Health Lab, Department of Animal Health and Management, Alagappa University, Science Block, 6th Floor, Burma Colony, Karaikudi 630004, Tamil Nadu, India; govindamalmuthukumar06@gmail.com (M.A.); shanthisathappan@gmail.com (S.S.)
- <sup>2</sup> Poultry Production and Product Safety Research Unit, ARS, USDA, Center of Excellence for Poultry Science, University of Arkansas, 1260 W Maple St., Fayetteville, AR 72701, USA; palmyjesudhasan@usda.gov
- <sup>3</sup> Department of Zoology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia; kghanim@ksu.edu.sa
- <sup>4</sup> Unit of Vector Control, Phytochemistry and Nanotechnology, Department of Zoology, Annamalai University, Annamalai Nagar 608002, Tamil Nadu, India; drgovind1979@gmail.com
- <sup>5</sup> Department of Zoology, Government College for Women (Autonomous), Kumbakonam 612001, Tamil Nadu, India
- \* Correspondence: vaseeharanb@gmail.com

**Abstract:** Bacterial fish disease outbreaks are a key concern for aquaculture. Complementary feed additives such as immunostimulants can serve as an ideal solution for disease prevention. Herein, we scrutinized the efficacy of exopolysaccharides (EPSs) from probiotic *Bacillus licheniformis* and EPS-mediated zinc oxide nanoparticles (EPS-ZnO NPs) for a diet to evaluate growth parameters, antioxidant enzyme activities, and immune stimulation together with disease resistance against *Aeromonas hydrophila* and *Vibrio parahaemolyticus* in Mozambique tilapia *Oreochromis mossambicus*. Fish were separated into seven groups, with six experimental groups fed with EPS and EPS-ZnO NPs at 2, 5, and 10 mg/g and a control fed a basal diet. The fish ingesting feed supplemented with EPS and EPS-ZnO NPs at 10 mg/g showed improved growth performance. Cellular and humoral-immunological parameters were tested in serum and mucus after 15 and 30 days of feeding. These parameters were substantially enriched with a 10 mg/g diet ( $p < 0.05$ ) of EPS and EPS-ZnO NPs in comparison with the control. Furthermore, the EPS and EPS-ZnO NP supplemental diet actively enhanced the antioxidant response (glutathione peroxidase, superoxide dismutase, and catalase). In addition, the supplemental diet of EPS and EPS-ZnO NPs lowered the death rate and improved the disease resistance of *O. mossambicus* following assessment with *A. hydrophila* and *V. parahaemolyticus* at 50  $\mu$ L. Hence, the overall results suggest that the supplemental diet of EPS and EPS-ZnO NPs might be used to ensure aquaculture feed additives.

**Keywords:** *Bacillus licheniformis*; zinc nanoparticle; immune parameters; antioxidant response; growth performance; aquatic pathogens



**Citation:** Abinaya, M.; Shanthi, S.; Palmy, J.; Al-Ghanim, K.A.; Govindarajan, M.; Vaseeharan, B. Exopolysaccharides-Mediated ZnO Nanoparticles for the Treatment of Aquatic Diseases in Freshwater Fish *Oreochromis mossambicus*. *Toxics* **2023**, *11*, 313. <https://doi.org/10.3390/toxics11040313>

Academic Editor: Antonia Concetta Elia

Received: 18 February 2023

Revised: 22 March 2023

Accepted: 24 March 2023

Published: 27 March 2023



**Copyright:** © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

## 1. Introduction

Aquaculture is the most important agriculture industry, and it has grown far faster than other food sectors to satisfy fish market requirements. Around 15% of the protein ingested by three billion people comes from fish. In emerging nations such as South Asia, Southeast Asia, China, and Japan, fish is considered a primary nutrition source, creating a massive demand for intensive farming. With the expansion of industry and increased yields, intensive culture practices have been adopted that lead to environmental stress and disease prevalence, which are considered essential challenges for the sector [1–4]. Bacterial

infections are the main issue in Indian aquaculture. *Vibrio* spp. and *Aeromonas hydrophila* are the most prevalent and harmful bacterial pathogens that challenge survival rates in both commercially important marine and freshwater fish [5–10].

The utilization of antibiotics and chemotherapeutics to suppress infections in fish is commonly criticized for its adverse impacts. The excess use of these agents has brought up a few concerns, such as antibiotic resistance and the appearance of residue in tissues and the environment. Likewise, many traditional ways of preventing infectious illnesses and improving fish growth parameters in aquaculture have been explored [11–14].

Aquaculture applications pay much attention to natural products that build up the immune systems of fish in order to enrich their growth and resilience to infection [15]. In aquaculture, some immune-stimulant plants, prebiotics, and probiotics can serve as ideal alternative feed additives for disease prevention without any hazardous effects on fish, human health, or the environment [16–19].

Nanotechnology is well thought out as a newer way to synthesize affordable materials for the remediation of water bodies [20]. Mainly, the level of zinc in a diet may undesirably deteriorate the condition of an additional element such as Cu (copper), Fe (iron), or Cd (cadmium) [21]. It increases the feed cost and subsidizes the minerals in the marine environment [22]. Consequently, the usage of trace minerals at the nano-dimension is a creative solution, which has led to fewer quantities being used to promote growth and immunity against the influence of antioxidants [23,24]. Likewise, nano-sized minerals have catalytic proficiency, more robust adsorbing capacity, enriched bactericidal ability, and greater bioavailability when compared with normal-sized particles [25,26].

In recent decades, numerous polysaccharides have been identified as biological response modifiers to potent immunostimulatory factors and used as biomaterials to prepare eco-friendly nanoparticles (NPs). Compared with polymers from other sources, microbial EPS can be formulated under precise constraints with enduring stability in the GI tract to enrich the colonization of useful microorganisms. They can also effectively act as a strong reducing and stabilizing agent for the production of metal NPs. Due to their properties and structures with many functional groups, EPSs are ideal and efficient for producing metal NPs destined to be applied in the biomedical sciences. Even though EPSs are initiated by macrophages and have a host-mediated anticancer effect, they have remarkable immunomodulatory capabilities [27]. However, limited information can be found on the impact of EPSs on bioactivities, e.g., fish immunomodulation [28]. In this study, we examined the anti-infective potential of exopolysaccharide-mediated zinc nanoparticles against *A. hydrophila* and *V. parahaemolyticus* using *O. mossambicus*.

## 2. Materials and Methods

### 2.1. Experimental Animal

*Oreochromis mossambicus* (average weight:  $7.7 \pm 1.2$  g and length:  $8 \pm 0.5$  cm) was bought from Victoria fish farm and aquarium in Karaikudi, Tamil Nadu, India and was acclimatized for 2 weeks before the feeding trial. According to the method of Gobi et al. [29], fish were sustained in a 300 L FRP tank and provided with a commercial feed (ad libitum; Tairoun Feed Company, Taipei, Taiwan) twice/day at 2% of their body weight. Water temperature was sustained at 29 °C, pH at  $6.91 \pm 1.44$ , salinity at  $0.25 \pm 0.05$  ppt, and DO at  $6.63 \pm 7.78$  mg<sup>-1</sup>.

### 2.2. Methods for EPS Extraction and EPS-ZnO NP Synthesis

We extracted exopolysaccharides (EPS) from *B. licheniformis* Dahb1 using ice-cold ethyl alcohol precipitation [30]. Inoculated *B. licheniformis* Dahb1 cultures were fermented at 37 °C for 72 h in 500 mL of nutrient broth medium. After 10 min at 8000 g, the enzymes were inactivated, and the cells were collected. Adding three liters of cold 95% ethanol to the supernatant solution containing EPS and cooling it overnight precipitated the EPS (20 °C). EPS was precipitated after 10 min of centrifuging at  $8000 \times g$ , washing twice with distilled water, and storing in a desiccator. The crude EPS powder was utilized to make ZnONPs.

Coprecipitation was used to biosynthesize EPS-mediated zinc oxide nanoparticles (EPS-ZnO NPs) [31]. An amount of 0.5 g of EPS crude powder was added to an aqueous solution (0.02 M) of zinc acetate and NaOH (2 M) at 37 °C while stirring quickly. The white material separated after 10 min of centrifuging at 8000 g and was washed thrice with clean water and 100% ethanol. Before further examination, the precipitate was washed and dried at 70 °C.

### 2.3. Experimental Diet Formulation

In the diet formulation, powdered samples were used for experimental purposes. The commercial diet was blended with EPS and EPS-ZnO NPs dissolved in distilled water (D.H<sub>2</sub>O) at the desired concentrations (control (0), 2, 5, and 10 mg/g). The diet pellets were left to dry before being stored at 4 °C [17,32].

### 2.4. Experimental Design and Sample Collection

At the pretest, fish were selected randomly and split into seven groups with three replicate tanks. Each glass tank (300 L) contained 30 fish with aeration-filtration, and water parameters (temperature, DO, pH, and photoperiod) were scrutinized daily. Each treatment group was fed a different concentration of EPS and EPS-ZnO NPs (control, 2, 5, and 10 mg/g). Fish were fed twice a day for 30 days. The tanks were cleaned biweekly. At 15 and 30 days, five fish were selected randomly from each tank in triplicate to measure the growth performance. After measuring growth parameters, liver tissue, blood serum, and mucus were collected according to the guidelines for the Use of Fish in Research to analyze non-specific cellular and humoral immune parameters and antioxidant enzyme activities [33].

#### 2.4.1. Liver Tissue Collection

The total weight of 5 fish livers (1.0 g) was pulverized into a buffer (10 mL) containing sodium phosphate (25 mM; pH 7.4); protease inhibitor (0.1 mM); dithiothreitol (DTT; 0.1 mM); EDTA (1.0 mM); and phenylthiourea (0.1 mM). Afterward, it was centrifuged at 30 min for 10,000× g. The supernatants were excluded and kept at −20 °C for further investigation.

#### 2.4.2. Blood and Serum Collection

Two mL of fish blood was drawn through a caudal puncture in which ≈one mL of blood was imported to a heparinized vacutainer tube and centrifuged at 10 min for 5000× g. Later, the excluded serum was retained at −20 °C for further investigation.

#### 2.4.3. Skin Mucus Collection

Each fish was imported into a polyethylene bag containing NaCl (10 mL; 50 Mm) and gently shaken for 2 min. Then, to collect the pooled mucus, the bag was centrifuged at 10 min for 1500× g. Afterward, the supernatant was filtered (0.45 μm) and retained for later use at −20 °C.

### 2.5. Growth Performance

After 30 days of the experimental diet, growth rates and feed intake were arbitrated as follows:

$$\text{Weight gain (WG; g/fish)} = W_t - W_0$$

$$\text{Specific growth rate (SGR)} = 100 \times [(W_t - W_0)/t]$$

$$\text{Feed conversion ratio (FCR)} = \text{FI}/(W_t - W_0)$$

where  $W_t$  is the final weight;  $W_0$  is the initial weight;  $t$  is the interval of feeding, and FI is the feed ingestion.

## 2.6. Immune Response

### 2.6.1. Cellular-Immunological Parameters

#### Myeloperoxidase (MPO) Activity

With minor changes, the MPO action of the fish serum and mucus was determined as per the technique illustrated by Kumari and Sahoo [34]. In brief, 20  $\mu\text{L}$  sample (serum and mucus) was diluted with HBSS, and 50  $\mu\text{L}$  freshly prepared myeloperoxidase substrate (5 mM of hydrogen peroxide and 20 mM of 3, 3', 5, 5'- tetramethylbenzidine hydrochloride) was placed for 30 min at 30 °C. Thenceforth, the reaction was ended by including 35  $\mu\text{L}$  sulphuric acid (4 M), and OD<sub>450</sub> nm was inferred with an ELISA plate.

