Effects of dietary supplementation of vitamin-E and commercial probiotics on the innate immunity of *Labeo rohita* against *Aeromonas hydrophila* infection

Leesa Priyadarsani a,*, Thangapalam Jawahar Abraham a, Harresh Adikesavalu a, Gadadhar Dash a, Talagunda Srinivasan Nagesh b

**ARTICLE INFO**

**Keywords:**
Labeo rohita
Immunomodulation
Innate immunity
*Aeromonas hydrophila*
Vitamin-E
Probiotics

**ABSTRACT**

Immunomodulation is one of the useful tools to prevent diseases in aquaculture. In this study, the immunomodulatory effects of vitamin-E (100 mg/kg feed) and commercial probiotic consortia, Rhodomax™ (5 g/kg feed) on the innate immunity of *Labeo rohita* and their protective effect against *Aeromonas hydrophila* infection were evaluated and compared. Three groups of fish at 30 numbers/tank were fed with vitamin-E, probiotic and control diets at 3% body weight for 30 days, in triplicate. Following this, the fish of all groups were injected intramuscularly with *A. hydrophila* N109 at 2.40 × 10⁷ cells/fish. The growth indices like specific growth rate (SGR) and feed conversion ratio (FCR) during the feeding and the non-specific immune responses during the feeding and post-challenge regimen were recorded. The dietary supplementation of vitamin-E and/or commercial probiotics caused significant improvements in the innate immunity of *L. rohita* compared to control. Nevertheless, the vitamin-E diet offered markedly better results in terms of SGR, FCR, ceruloplasmin, anti-protease, myeloperoxidase and phagocytic activities of *L. rohita* during the feeding and post-challenge regimen. While the respiratory oxidative burst activity was enhanced in probiotic diet-fed *L. rohita* only during the feeding regimen. All the immune parameters reached normalcy on day 15 post-injection with *A. hydrophila*. These findings revealed that supplementation vitamin-E at 100 mg/kg feed may improve the growth indices, prime the non-specific immune responses of *L. rohita* against *A. hydrophila* infection and enhance the overall health status than the tested commercial probiotics.

1. Introduction

The demand for fish is ever increasing because of its nutritive value and as a source of cheap animal proteins. Carps are the major group of freshwater fish that have a global significance as a source of food and as experimental models for research. The major carps are the most preferred farm fish because of their fast growth and higher acceptability to consumers [1]. The technologies of induced carp breeding and polyculture in static ponds and tanks have brought about the remarkable upward trend in aquaculture productivity and turned the sector into a fast-growing industry. In India, the states Andhra Pradesh and West Bengal are the top producers of aquaculture freshwater fish [2]. With the intensification of cultural practices, diseases have become the primary constraint in carp aquaculture. Cultured carps are susceptible to various kinds of bacterial, fungal, viral, parasitic and nutritional diseases, which impedes aquaculture production, economic and social advancement in the country [3,4]. *Aeromonas hydrophila* infections are probably the most common bacterial disease diagnosed in cultured warm-water fish [5].

Immunomodulators comprise a group of biological and synthetic compounds that enhance the non-specific cellular and humoral defense mechanism [6]. In aquaculture, immunomodulation is one of the useful tools where vaccination and/or treatment by injections are difficult and laborious processes, and repeated chemotherapy poses a problem of developing drug-resistant strains of pathogens [3,6]. Immunostimulants...
are particularly important during the larval and juvenile stages as they can strengthen the activity of non-specific defense mechanisms of the immune system and confer protection against disease [7]. The immunostimulants mainly facilitate the functions of phagocytic cells, increase their bactericidal activities, and stimulate the natural killer cells, complement system, lysozyme activity and antibody responses in fish, which confer enhanced protection from infectious diseases [8]. They are valuable for the prevention and control of fish diseases in aquaculture as they represent an alternative and supplementary treatment to vaccination. The immunostimulants also have additional effects such as growth enhancement and an increase in the survival rates of the fish under stress [9]. Vitamin-E is essential for normal immune function and it is only obtained through diet as fish cannot synthesize vitamin-E [10]. The lipid peroxidation can cause damage to cells if the oxidative process is not kept in check and such damage appears to be exacerbated in animals fed diets deficient in vitamin-E [11]. Vitamin-E can reduce mortality and improve fish performance while increasing specific and non-specific immune responses [12]. Also, probiotics, as dietary supplements, beneficially affect the host by improving its intestinal balance to enhance and an increase in the survival rates of the fish under stress [13]. They are also effective in mitigating the effects of stress and augmenting fish production [14]. In Indian aquaculture, the use of immunomodulators like β-glucans [15] vitamin-C [16] and Allium sativum and Magnifera indica [17] and probiotics [18, 19] have been documented. However, the efficacy and actual benefits of many other immunomodulators and probiotics are largely unknown. The present investigation assessed and compared the effects of vitamin-E and commercial probiotic consortia, Rhodomax™ on the innate immunity of Labeo rohita as well as their protective effect against Aeromonas hydrophila infection.