#### Reactive Oxygen Species (ROS) Activity

The ROS activity was investigated with the technique illustrated by Secombes [35]. In short, fish blood regulated with an anticoagulant (heparin) solution was loaded into ninety-six-well plates and incubated for 30 °C at 60 min. After incubation, PBS-pH 7.2 was used to rinse the wells to eradicate non-adhered cells. Afterward, 50  $\mu\text{L}$  of NBT was added to incubate at the same interval. For the cell fixation, 30% of CH<sub>3</sub>OH was added and left to settle for three minutes. Lastly, potassium hydroxide (60  $\mu\text{L}$ ; 2 M) plus DMSO (70  $\mu\text{L}$ ) was filled in each well, and absorbance was read at OD<sub>540</sub> nm.

#### Reactive Nitrogen Species (RNS) Activity

For RNS activity, Griess reagent was used to determine the fish serum nitric oxide (NO) level [36]. Briefly, 2 mL of 10 mM sodium-nitroprusside was liquefied with PBS (0.5 mL; pH 7.4); to that, serum (100  $\mu\text{L}$ ) was added prior to incubation at 25 °C. After 2 h, the Griess reagent (0.5 mL) was added and left at 37 °C for 30 min to infer the absorbance at OD<sub>546</sub> nm.

### 2.6.2. Humoral-Immunological Parameters

#### Antiprotease Activity

The serum antiprotease assay was performed as described by Bowden et al. [37] with subtle changes. Concisely, serum (10  $\mu\text{L}$ ) was added among 20  $\mu\text{L}$  trypsin, followed by BAPNA substrate (500  $\mu\text{L}$ ), and the concentration was made again with Tris-HCl (1 mL; 0.1 M). After incubation for 25 min at 22 °C, the action was ended by including acetic acid (30%; 150  $\mu\text{L}$ ), and the proportion of trypsin inhibition was measured at OD<sub>415</sub> nm.

#### Lysozyme-LYZ Activity

Lysozyme activity of the fish serum and mucus was assessed using Gram+ *Micrococcus lysodeikticus* bacteria (Sigma USA) [38]. In this, *M. lysodeikticus* (1 mL; 0.2 mg/mL) was kept in PBS (pH:6.2; 0.05 M). To this, 100  $\mu\text{L}$  of the sample (serum and mucus) was added and left for 1 h to interpret the absorbance at OD<sub>530</sub> nm.

#### Alkaline Phosphatase-ALP Activity

The alkaline phosphatase assay was performed with the following procedure of Sanchooli et al. [39]. Concisely, the serum and mucus (50  $\mu\text{L}$ ) were incubated with NH<sub>4</sub>HCO<sub>3</sub> (150  $\mu\text{L}$ ; pH: 7.8; and 100 mM) containing MgCl<sub>2</sub> (1 mM) for 15 min at 30 °C. Afterward, 50  $\mu\text{L}$  of 4 mM p-nitrophenyl phosphate (substrate) was mixed, and the absorbance was interpreted at OD<sub>405</sub> nm.

#### Hemolytic Complement Activity

The hemolytic complement ability of the fish serum was evaluated using Human RBC as elucidated by Cuesta et al. [40]. For this assay, serum was diluted 2-fold with 100  $\mu\text{L}$  of HBSS containing 1 mM Mg<sup>2+</sup>, 10 mM EGTA, and 6.7 mM of HEPES. Afterward, 100  $\mu\text{L}$  HuRBCs was added and left at 22 °C for 90 min. The reaction was then terminated using 1 mL HBSS containing 10 mM EDTA. Finally, a centrifuge of the non-lysed HuRBCs (500 $\times$  g; 5 min) and the supernatant were used to read the activity at OD<sub>405</sub> nm.

## 2.7. Antioxidant Enzymes Activity

### 2.7.1. Superoxide Dismutase Activity

This assay was completed with the spectrophotometric technique clarified by Suzuki et al. [41] with slight changes. Briefly,  $O_2^-$  producer (xanthine oxidase; 0.1  $\mu$ M/mL in 2 M  $(NH_4)_2SO_4$ ), 50 mM sodium carbonate (xanthine: 0.1 mM; NBT-indicator: 0.025 mM and EDTA: 0.1 mM), and serum/blank-PBS were assorted and calculated at OD<sub>560</sub> nm.

### 2.7.2. Catalase Activity

The catalase assay was performed using the procedure of Takahara et al. [42] with subtle changes. In brief, serum (100  $\mu$ L) was dispersed with phosphate buffer (1.2 mL), followed by 1 mL of hydrogen peroxide suspension. After 5 min, the absorbance diminution was read at OD<sub>240</sub> nm.

### 2.7.3. Glutathione Peroxidase Activity

The protocol of Rotruck et al. [43] was modified to determine the GPx activity. Briefly, a mixture of 0.1 mL serum, 0.1 mL  $H_2O_2$ , 0.2 mL EDTA, 0.1 mL sodium azide, 0.4 mL phosphate buffer, and 0.2 mL GSH was incubated for 10 min at 37 °C. To detain the response, trichloroacetic acid (TCA; 0.5 mL) was added and centrifuged at 2000 $\times$  g. Afterward, 1 mL of 5,5'-dithiobis (2-nitrobenzoic acid DTNB) with 3 mL of  $Na_2HPO_4$  were added on to the obtained supernatant and read at OD<sub>420</sub> nm.

## 2.8. Challenge Test with *A. hydrophila* and *V. parahaemolyticus*

This investigation explored the possibility of using EPS and EPS-ZnO NPS-supplement diets to combat against *A. hydrophila* and *V. parahaemolyticus* infection in *O. mossambicus*. Ten fish were injected intraperitoneally from all groups with an *A. hydrophila* and *V. parahaemolyticus* bacterial cell suspension (50  $\mu$ L;  $1 \times 10^7$  cells/mL). The tested fish were scrutinized daily for 2 weeks to document mortality [44].

## 2.9. Statistical Analyses

A statistical study was performed by applying 1-way ANOVA with SPSS version 16.0. Tukey's multiple comparisons post hoc was applied to relate the means of different groups, where the statistical implication was accepted at  $p < 0.05$ . The outcomes were displayed as means  $\pm$  SD.

## 3. Results

### 3.1. Growth Performance

Table 1 shows the impact of the EPS and EPS-ZnO NPs diet on the body mass of *O. mossambicus*. After a 30-days trial in *O. mossambicus*, appreciable mass differences in growth rates were noticed when compared with the control (without diet). However, compared to EPS, the highest effect on Wt, SGR, and FCR was noticed at 10 mg/g of EPS-ZnO NPs fed to fish. In the control, the diet failed to prompt any substantial growth parameters.

**Table 1.** Growth performance of *O. mossambicus* fed with the experimental diet (EPS and EPS-ZnO NPs) after 30 days of feeding trial. Different letters correspond to the statistical difference between treated groups.

Parameters	30 Days						
	Control	EPS-2 mg/g	EPS-5 mg/g	EPS-10 mg/g	EPS-ZnO NPs-2 mg/g	EPS-ZnO NPs-5 mg/g	EPS-ZnO NPs-10 mg/g
Initial weight (g)	7.69 $\pm$ 1.2 <sup>a</sup>	7.72 $\pm$ 1.4 <sup>a</sup>	7.70 $\pm$ 1.1 <sup>a</sup>	7.75 $\pm$ 1.8 <sup>a</sup>	7.68 $\pm$ 1.4 <sup>a</sup>	7.73 $\pm$ 1.3 <sup>a</sup>	7.67 $\pm$ 1.9 <sup>a</sup>
Final weight (g)	17.50 $\pm$ 0.9 <sup>b</sup>	22.73 $\pm$ 0.2 <sup>bc</sup>	25.41 $\pm$ 0.4 <sup>bc</sup>	27.25 $\pm$ 0.1 <sup>cd</sup>	24.61 $\pm$ 0.3 <sup>cd</sup>	26.80 $\pm$ 0.5 <sup>cd</sup>	34.42 $\pm$ 1.8 <sup>d</sup>
Specific growth rate (SGR)	1.56 $\pm$ 0.3 <sup>a</sup>	2.12 $\pm$ 0.6 <sup>bc</sup>	2.30 $\pm$ 0.2 <sup>bc</sup>	2.40 $\pm$ 0.6 <sup>cd</sup>	2.23 $\pm$ 0.5 <sup>bc</sup>	2.36 $\pm$ 0.4 <sup>bc</sup>	2.57 $\pm$ 0.8 <sup>d</sup>
Feed conversion ratio (FCR)	1.32 $\pm$ 0.2 <sup>a</sup>	1.22 $\pm$ 0.1 <sup>ab</sup>	1.18 $\pm$ 0.3 <sup>ab</sup>	1.03 $\pm$ 0.4 <sup>bc</sup>	1.08 $\pm$ 0.3 <sup>bc</sup>	0.93 $\pm$ 0.2 <sup>cd</sup>	0.76 $\pm$ 0.1 <sup>d</sup>

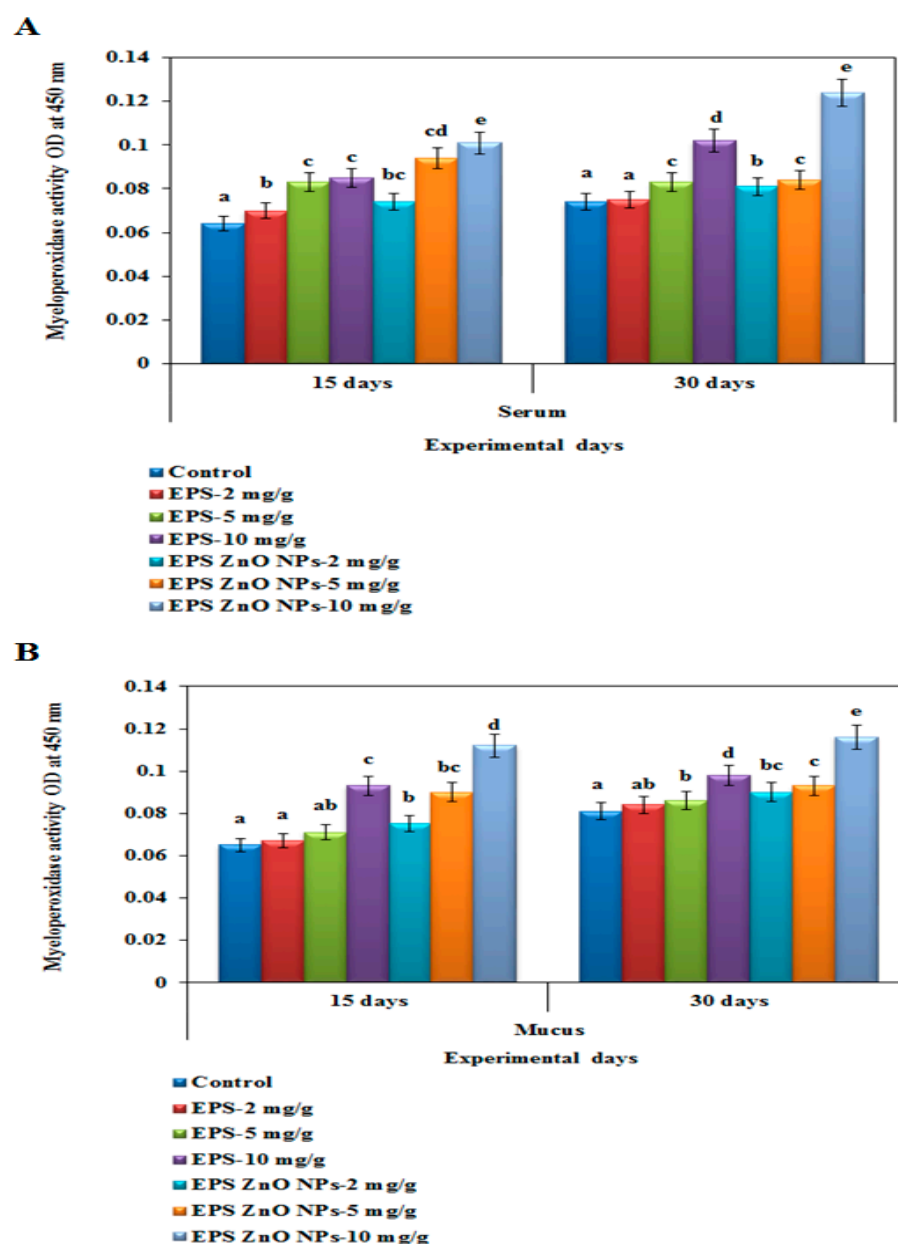


### 3.2. Immune Response

#### 3.2.1. Cellular Immunological Parameters

##### Myeloperoxidase-MOP Activity

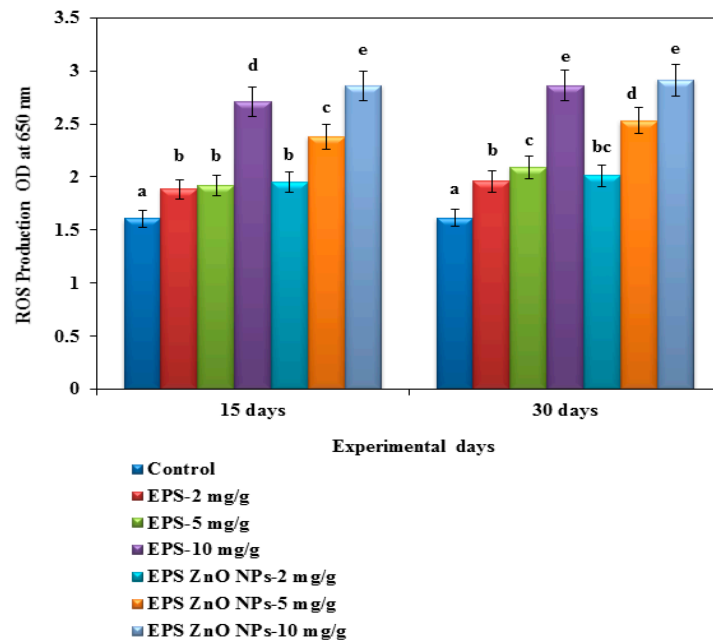
After thirty days of the trial diet with EPS and EPS-ZnO NPs, serum and mucus MOP activity were elevated significantly more than in the control (Figure 1A,B). In fish serum on day 15, the fish fed with EPS and EPS-ZnO NPs (10 mg/g) showed 0.085 U and 0.101 U, whereas on day 30, the MOP level increased to 0.102 U and 0.124 U, respectively. In fish mucus on day 15, fish fed with EPS and EPS-ZnO NPs (10 mg/g) showed 0.093 U and 0.112 U, whereas on day 30, the MOP level increased to 0.098 U and 0.116 U, respectively. Our findings show that a diet containing EPS-ZnO NPs was more potent than EPS in enhancing the MOP activity of mucus and was more significant in both diet groups than the serum of treated fish. In comparison, the control groups showed no improvement.



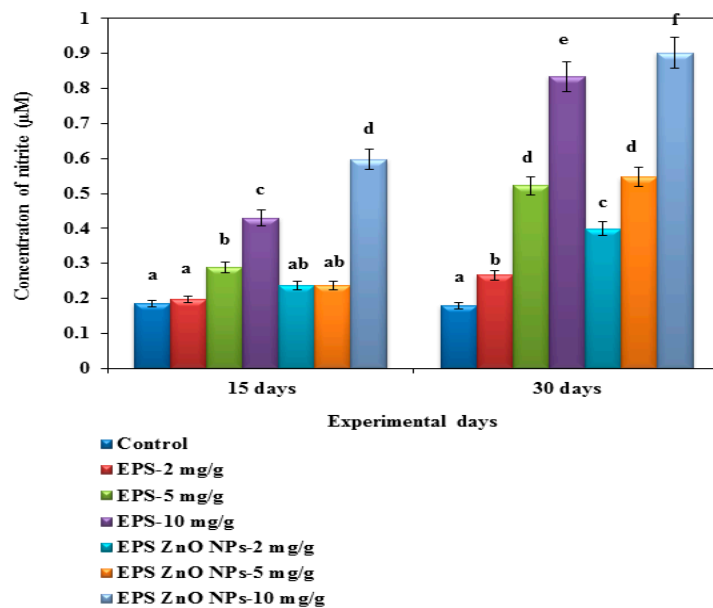
**Figure 1.** Myeloperoxidase activity of *O. mossambicus* fed with EPS and EPS-ZnO NPs: (A) serum and (B) mucus. T-bars indicate three replicates of mean  $\pm$  SD, and different letters correspond to the statistical difference between treated groups.

ROS and RNS Activity

Figures 2 and 3 present the efficacy of the EPS and EPS-ZnO NPs diet on ROS and RNS activity. On day 30, fish fed with 2, 5, and 10 mg/g of EPS- ZnO NPs showed increased ROS activity at 2.013 U, 2.532 U, and 2.912 U, and those on the EPS diet showed 1.614 U, 1.959 U, and 2.093 U, respectively. Conversely, 10 mg/g of the EPS-ZnO NPs diet showed greater RNS activity at 0.902 U, whereas it was significantly lower in the group fed with 10 mg/g of EPS at 0.834 U. A significant difference was not noticeable in the control group.



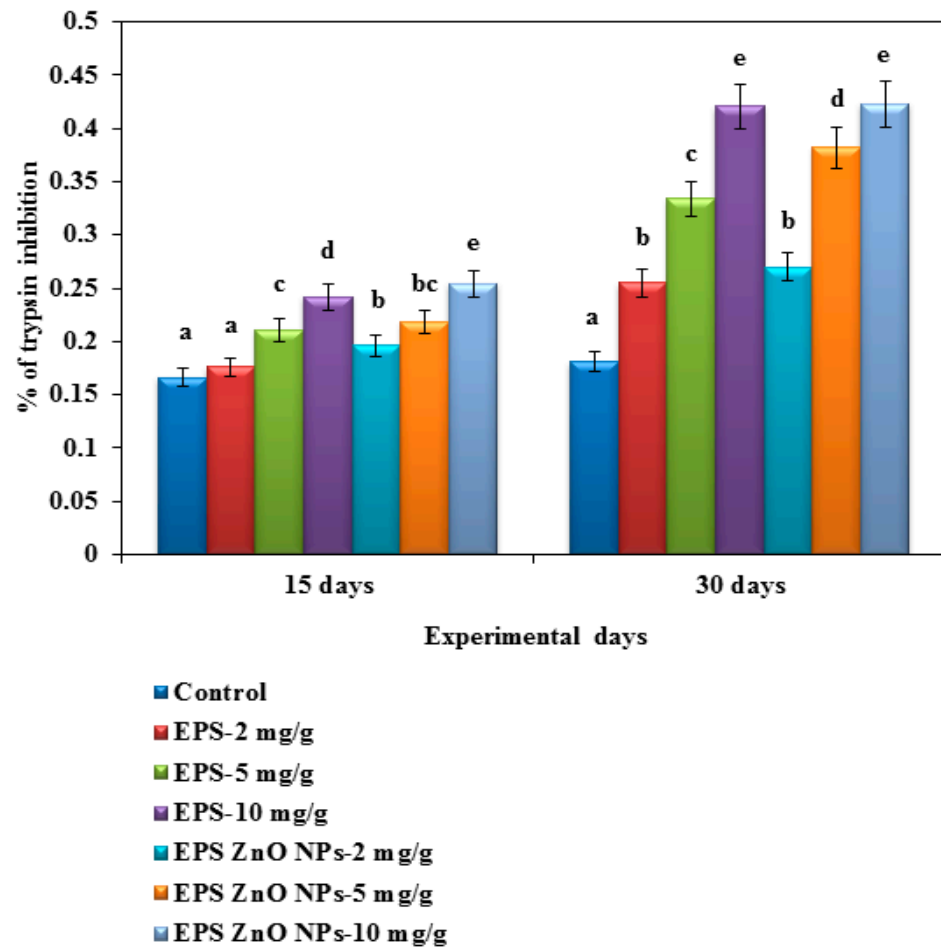
**Figure 2.** Respiratory burst activity/reactive oxygen species production/nitroblue tetrazolium (NBT) assay of *O. mossambicus* fed with EPS and EPS-ZnO NPs. T-bars indicate three replicates of mean ± SD, and different letters correspond to the statistical difference between treated groups.



**Figure 3.** Reactive nitrogen species of *O. mossambicus* fed with EPS and EPS-ZnO NPs. T-bars indicate three replicates of mean ± SD, and different letters correspond to the statistical difference between treated groups.

### 3.2.2. Humoral Immunological Parameters Antiprotease Activity

In this assay, the effect of different concentrations of the EPS and EPS-ZnO NP-supplemented diet (2, 5, and 10 mg/g) on the serum antiprotease of experimental fish was illustrated on day 15 and 30 (Figure 4). For day 30, fish fed with EPS-ZnO NPs were elevated at 0.270, 0.382, and 0.423 U, whereas those fed with EPS were 0.255, 0.334, and 0.421 U, respectively. However, fish fed with both diets had intensified serum antiprotease action levels, and a significant difference was not noticeable in the control.