2. Materials and methods

2.1. Preparation of vitamin-E and probiotic diets

The vitamin-E (100 mg DL-α-tocopherol acetate; HiMedia, India) was first added to 20 mL soya bean oil and mixed thoroughly. This mixture was then admixed with 1 kg commercial pellet feed (Charean Pokphand Foods, India Private Limited; Product code: CP 9951), containing 28% crude protein, mixed thoroughly for 30 min for the uniform distribution of vitamin-E and air-dried overnight at room temperature [20]. The air-dried feed was stored in an air-tight container and designated as a vitamin-E diet. Likewise, 5 g of commercial probiotic consortia, Rhodomax™ (TIL Biosciences, India) was initially mixed thoroughly with 20 mL soya bean oil. The mixture was then admixed with 1 kg commercial pellet feed for 30 min for uniform distribution and air-dried overnight at room temperature. This feed was designated as a probiotic diet and stored in an airtight container. Each kilogram of Rhodomax™ contained 2.5 g Streptococcus faecalis, 2.5 g Bacillus mesentericus and 1.0 g Clostridium butyricum. The control diet was prepared as above without vitamin-E or Rhodomax™. The fish were fed with respective experimental diets at 3% body weight (BW) twice daily for 30 days.

2.2. Experimental design

The experimental carp Labeo rohita (236.22±24.07 g) were procured from a commercial fish breeder of Naihati (Lat. 22°54′10″ N and Long. 88°25′01″ E), North 24 Parganas district, West Bengal, India to assess and compare the immunomodulatory effect of the vitamin-E and probiotic diets. Healthy L. rohita (n = 270) after 15 days of acclimatization were divided into nine groups, viz., group 1, group 2 and group 3, in triplicate. Group 1 was fed with the control diet. The fish of groups 2 and 3 were fed with vitamin-E and probiotic diets, respectively at 3% BW twice daily for 30 days. The wastes and feces were removed every 3rd day followed by an exchange of 25% water. The rearing water temperature varied from 24 to 28 °C. All applicable guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, New Delhi were followed. All efforts were made to minimize the suffering of the fish.

2.3. Sampling of blood and collection of serum

The experimental fish were first anaesthetized with clove oil (Dabur, India) at 50 μL/L of water during handling and bled by caudal vein puncture using a 2 mL sterile plastic syringe with 22 G 1-inch needle. The blood and serum were collected on the 0th day, 15th day and 30th day of feeding. The blood was allowed to clot at room temperature (∼28 °C) by keeping the syringe in a slanting position followed by overnight incubation at 4 °C. The serum samples were collected by centrifuging at 700 x g for 10–15 min at 4 °C. The serum samples of two fish from each of the three replicate were pooled separately, labelled and stored at −20 °C until use.

2.4. Pathogenicity of Aeromonas hydrophila N10P

Ten glass aquaria (60 cm L x 45 cm B x 30 cm H) of 50 L capacity filled with clean water to a volume of 30 L each were used for this experiment. The experimental fish (n = 100) were grouped into five groups namely A, B, C, D and E in duplicate. After three days, the glass aquaria were stocked with experimental fish at 10 fish/aquarium from the acclimatized stocks. Aliquots (0.1 mL) of A. hydrophila N10P cell suspensions from 10° to 10−3 dilutions were injected intramuscularly [21] to administer 10−6 cells/fish. Control fish received 0.1 mL of 0.85% physiological saline. The challenged fish of each group were maintained in the respective aquaria for 20 days. Mortality, external signs of infection and behavioural abnormalities were recorded daily. The experiment was carried out in duplicate. The lethal dose (LD50) was calculated according to Reed and Muench [22].

2.5. Experimental challenge with Aeromonas hydrophila N10P

Following 30 days of feeding, the fish of all groups were injected intramuscularly with a sub-lethal dose of A. hydrophila N10P (NCBI accession number KC914628) at 2.40 x 103 cells/fish and maintained in their respective tanks. The selection of sub-lethal dose was based on the preliminary study on LD50 determination. Mortality, external signs of infection and behavioural abnormalities were recorded daily for 15 days. The blood and serum were collected on 6-hours post-injection (hpi), 7-days post-injection (dpi), and 15-dpi with A. hydrophila from fish of each group as described above. The serum samples of two fish/tank from three replicate were pooled separately, labelled and stored at −20 °C until use. An aliquot of blood was heparinized using 2.7% EDTA and processed for the measurement of respiratory oxidative burst (ROB) activity within an hour of collection.