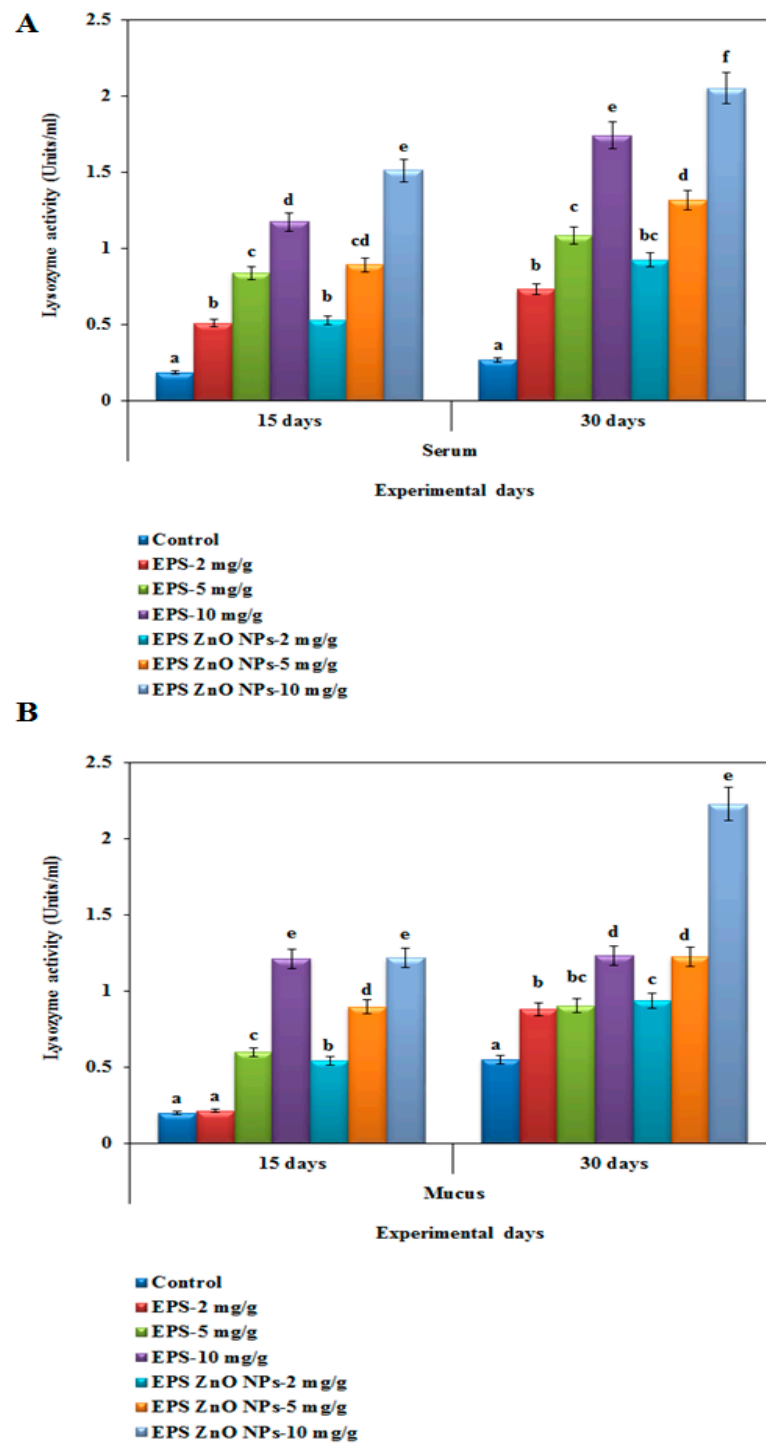


**Figure 4.** Antiprotease activity of *O. mossambicus* fed with EPS and EPS-ZnO NPs. T-bars indicate three replicates of mean  $\pm$  SD, and different letters correspond to the statistical difference between treated groups.

### Lysozyme-LYZ Activity

In this assay, the mucus and serum of LYZ activity were increased substantially ( $p < 0.05$ ) in the EPS and EPS-ZnO NPs experimental diet groups and compared to control groups after 15 and 30 days of the feeding trial (Figure 5A,B). In fish serum on day 15, those fed EPS and EPS-ZnO NPs (10 mg/g) showed 1.173 U and 1.511 U, whereas on day 30, lysozyme activity was increased to 1.74 U and 2.049 U, respectively. In fish mucus on day 15, those fed with EPS and EPS-ZnO NPs (10 mg/g) showed 1.21 U and 1.217 U, whereas on day 30, the level of myeloperoxidase was increased to 1.23 U and 2.227 U, respectively. The mucus showed the highest LYZ level, which correlated with serum after 30 days of the diet.

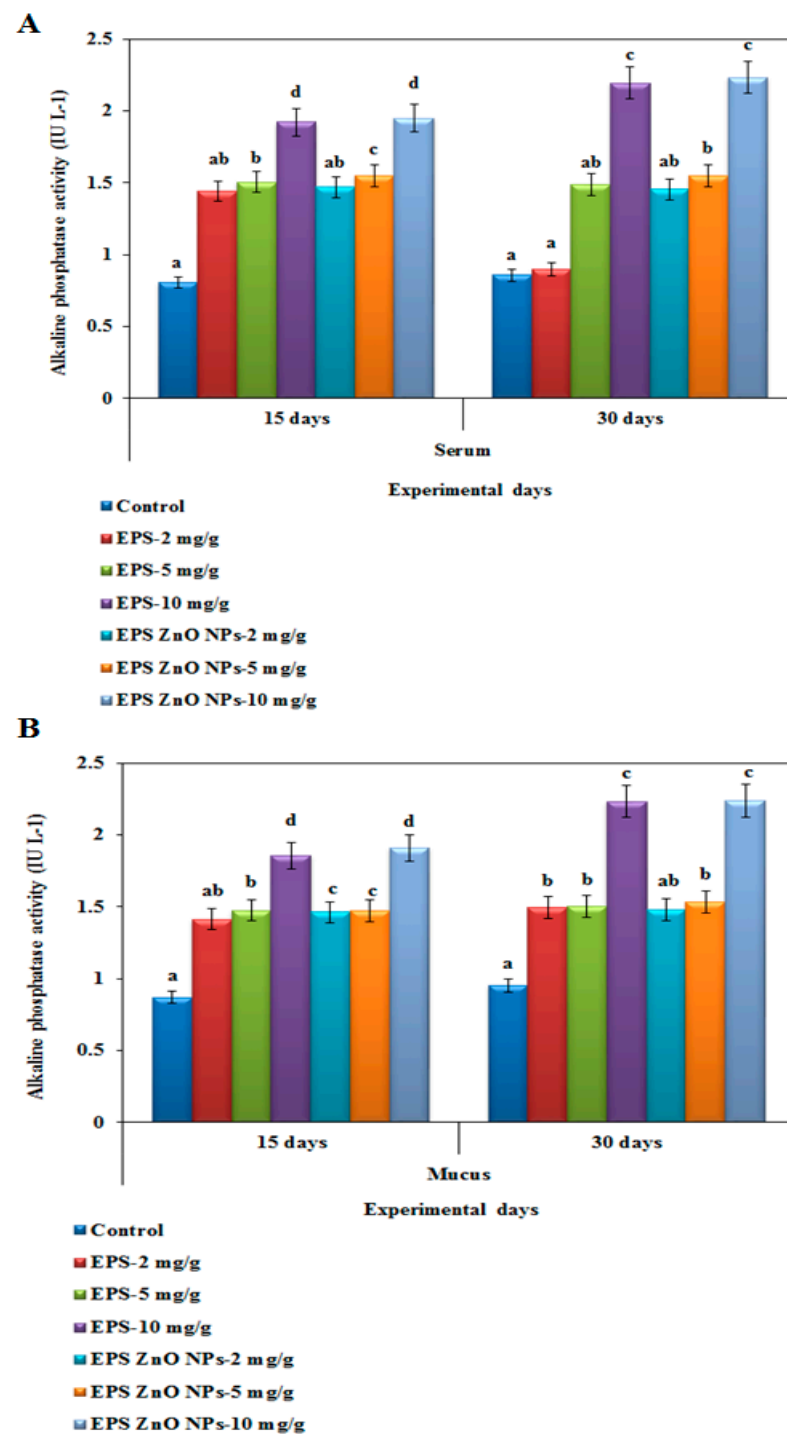




**Figure 5.** Lysozyme activity of *O. mossambicus* fed with EPS and EPS-ZnO NPs: (A) serum and (B) mucus. T-bars indicate three replicates of mean  $\pm$  SD, and different letters correspond to the statistical difference between treated groups.

#### Alkaline Phosphatase-ALP Activity

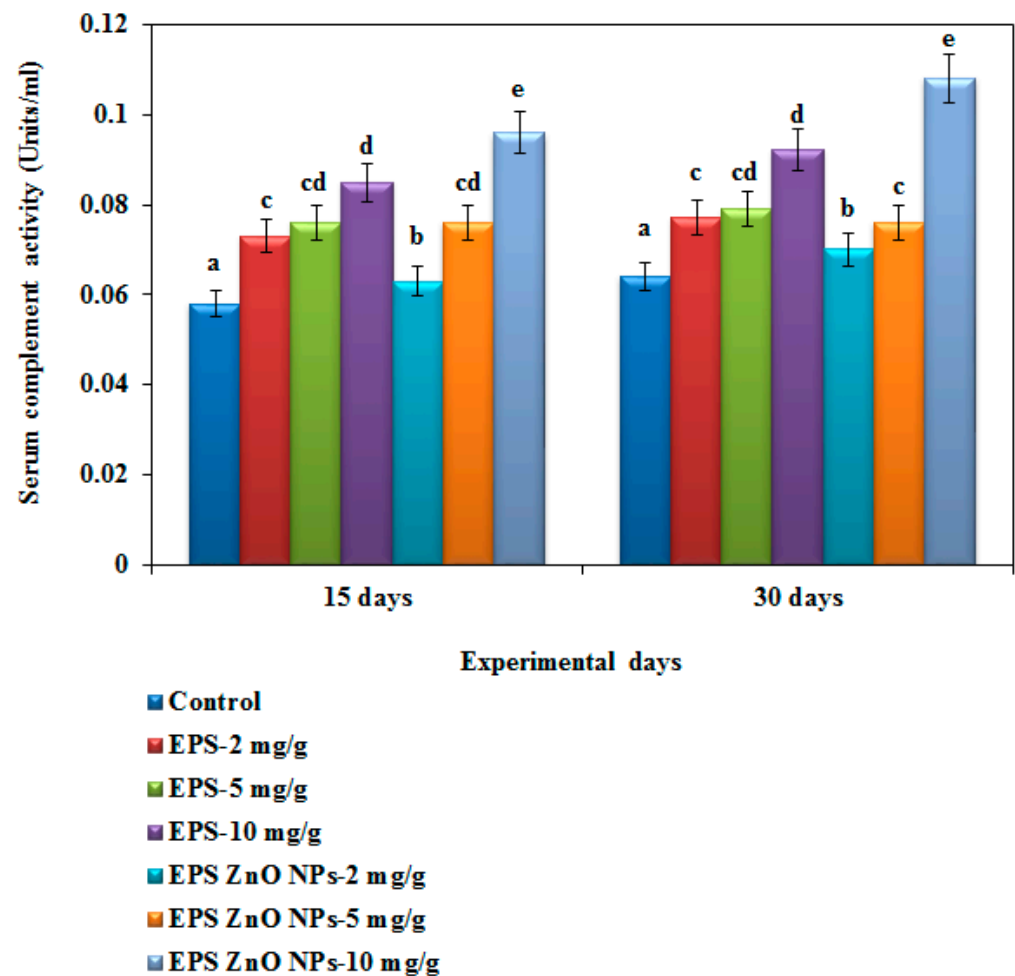
Figure 6A,B show the ALP activity of serum and mucus in fish fed with EPS and EPS-ZnO NPs. Higher alkaline phosphatase activity was noted at 10 mg/g of the EPS-ZnO NPs diet (0.421 U), which corresponded to EPS (0.423 U) on day 30. Conversely, lower activity was shown with 2 mg/g of EPS (0.255 U) on day 30.



**Figure 6.** Alkaline phosphatase activity of *O. mossambicus* fed with EPS and EPS-ZnO NPs: (A) serum and (B) mucus. T-bars indicate three replicates of mean  $\pm$  SD, and different letters correspond to the statistical difference between treated groups.

### Hemolytic Complement Activity

The hemolytic complement activity of the serum of fish fed with the EPS and EPS-ZnO NPs dietary supplement is revealed in Figure 7. However, the EPS-ZnO NPs diet showed a drastically elevated complement action of 0.096 U on day 15 and 0.108 U on day 30, in contrast to the EPS diet with 0.085 U on day 15 and 0.092 U on day 30. The control had no intensified complement action (0.058 on day 15 and 0.064 on day 30).



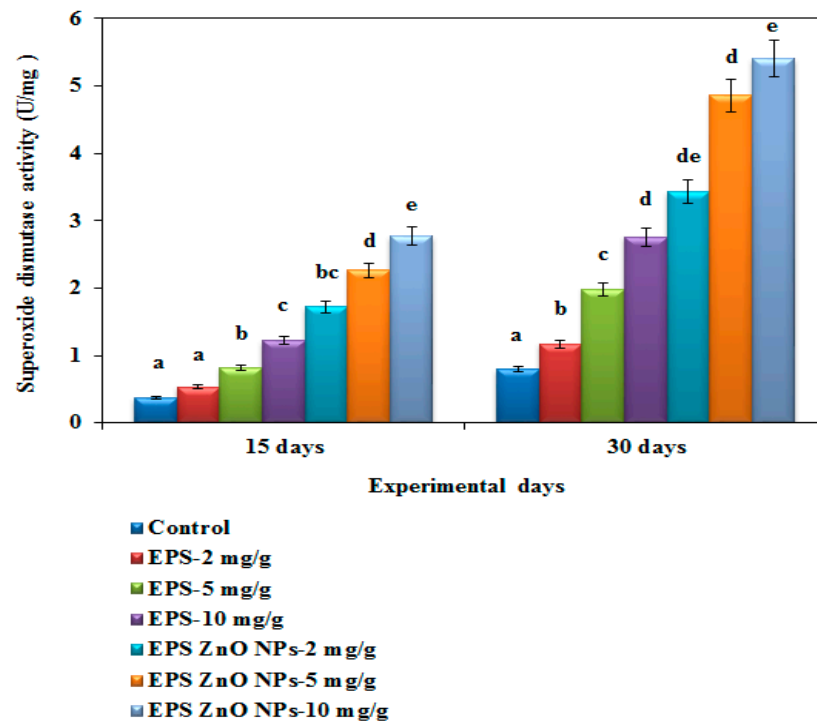
**Figure 7.** Serum innate immune complement hemolytic activity of *O. mossambicus* fed with EPS and EPS-ZnO NPs. T-bars indicate three replicates of mean  $\pm$  SD, and different letters corresponding to the statistical difference between treated groups.

### 3.3. Antioxidant Enzyme Activity

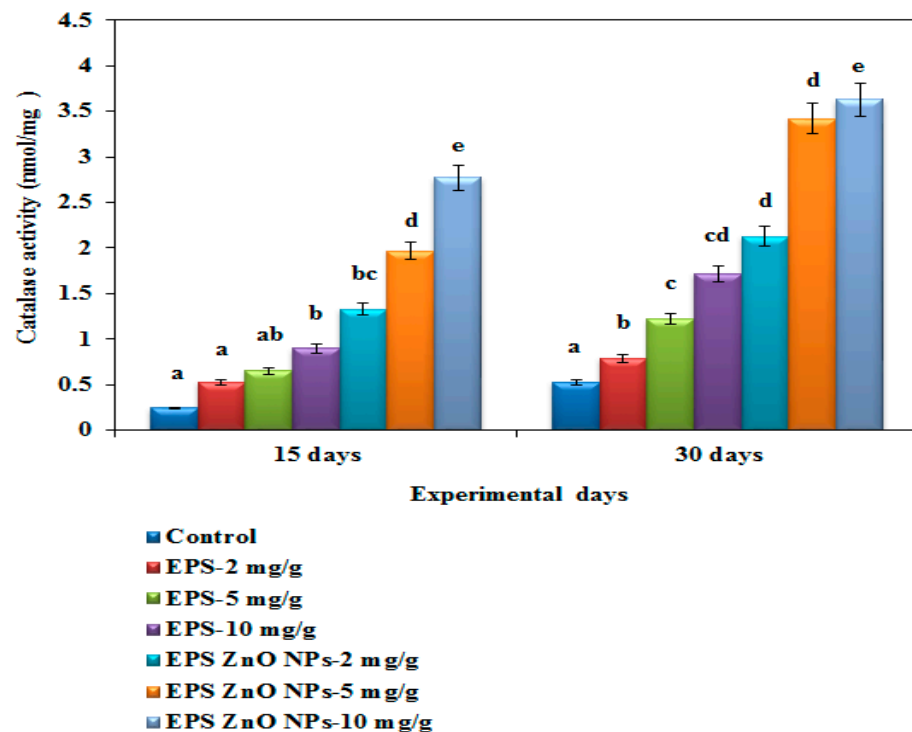
The SOD activity of the fish fed with the EPS-ZnO NPs diet was extensively higher, at 0.897 U/mg and 1.72 U/mg on day 15 and 30, respectively, compared to those fed with EPS (2.77 U/mg on day 15 and 3.63 U/mg on day 30) (Figure 8). The catalase activity of fish fed with the EPS and EPS-ZnO NPs diet was drastically higher compared with the control: on day 30, fish fed with 10 mg/g of EPS and EPS-ZnO NPs showed 2.77 U and 5.40 U of catalase activity (Figure 9). In glutathione peroxidase activity, fish fed with the EPS and EPS-ZnO NP-supplemented diet were elevated with the highest dose (10 mg), and the values observed were at 0.52 U and 0.902 U (Figure 10). Finally, SOD, CAT, and GPx were enriched pointedly at  $p < 0.05$  in the EPS and EPS-ZnO NPs diet in comparison to the control group.

### 3.4. Challenge Test with *A. hydrophila* and *V. parahaemolyticus*

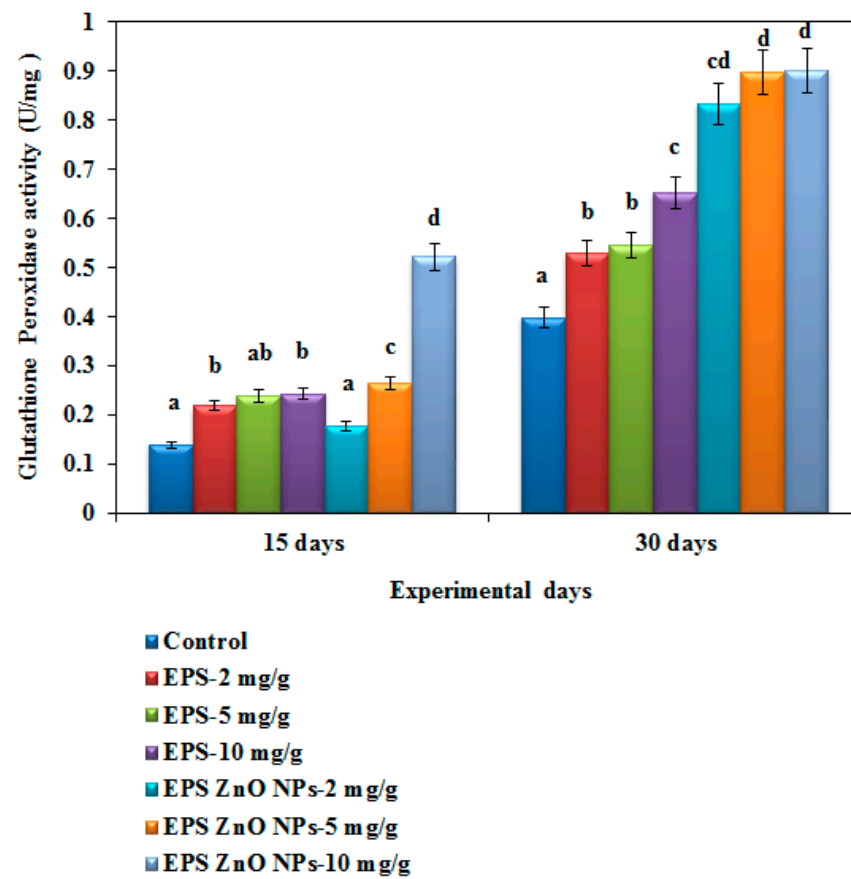
When challenged with *A. hydrophila* and *V. parahaemolyticus*, fish fed with the EPS and EPS-ZnO NPs diet survived substantially more than the control. Fish fed with the EPS-ZnO NPs diet displayed a higher rate of survival (93% in *A. hydrophila* and 85% in *V. parahaemolyticus*) compared to those fed with EPS (76% in *A. hydrophila* and 79% in *V. parahaemolyticus*) (Figure 11). Compared to the control, *O. mossambicus* fish had lower mortality with the experimental diet.



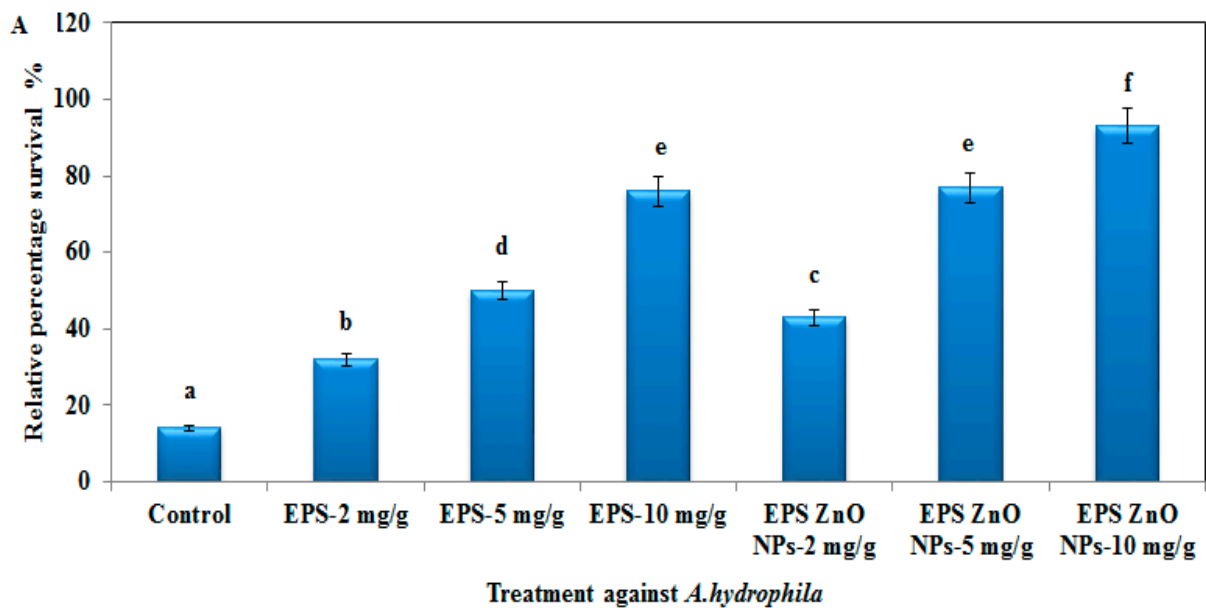
**Figure 8.** Superoxide dismutase activity (SOD) of *O. mossambicus* fed with EPS and EPS-ZnO NPs. T-bars indicate three replicates of mean  $\pm$  SD, and different letters correspond to the statistical difference between treated groups.