2.6. Growth indices

The specific growth rate (SGR) and feed conversion ratio (FCR) were calculated as described in Ricker [23] and Maqbool et al. [24], respectively.

2.7. Assessment of non-specific immune responses

2.7.1. Serum ceruloplasmin activity

The serum ceruloplasmin activity was determined as p-phenylenediamine (PPD) (HiMedia, India) oxidase activity as described by Sahoo et al. [25, 26] with slight modification. Briefly, 25 μL of serum was mixed with 0.5 mL acetate buffer (1.2 M, pH 5.0) containing 0.1% PPD as a substrate. At the same time, a blank was taken by adding 0.5 mL 0.5% sodium azide (NaNO2) to 25 μL of each serum sample. Both the
mixtures were incubated for 30 min at 30 °C. The reaction was stopped by the addition of 0.5 mL 0.5% NaN₃. The absorbance of the samples and blanks was measured at 540 nm using a microplate reader (Dynamaica, Australia). One unit of ceruloplasmin was defined as the amount of oxidase that catalysed PPD and caused a decreased spectral absorbance of 0.001/min at 550 nm.

2.7.2. Serum anti-protease activity

The anti-protease activity of serum was measured following the protocol described by Sahoo et al. [26]. Ten microlitres of serum were added to 100 μL of trypsin type I from bovine pancreas (Sigma, USA) suspended in tri-s-HCL (50 mM, pH 8.2). Two blanks were made with 110 μL PBS and three references were made by mixing 10 μL PBS with 100 μL of trypsin. All mixtures were incubated at 25 °C for 30 min, followed by the addition of 1 mL 0.2% casein (HiMedia, India) in PBS and further incubation for 15 min. The reaction was stopped by adding 500 μL of 10% trichloroacetic acid (TCA). The mixture was centrifuged at 10,000 x g for 15 min at 25 °C. The supernatant was collected separately and read the optical density (OD) at 280 nm. Percentage inhibition was calculated as given below:

\[
\text{Percent inhibition} = \frac{\text{OD of reference} - \text{OD of sample}}{\text{OD of reference}} \times 100
\]

2.7.3. Serum myeloperoxidase (MPO) activity

The serum MPO activity was determined as per Sahoo et al. [26]. About 10 μL of serum was diluted with 90 μL of Hank’s balanced salt solution (HBSS) without Ca²+ or Mg²+ in 96-well plates. To which added was 35 μL of a mixture of freshly prepared 20 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride [TMB] (Genei, India) and 5 mM hydrogen peroxide in 1:20 dilution. The color change reaction was stopped after 2 min by adding 35 μL of 4 M sulphuric acid. The OD was read at 450 nm in a microplate reader.

2.7.4. Respiratory oxidative burst activity

The respiratory oxidative burst activity by neutrophils was determined by the reduction of nitroblue tetrazolium (NBT) to formazan according to the protocol described by Sahoo et al. [26]. Ten microlitres of serum were added to 100 μL of NBT solution (0.1% in PBS) and 100 μL of NBT reagent (0.1% in PBS) in a glass tube and centrifuged at 5000 x g for 15 min at 30 °C. From the resultant suspension, 50 μL was mixed with 5 mL of 0.5% NaN₃ and incubated at 30 °C for 30 min, followed by the addition of 1 mL 0.5% NaN₃ in PBS and further incubation for 24 h at 30 °C. The respiratory oxidative burst activity by neutrophils was determined by the reduction of nitroblue tetrazolium (NBT) to formazan in a microplate well, 0.1 mL of the blood sample was placed. To which 0.1 mL of a mixture of freshly prepared 20 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride [TMB] (Genei, India) and 5 mM hydrogen peroxide in 1:20 dilution. The color change reaction was stopped after 2 min by adding 35 μL of 4 M sulphuric acid. The OD was read at 562 nm using a microplate reader.