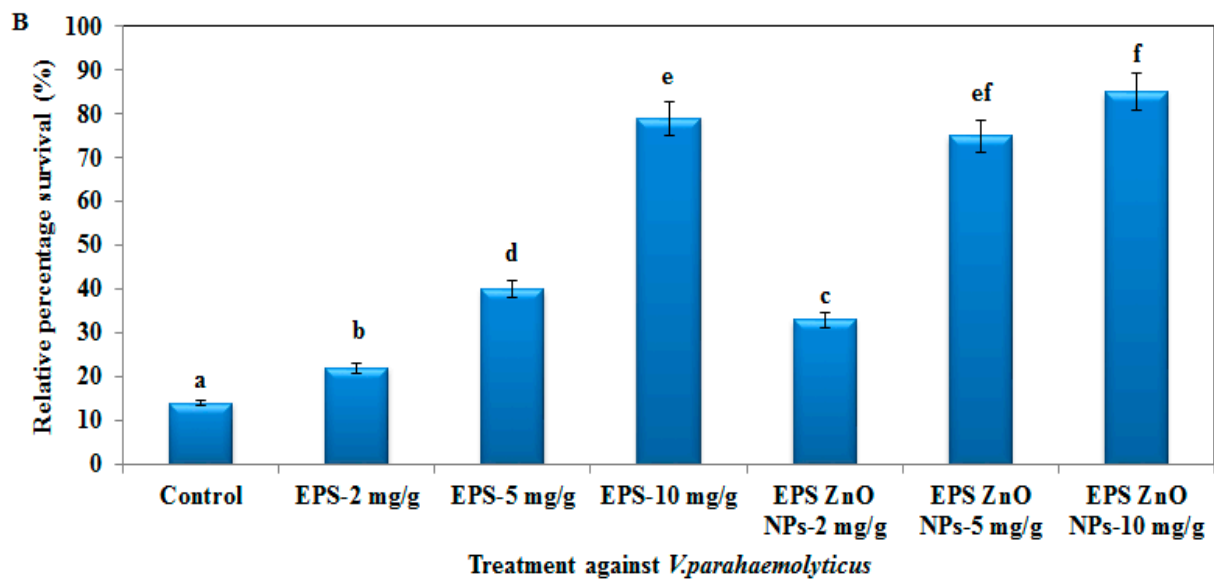
**Figure 9.** Catalase activity (CAT) of *O. mossambicus* fed with EPS and EPS-ZnO NPs. T-bars indicate three replicates of mean  $\pm$  SD, and different letters correspond to the statistical difference between treated groups.



**Figure 10.** Glutathione peroxidase activity (GPx) of *O. mossambicus* fed with EPS and EPS-ZnO NPs. T-bars indicate three replicates of mean  $\pm$  SD, and different letters correspond to the statistical difference between treated groups.



**Figure 11.** Cont.



**Figure 11.** The survival rate of *O. mossambicus* fed with EPS and EPS-ZnO NPs against *A. hydrophila* (A) and *V. parahaemolyticus* (B). T-bars indicate three replicates of mean  $\pm$  SD, and different letters correspond to the statistical difference between treated groups.

#### 4. Discussion

In the last decade, aquaculture has faced numerous difficulties with the emergence of pathogens and drug consumption limits for disease treatment [45]. Medicinal plants and bioactive compounds have been used for animal health [46–50]. The role of probiotics in enriching fish growth parameters, immune response feed efficiency, and modulating gut microflora was already well known [18,51,52]. Several studies have been published on the proficiency of *Bacillus* spp. [53–56] to produce extracellular enzymes in the GI (gastrointestinal) tracts of freshwater fish such as *Labeo rohita* [57,58], *Oreochromis niloticus* [59], and *Ephinephelus bruneus* [60]. However, there seems to be no research into the efficacy of dietary microbial polysaccharides on *O. mossambicus*. The anti-infective potential of EPS and EPS-ZnO NPs with regard to the growth performance, immunity, and disease resistance of Mozambique tilapia *O. mossambicus* against aquatic pathogens was investigated in this study.

Regarding growth parameters, our findings revealed that fish fed with EPS and EPS-ZnO NPs had an increased growth rate and noticeable mass differences with an amount of 10 mg/g after 30 days. In the same way, significant improvements in the growth of *Oncorhynchus mykiss* [61] and *Rutilus frisii kutum* [62] fed with probiotic bacteria have been reported.

In fish, cellular-immunological parameters are the foremost line of defense against intruding pathogenic microbes [63]. Our results strongly demonstrate that feeding trials with EPS and EPS-ZnO NPs were substantial at  $p < 0.05$  and increased both serum and mucus MOP activity after 15 and 30 days. EPS-ZnO NPs were more effective than EPS in enhancing the MOP activity of mucus, which was greater in both diet groups as shown in the treated fish serum. These results correlated with the reports on increased MOP activity in serum, which includes *Oncorhynchus mykiss* fed *B. subtilis* [64]; *P. hypophthalmus* fed *B. licheniformis* Dahb1 [30]; catla fingerlings fed *B. amyloliquefaciens* [52]; and gilthead seabream fed a dietary mix of *Bacillus* spp. and date palm fruit [65]. The ROS and RNS investigation showed higher production in the fish serum of the group fed with EPS and EPS-ZnO NPs at 10 mg/g. Our results were comparable with Wu et al. [66], who reported enriched ROS and RNS generation in *Sophora flavescens*. Likewise, the dietary feed of *T. cordifolia* [67], *S. trilobatum* [68], and *E. alba* [69] in *O. mossambicus* has been reported.



Humoral-immunological parameters are mediated via serum antibodies, which must be secreted with plasma cells and bind to antigens to eliminate them [70]. The maximum lysozyme activity was seen in the mucus following a 30-day immune-boosting diet, which was correlated with the serum. In fish serum on day 30, those fed with EPS and EPS-ZnO NPs (10 mg/g) showed 1.74 U and 2.049 U, and in fish mucus on day 30, it was increased to 2.3 U and 2.22 U, respectively. Our results correlated with the serum of *O. mykiss* fed with *Lactobacillus rhamnosus* [71] and the mucus of *O. niloticus* fed a diet containing probiotics [72]. In addition, higher alkaline phosphatase activity was observed at 10 mg/g of the EPS-ZnO NPs diet (0.421 U), which correlated with those fed with EPS (0.423 U) at day 30. Conversely, lower activity was seen with 2 mg/g of EPS (0.255 U) on day 30. In contrast, *Cyprinus carpio* fed with leaf extract of *Euphorbia hirta* revealed a significant enhancement in the ALP compared to a control [73], and ALP was also increased intensely in *L. rohita* fed with 0.01–0.05% *Achyranthes aspera* [74]. In the serum antiprotease assay, fish fed with EPS-ZnO NPs showed elevated levels of 0.270, 0.382, and 0.423 U, compared to those fed with EPS, which showed 0.255, 0.334, and 0.421 U at day 30. However, both diets showed intensified serum antiprotease action, and a significant difference was not noticeable in the control. In line with other studies in fish, enhanced protease and antiprotease activities have been reported following probiotic feeding [75,76].

For an antioxidant assessment, in the current study, *O. mossambicus* fed with the EPS and EPS-ZnO NPs diet showed pointedly elevated SOD, CAT, and GPx with the experimental diet ( $p < 0.05$ ) compared to the control. A study by Harikrishnan et al. [77] supports these results; enhanced SOD and GPx activity were seen in *E. bruneus* fed with a *P. linteus* diet.

On the other hand, pathogen challenge tests are appropriate to examine the perceptible effect of EPS and EPS-ZnO NPs on the defense tolerance against pathogens. After being exposed to *A. hydrophila* and *V. parahaemolyticus*, fish fed with the EPS and EPS-ZnO NPs diet survived substantially more than the control. Compared to EPS (76% in *A. hydrophila* and 79% in *V. parahaemolyticus*), EPS-ZnO NP-fed fish had a higher survival rate (93% in *A. hydrophila* and 85% in *V. parahaemolyticus*). In agreement with our observations, similar observations were described with Nile tilapia fed a probiotics diet and exposed to *S. agalactiae* [78]; goldfish fed with *E. acetylicum* S01 and exposed to *A. hydrophila* [79]; *O. mossambicus* fed with *B. licheniformis* and exposed to *A. hydrophila* [18]; *L. plantarum* fed a VSG3 diet and exposed to *A. hydrophila* [80]; *L. rohita* fed with VSG2 and exposed to *P. aeruginosa* [81]; and *O. mykiss* fed with combined inactivated *E. faecilis* and mannon oligosaccharides and exposed to *A. salmonicida* [82].

## 5. Conclusions

This study concludes that EPS and EPS-ZnO NPs diets can invoke an immune response, enrich growth, and increase the disease resistance of *O. mossambicus* against *A. hydrophila* and *V. parahaemolyticus* infection. Furthermore, *O. mossambicus* fed with this diet had the highest post-challenge survival rate, indicating an immunostimulant impact. Hence, our evidence suggests that EPS and EPS-ZnO NPs might be good diet supplements for aquaculture environments. This study also paves the way for finding solutions to microbial diseases in aquatic environments, and the diets described here might be a way for aquafarmers to increase the production rate of cultured fish. The study provides more perspectives in the areas of nanotechnology, aquaculture, and immunology.

**Author Contributions:** Conceptualization, M.A. and B.V.; methodology, M.A. and B.V.; software, M.G., S.S. and M.A.; validation, M.A., M.G. and B.V.; formal analysis, M.A.; investigation, M.A.; resources, K.A.A.-G.; writing—original draft preparation, M.A.; writing—review and editing, J.P., K.A.A.-G. and M.G.; supervision, B.V.; funding acquisition, K.A.A.-G. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by King Saud University, grant number RSP2023R48.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** The authors express their sincere appreciation to the Researchers Supporting Project Number (RSP2023R48), King Saud University, Riyadh, Saudi Arabia. The authors enthusiastically acknowledge the DST-INSPIRE fellowship-IF160623, New Delhi, India. The authors give their sincere thanks to a RUSA phase 2.0 grant (ref-24–51/2014—U, Policy) TN. Multi-Gen, Depart of Edn, Govt. Of India.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

- Wang, J.L.; Meng, X.L.; Lu, R.H.; Wu, C.; Luo, Y.T.; Yan, X.; Li, X.J.; Kong, X.H.; Nie, G.X. Effects of *Rehmannia glutinosa* on growth performance, immunological parameters and disease resistance to *Aeromonas hydrophila* in common carp (*Cyprinus carpio* L.). *Aquaculture* **2015**, *435*, 293–300. [[CrossRef](#)]
- Aboyadak, I.M.; Sabry, N.M.; Ali, N.G.; El-Sayed, H.S. Isolation of *Staphylococcus epidermidis*, *Bacillus cereus* and *Pseudomonas stutzeri* from diseased European sea bass (*Dicentrarchus labrax*) for the first time in Egypt. *Egypt. J. Aquat. Biol. Fish.* **2016**, *20*, 103–114. [[CrossRef](#)]
- Gallage, S.; Katagiri, T.; Endo, M.; Maita, M. Comprehensive evaluation of immunomodulation by moderate hypoxia in *S. agalactiae* vaccinated Nile tilapia. *Fish Shellfish Immunol.* **2017**, *66*, 445–454. [[CrossRef](#)] [[PubMed](#)]
- FAO. *Global Aquaculture Production*; United Nations Fisheries and Aquaculture Department: Rome, Italy, 2018.
- Austin, B.; Austin, D.A. Vibrionaceae representatives. In *Bacterial Fish Pathogens*; Springer: Dordrecht, The Netherlands, 2012; pp. 357–411. [[CrossRef](#)]
- Shoemaker, C.; Xu, D.H.; LaFrentz, B.; LaPatra, S. Overview of fish immune system and infectious diseases. In *Dietary Nutrients, Additives and Fish Health*; Wiley: Toronto, ON, Canada, 2015; pp. 1–24. [[CrossRef](#)]
- Huang, L.; Qin, Y.; Yan, Q.; Lin, G.; Huang, L.; Huang, B.; Huang, W. MinD plays an important role in *Aeromonas hydrophila* adherence to *Anguilla japonica* mucus. *Gene* **2015**, *565*, 275–281. [[CrossRef](#)] [[PubMed](#)]
- Wang, W.; Sun, J.; Liu, C.; Xue, Z. Application of immunostimulants in aquaculture: Current knowledge and future perspectives. *Aquac. Res.* **2017**, *48*, 1–23. [[CrossRef](#)]
- Elsheshtawy, A.; Yehia, N.; Elkemary, M.; Soliman, H. Direct detection of unamplified *Aeromonas hydrophila* DNA in clinical fish samples using gold nanoparticle probe-based assay. *Aquaculture* **2019**, *500*, 451–457. [[CrossRef](#)]
- Li, H.; Tian, X.; Zhao, K.; Jiang, W.; Dong, S. Effect of *Clostridium butyricum* in different forms on growth performance, disease resistance, expression of genes involved in immune responses and mTOR signaling pathway of *Litopenaeus vannamei*. *Fish Shellfish Immunol.* **2019**, *87*, 13–21. [[CrossRef](#)] [[PubMed](#)]
- Alderman, D.J.; Hastings, T.S. Antibiotic use in aquaculture: Development of antibiotic resistance—potential for consumer health risks. *Int. J. Food Sci. Technol.* **1998**, *33*, 139–155. [[CrossRef](#)]
- Teuber, M. Veterinary use and antibiotic resistance. *Curr. Opin. Microbiol.* **2001**, *4*, 493–499. [[CrossRef](#)]
- Xiong, W.; Sun, Y.; Zhang, T.; Ding, X.; Li, Y.; Wang, M.; Zeng, Z. Antibiotics, antibiotic resistance genes, and bacterial community composition in fresh water aquaculture environment in China. *Microb. Ecol.* **2015**, *70*, 425–432. [[CrossRef](#)]
- Liu, X.; Steele, J.C.; Meng, X.Z. Usage, residue, and human health risk of antibiotics in Chinese aquaculture: A review. *Environ. Pollut.* **2017**, *223*, 161–169. [[CrossRef](#)] [[PubMed](#)]
- Ji, S.C.; Takaoka, O.; Jeong, G.S.; Lee, S.W.; Ishimaru, K.; Seoka, M.; Takii, K. Dietary medicinal herbs improve growth and some non-specific immunity of red sea bream *Pagrus major*. *Fish Sci.* **2007**, *73*, 63–69. [[CrossRef](#)]
- Bulfon, C.; Volpatti, D.; Galeotti, M. Current research on the use of plant-derived products in farmed fish. *Aquac. Res.* **2015**, *46*, 513–551. [[CrossRef](#)]
- Gobi, N.; Ramya, C.; Vaseeharan, B.; Malaikozhundan, B.; Vijayakumar, S.; Murugan, K.; Benelli, G. *Oreochromis mossambicus* diet supplementation with *Psidium guajava* leaf extracts enhance growth, immune, antioxidant response and resistance to *Aeromonas hydrophila*. *Fish Shellfish Immunol.* **2016**, *58*, 572–583. [[CrossRef](#)] [[PubMed](#)]
- Gobi, N.; Vaseeharan, B.; Chen, J.C.; Rekha, R.; Vijayakumar, S.; Anjugam, M.; Iswarya, A. Dietary supplementation of probiotic *Bacillus licheniformis* Dahb1 improves growth performance, mucus and serum immune parameters, antioxidant enzyme activity as well as resistance against *Aeromonas hydrophila* in tilapia *Oreochromis mossambicus*. *Fish Shellfish Immunol.* **2018**, *74*, 501–508. [[CrossRef](#)] [[PubMed](#)]
- Talpur, A.D.; Ikhwanuddin, M.H.D. Dietary effects of garlic (*Allium sativum*) on haemato-immunological parameters, survival, growth, and disease resistance against *Vibrio harveyi* infection in Asian sea bass, *Lates calcarifer* (Bloch). *Aquaculture* **2012**, *364*, 6–12. [[CrossRef](#)]
- Dimapilis, E.A.S.; Hsu, C.S.; Mendoza, R.M.O.; Lu, M.C. Zinc oxide nanoparticles for water disinfection. *Sustain. Environ. Res.* **2018**, *28*, 47–56. [[CrossRef](#)]

21. Heijerick, D.G.; de Schampelaere, K.A.; Janssen, C.R. Predicting acute zinc toxicity for *Daphnia magna* as a function of key water chemistry characteristics: Development and validation of a biotic ligand model. *Environ. Toxicol. Chem.* **2002**, *21*, 1309–1315. [[CrossRef](#)]
22. Wekell, J.C.; Shearer, K.D.; Gauglitz, E.J., Jr. Zinc supplementation of trout diets: Tissue indicators of body zinc status. *Prog. Fish. Cult.* **1986**, *48*, 205–212. [[CrossRef](#)]
23. Swain, P.S.; Rao, S.B.; Rajendran, D.; Dominic, G.; Selvaraju, S. Nano zinc, an alternative to conventional zinc as animal feed supplement: A review. *Anim. Nutr.* **2016**, *2*, 134–141. [[CrossRef](#)]
24. Nasr-Eldahan, S.; Nabil-Adam, A.; Shreadah, M.A.; Maher, A.M.; El-Sayed Ali, T. A review article on nanotechnology in aquaculture sustainability as a novel tool in fish disease control. *Aquac. Int.* **2021**, *29*, 1459–1480. [[CrossRef](#)] [[PubMed](#)]
25. Albrecht, M.A.; Evans, C.W.; Raston, C.L. Green chemistry and the health implications of nanoparticles. *Green Chem.* **2006**, *8*, 417–432. [[CrossRef](#)]
26. Rajendran, D. Application of nano minerals in animal production system. *Res. J. Biotechnol.* **2013**, *8*, 1–3.
27. Welman, A.D.; Maddox, I.S. Exopolysaccharides from lactic acid bacteria: Perspectives and challenges. *Trends Biotechnol.* **2003**, *21*, 269–274. [[CrossRef](#)]
28. Marudhupandi, T.; Inbakandan, D. Polysaccharides in aquatic disease management. *Fish. Aquac. J.* **2015**, *6*, 1–3. [[CrossRef](#)]
29. Gobi, N.; Malaikozhundan, B.; Sekar, V.; Shanthi, S.; Vaseeharan, B.; Jayakumar, R.; Nazar, A.K. GFP tagged *Vibrio parahaemolyticus* Dahv2 infection and the protective effects of the probiotic *Bacillus licheniformis* Dahb1 on the growth, immune and antioxidant responses in *Pangasius hypophthalmus*. *Fish Shellfish Immunol.* **2016**, *52*, 230–238. [[CrossRef](#)]
30. Abinaya, M.; Vaseeharan, B.; Divya, M.; Vijayakumar, S.; Govindarajan, M.; Alharbi, N.S.; Khaled, J.M.; Al-Anbr, M.N.; Benelli, G. Structural characterization of *Bacillus licheniformis* Dahb1 exopolysaccharide—Antimicrobial potential and larvicidal activity on malaria and Zika virus mosquito vectors. *Environ. Sci. Pollut. Res.* **2018**, *25*, 18604–18619. [[CrossRef](#)]
31. Abinaya, M.; Vaseeharan, B.; Divya, M.; Sharmili, A.; Govindarajan, M.; Alharbi, N.S.; Kadaikunnan, S.; Khaled, J.M.; Benelli, G. Bacterial exopolysaccharide (EPS)-coated ZnO nanoparticles showed high antibiofilm activity and larvicidal toxicity against malaria and Zika virus vectors. *J. Trace Elem. Med. Biol.* **2018**, *45*, 93–103. [[CrossRef](#)]
32. Pakravan, S.; Hajimoradloo, A.; Ghorbani, R. Effect of dietary willow herb, *Epilobium hirsutum* extract on growth performance, body composition, haematological parameters and *Aeromonas hydrophila* challenge on common carp, *Cyprinus carpio*. *Aquac. Res.* **2012**, *43*, 861–869. [[CrossRef](#)]
33. Jenkins, J.A.; Bart, H.L., Jr.; Bowker, J.D.; Bowser, P.R.; MacMillan, J.R.; Nickum, J.G. *Guidelines for the Use of Fishes in Research*; American Fisheries Society: Bethesda, MA, USA, 2014.
34. Kumari, J.; Sahoo, P.K. Dietary  $\beta$ -1, 3 glucan potentiates innate immunity and disease resistance of Asian catfish, *Clarias batrachus* (L.). *J. Fish Dis.* **2006**, *29*, 95–101. [[CrossRef](#)]
35. Secombes, C. Isolation of salmonid macrophages and analysis of their killing activity. *Techn. Fish Immunol.* **1990**, *1*, 137–154.
36. Green, L.C.; Wagner, D.A.; Glogowski, J.; Skipper, P.L.; Wishnok, J.S.; Tannenbaum, S.R. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. *Anal. Biochem.* **1982**, *126*, 131–138. [[CrossRef](#)]
37. Bowden, T.J.; Butler, R.; Bricknell, I.R.; Ellis, A.E. Serum trypsin-inhibitory activity in five species of farmed fish. *Fish Shellfish Immunol.* **1997**, *7*, 377–385. [[CrossRef](#)]
38. Ellis, A.I. Lysozyme assays. *Techn. Fish Immunol.* **1990**, *1*, 101–103.
39. Sanchooli, O.; Hajimoradloo, A.; Ghorbani, R. Measurement of alkaline phosphatase and lysozyme enzymes in epidermal mucus of different weights of *Cyprinus carpio*. *World J. Fish Mar. Sci.* **2012**, *4*, 521–524. [[CrossRef](#)]
40. Cuesta, A.; Rodríguez, A.; Esteban, M.A.; Meseguer, J. In vivo effects of propolis, a honeybee product, on gilthead seabream innate immune responses. *Fish Shellfish Immunol.* **2005**, *18*, 71–80. [[CrossRef](#)] [[PubMed](#)]
41. Suzuki, K. Measurement of Mn-SOD and Cu, Zn-SOD. *Exp. Protoc. React. Oxyg. Nitrogen Species* **2000**, *1*, 91–95.
42. Takahara, S.; Hamilton, H.B.; Neel, J.V.; Kobara, T.Y.; Ogura, Y.; Nishimura, E.T. Hypocatalasemia: A new genetic carrier state. *J. Clin. Investig.* **1960**, *39*, 610–619. [[CrossRef](#)] [[PubMed](#)]
43. Rotruck, J.T.; Pope, A.L.; Ganther, H.E.; Swanson, A.B.; Hafeman, D.G.; Hoekstra, W. Selenium: Biochemical role as a component of glutathione peroxidase. *Science* **1973**, *179*, 588–590. [[CrossRef](#)]
44. Misra, C.K.; Das, B.K.; Mukherjee, S.C.; Meher, P.K. The immunomodulatory effects of tuftsin on the non-specific immune system of Indian Major carp, *Labeo rohita*. *Fish Shellfish Immunol.* **2006**, *20*, 728–738. [[CrossRef](#)]
45. Dawood, M.A.; Koshio, S.; Esteban, M.Á. Beneficial roles of feed additives as immunostimulants in aquaculture: A review. *Rev. Aquac.* **2018**, *10*, 950–974. [[CrossRef](#)]
46. Reverter, M.; Bontemps, N.; Lecchini, D.; Banaigs, B.; Sasal, P. Use of plant extracts in fish aquaculture as an alternative to chemotherapy: Current status and future perspectives. *Aquaculture* **2014**, *433*, 50–61. [[CrossRef](#)]
47. Shen, B. A new golden age of natural products drug discovery. *Cell* **2015**, *163*, 1297–1300. [[CrossRef](#)] [[PubMed](#)]
48. Bilen, S.; Ünal, S.; Güvensoy, H. Effects of oyster mushroom (*Pleurotus ostreatus*) and nettle (*Urtica dioica*) methanolic extracts on immune responses and resistance to *Aeromonas hydrophila* in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **2016**, *454*, 90–94. [[CrossRef](#)]
49. Yousefi, M.; Hoseini, S.M.; Vatnikov, Y.A.; Kulikov, E.V.; Drukovsky, S.G. Rosemary leaf powder improved growth performance, immune and antioxidant parameters, and crowding stress responses in common carp (*Cyprinus carpio*) fingerlings. *Aquaculture* **2019**, *505*, 473–480. [[CrossRef](#)]

50. Zoral, M.A.; Futami, K.; Endo, M.; Maita, M.; Katagiri, T. Anthelmintic activity of *Rosmarinus officinalis* against *Dactylogyrus minutus* (Monogenea) infections in *Cyprinus carpio*. *Vet. Parasitol.* **2017**, *247*, 1–6. [[CrossRef](#)]
51. Das, A.; Nakhro, K.; Chowdhury, S.; Kamilya, D. Effects of potential probiotic *Bacillus amyloliquifaciens* FPTB16 on systemic and cutaneous mucosal immune responses and disease resistance of catla (*Catla catla*). *Fish Shellfish Immunol.* **2013**, *35*, 1547–1553. [[CrossRef](#)]
52. Salinas, I.; Abelli, L.; Bertoni, F.; Picchietti, S.; Roque, A.; Furones, D.; Cuesta, A.; Meseguer, J.; Esteban, M.Á. Monospecies and multispecies probiotic formulations produce different systemic and local immunostimulatory effects in the gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol.* **2008**, *25*, 114–123. [[CrossRef](#)]
53. Bairagi, A.; Ghosh, K.S.; Sen, S.K.; Ray, A.K. Enzyme producing bacterial flora isolated from fish digestive tracts. *Aquac. Int.* **2002**, *10*, 109–121. [[CrossRef](#)]
54. Banerjee, G.; Ray, A.K.; Askarian, F.; Ringø, E. Characterisation and identification of enzyme-producing autochthonous bacteria from the gastrointestinal tract of two Indian air-breathing fish. *Benef. Microbes* **2013**, *4*, 277–284. [[CrossRef](#)]
55. Mondal, S.; Roy, T.; Ray, A.K. Characterization and identification of enzyme-producing bacteria isolated from the digestive tract of bata, *Labeo bata*. *J. World Aquac. Soc.* **2010**, *41*, 369–377. [[CrossRef](#)]
56. Ray, A.K.; Roy, T.; Mondal, S.; Ringø, E. Identification of gut-associated amylase, cellulase and protease-producing bacteria in three species of Indian major carps. *Aquac. Res.* **2010**, *41*, 1462–1469. [[CrossRef](#)]
57. Nayak, S.K.; Swain, P.; Mukherjee, S.C. Effect of dietary supplementation of probiotic and vitamin C on the immune response of Indian major carp, *Labeo rohita* (Ham.). *Fish Shellfish Immunol.* **2007**, *23*, 892–896. [[CrossRef](#)]
58. Giri, S.S.; Sukumaran, V.; Dangi, N.K. Characteristics of bacterial isolates from the gut of freshwater fish, *Labeo rohita* that may be useful as potential probiotic bacteria. *Probiotics Antimicrob. Proteins* **2012**, *4*, 238–242. [[CrossRef](#)]
59. Aly, S.M.; Ahmed, Y.A.G.; Abdel-Aziz, A. Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections. *Fish Shellfish Immunol.* **2008**, *25*, 128–136. [[CrossRef](#)]
60. Harikrishnan, R.; Balasundaram, C.; Heo, M.S. *Lactobacillus sakei* BK19 enriched diet enhances the immunity status and disease resistance to streptococcosis infection in kelp grouper, *Epinephelus bruneus*. *Fish Shellfish Immunol.* **2010**, *29*, 1037–1043. [[CrossRef](#)] [[PubMed](#)]
61. Merrifield, D.L.; Bradley, G.; Baker, R.T.M.; Davies, S.J. Probiotic applications for rainbow trout (*Oncorhynchus mykiss* Walbaum) II. Effects on growth performance, feed utilization, intestinal microbiota and related health criteria postantibiotic treatment. *Aquac. Nutr.* **2010**, *16*, 496–503. [[CrossRef](#)]
62. Azarin, H.; Aramli, M.S.; Imanpour, M.R.; Rajabpour, M. Effect of a probiotic containing *Bacillus licheniformis* and *Bacillus subtilis* and ferroin solution on growth performance, body composition and haematological parameters in Kutum (*Rutilus frisii kutum*) fry. *Probiotics Antimicrob. Proteins* **2015**, *7*, 31–37. [[CrossRef](#)]
63. Ellis, A.E. Innate host defense mechanisms of fish against viruses and bacteria. *Dev. Comp. Immunol.* **2001**, *25*, 827–839. [[CrossRef](#)]
64. Newaj-Fyzul, A.; Adesiyun, A.A.; Mutani, A.; Ramsubhag, A.; Brunt, J.; Austin, B. *Bacillus subtilis* AB1 controls *Aeromonas* infection in rainbow trout (*Oncorhynchus mykiss*, Walbaum). *J. Appl. Microbiol.* **2007**, *103*, 1699–1706. [[CrossRef](#)]
65. Cerezuela, R.; Guardiola, F.A.; Meseguer, J.; Esteban, M.A. Enrichment of gilthead seabream (*Sparus aurata* L.) diet with microalgae: Effects on the immune system. *Fish Physiol. Biochem.* **2012**, *38*, 1729–1739. [[CrossRef](#)] [[PubMed](#)]
66. Wu, Y.R.; Gong, Q.F.; Fang, H.; Liang, W.W.; Chen, M.; He, R.J. Effect of *Sophora flavescens* on non-specific immune response of tilapia (GIFT *Oreochromis niloticus*) and disease resistance against *Streptococcus agalactiae*. *Fish Shellfish Immunol.* **2013**, *34*, 220–227. [[CrossRef](#)] [[PubMed](#)]
67. Alexander, C.P.; Kirubakaran, C.J.W.; Michael, R.D. Water soluble fraction of *Tinospora cordifolia* leaves enhanced the non-specific immune mechanisms and disease resistance in *Oreochromis mossambicus*. *Fish Shellfish Immunol.* **2010**, *29*, 765–772. [[CrossRef](#)] [[PubMed](#)]
68. Christyapita, D.; Divyagnaneswari, M.; Michael, R.D. Oral administration of *Eclipta alba* leaf aqueous extract enhances the non-specific immune responses and disease resistance of *Oreochromis mossambicus*. *Fish Shellfish Immunol.* **2007**, *23*, 840–852. [[CrossRef](#)] [[PubMed](#)]
69. Divyagnaneswari, M.; Christyapita, D.; Michael, R.D. Enhancement of nonspecific immunity and disease resistance in *Oreochromis mossambicus* by *Solanum trilobatum* leaf fractions. *Fish Shellfish Immunol.* **2007**, *23*, 249–259. [[CrossRef](#)] [[PubMed](#)]
70. Lie, Ø.; Evensen, Ø.; Sorensen, A.; Froysadal, E. Study on lysozyme activity in some fish species. *Dis. Aquat. Org.* **1989**, *6*, 1–5. [[CrossRef](#)]
71. Panigrahi, A.; Kiron, V.; Kobayashi, T.; Puangkaew, J.; Satoh, S.; Sugita, H. Immune responses in rainbow trout *Oncorhynchus mykiss* induced by a potential probiotic bacteria *Lactobacillus rhamnosus* JCM 1136. *Vet. Immunol. Immunopathol.* **2004**, *102*, 379–388. [[CrossRef](#)]
72. Taoka, Y.; Maeda, H.; Jo, J.Y.; Kim, S.M.; Park, S.I.; Yoshikawa, T.; Sakata, T. Use of live and dead probiotic cells in tilapia *Oreochromis niloticus*. *Fish Sci.* **2006**, *72*, 755–766. [[CrossRef](#)]
73. Pratheepa, V.; Sukumaran, N. Effect of *Euphorbia hirta* plant leaf extract on immunostimulant response of *Aeromonas hydrophila* infected *Cyprinus carpio*. *PeerJ* **2014**, *2*, 671. [[CrossRef](#)]
74. Rao, Y.V.; Das, B.K.; Jyotirmayee, P.; Chakrabarti, R. Effect of *Achyranthes aspera* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. *Fish Shellfish Immunol.* **2006**, *20*, 263–273. [[CrossRef](#)]



75. Sharifuzzaman, S.M.; Austin, B. Influence of probiotic feeding duration on disease resistance and immune parameters in rainbow trout. *Fish Shellfish Immunol.* **2009**, *27*, 440–445. [[CrossRef](#)] [[PubMed](#)]
76. Cordero, H.; Morcillo, P.; Meseguer, J.; Cuesta, A.; Esteban, M.Á. Effects of *Shewanella putrefaciens* on innate immunity and cytokine expression profile upon high stocking density of gilthead seabream specimens. *Fish Shellfish Immunol.* **2016**, *51*, 33–40. [[CrossRef](#)] [[PubMed](#)]
77. Harikrishnan, R.; Balasundaram, C.; Heo, M.S. Diet enriched with mushroom *Phellinus linteus* extract enhances the growth, innate immune response, and disease resistance of kelp grouper, *Epinephelus bruneus* against vibriosis. *Fish Shellfish Immunol.* **2011**, *30*, 128–134. [[CrossRef](#)] [[PubMed](#)]
78. Van Doan, H.; Hoseinifar, S.H.; Khanongnuch, C.; Kanpiengjai, A.; Unban, K.; Srichaiyo, S. Host-associated probiotics boosted mucosal and serum immunity, disease resistance and growth performance of Nile tilapia (*Oreochromis niloticus*). *Aquaculture* **2018**, *491*, 94–100. [[CrossRef](#)]
79. Jinendiran, S.; Nathan, A.A.; Ramesh, D.; Vaseeharan, B.; Sivakumar, N. Modulation of innate immunity, expression of cytokine genes and disease resistance against *Aeromonas hydrophila* infection in goldfish (*Carassius auratus*) by dietary supplementation with *Exiguobacterium acetylicum* S01. *Fish Shellfish Immunol.* **2019**, *84*, 458–469. [[CrossRef](#)]
80. Giri, S.S.; Sukumaran, V.; Oviya, M. Potential probiotic *Lactobacillus plantarum* VSG3 improves the growth, immunity, and disease resistance of tropical freshwater fish, *Labeo rohita*. *Fish Shellfish Immunol.* **2013**, *34*, 660–666. [[CrossRef](#)]
81. Giri, S.S.; Sen, S.S.; Sukumaran, V. Effects of dietary supplementation of potential probiotic *Pseudomonas aeruginosa* VSG-2 on the innate immunity and disease resistance of tropical freshwater fish, *Labeo rohita*. *Fish Shellfish Immunol.* **2012**, *32*, 1135–1140. [[CrossRef](#)]
82. Rodriguez-Estrada, U.; Satoh, S.; Haga, Y.; Fushimi, H.; Sweetman, J. Effects of inactivated *Enterococcus faecalis* and mannan oligosaccharide and their combination on growth, immunity, and disease protection in rainbow trout. *N. Am. J. Aquac.* **2013**, *75*, 416–428. [[CrossRef](#)]

**Disclaimer/Publisher’s Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.