2.7.5. Serum phagocytic activity

Serum phagocytic activity was determined by using Staphylococcus aureus [27] with slight modification. Staphylococcus aureus was grown at 30±2 °C for 24 h in 30 mL tryptone soya broth (TSB). The cells were harvested by centrifugation at 6500 x g for 20 min at 25 °C, washed twice by centrifugation with sterile phosphate-buffered saline (PBS) and finally resuspended in 5 mL sterile PBS. From the crude cell suspension, 1 mL was drawn and suitably diluted to get 1 × 10⁶ cells of S. aureus in a microtiter plate well, 0.1 mL of the blood sample was placed. To which added was 0.1 mL of the suspension containing 1 × 10⁸ cells of S. aureus. The mixture was incubated for 30 min at room temperature. From the mixture, 50 μL was drawn, placed on a clean grease-free glass slide and prepared the smear. The smear was air-dried and fixed with 95% ethanol for 5 min. The slides with smear were stained with safranin (0.15%) for 10 min and observed under the light microscope. The number of phagocytosing cells and the number of bacteria engulfed by the phagocytes were counted. The phagocytic activity was calculated as given below:

\[
\text{Phagocytic activity} = \frac{\text{Number of phagocytic cells with engulfed bacteria}}{\text{Number of phagocytic cells}} \times 100
\]

2.8. Statistical analyses

Data were analyzed by one-way ANOVA and Tukey HSD post-hoc for the comparison of means. All the statistical analyses were done using Statistical Package for Social Sciences (IBM-SPSS) Version: 22.0, considering a probability level of \( P < 0.05 \).

3. Results

3.1. Determination of lethal dose (LD₅₀) of Aeromonas hydrophila Nᵢ₃P

The challenged L. rohita were observed to be under stress showing symptoms like inflammation or ulceration at the site of injection with A. hydrophila, petechial haemorrhages and fin/tail haemorrhages. The first mortality was observed within 16 h post-injection with A. hydrophila. All 10 fish died at the challenge dose of 6.37 × 10¹⁰ and 6.37 × 10⁹ A. hydrophila Nᵢ₃P cells/fish within 24 h of injection. At 6.37 × 10⁸ cells/fish, it affected 90% mortalities within 48 h of injection; while at 6.37 × 10⁷ cells/fish, 30% mortalities were recorded on day 3 post-injection. No mortalities were noticed in control. The lethal dose at which 50% of the experimental population die (LD₅₀) was calculated based on mortalities in all test concentrations over 15 days. The LD₅₀ value of A. hydrophila Nᵢ₃P was calculated as 2.37 × 10⁸ cells/fish.

3.2. Growth indices

The SGR of L. rohita fed with control, vitamin-E and probiotic diets were 0.50±0.06 g, 0.62±0.05 g and 0.56±0.04 g, respectively (Fig. 1a). A significant difference in SGR was noted only between control and vitamin-E diets \( (P < 0.05) \). The FCR of L. rohita fed with control, vitamin-E and probiotic diets were 2.74±0.09, 2.16±0.13 and 2.47±0.05, respectively (Fig. 1b), which differed significantly among the treatment groups \( (P < 0.05) \).

3.3. The gross and clinical signs, and mortalities in challenged fish

The gross and clinical signs, behavior and mortalities of the challenged L. rohita were recorded after injection with A. hydrophila. Lethargy, abnormal behavior, wandering around corners and erratic swimming were the first signs observed in challenged L. rohita. On 2-dpi with A. hydrophila Nᵢ₃P, 2 (6.67%), 4 (13.33%), and 6 (20%) fish/tank died in the vitamin-E, probiotic and control diet-fed groups, respectively. On subsequent days, no mortalities were observed.

3.4. Non-specific immune responses

3.4.1. Serum ceruloplasmin activity

The serum ceruloplasmin activity of control diet-fed L. rohita was 0.22±0.005 OD. The serum ceruloplasmin activities following 15-and 30-days post-feeding (dpf) with vitamin-E diet were 0.23±0.02 and 0.27±0.001 OD, respectively. In the probiotic diet group, the respective ceruloplasmin activities were 0.20±0.002 and 0.23±0.01 OD (Fig. 2a). Statistically significant differences in ceruloplasmin activities were observed among the treatment groups and days of feeding \( (P < 0.05) \). The observed ceruloplasmin levels in control diet-fed L. rohita following challenge with A. hydrophila Nᵢ₃P on 6-hpi and 15-dpi were 0.20±0.008 and 0.22±0.12 OD, respectively (Fig. 2b). A significant reduction in ceruloplasmin activity from 0.27±0.001 to 0.24±0.01 OD in vitamin-E diet-fed and A. hydrophila Nᵢ₃P challenged L. rohita on 6-hpi followed by a significant increase \( (P < 0.05) \) on 15-dpi (0.32±0.03 OD) was observed.
In the probiotic diet-fed group, the ceruloplasmin activity decreased significantly from $0.23 \pm 0.01$ to $0.21 \pm 0.004$ OD on 6-hpi followed by a significant increase ($0.36 \pm 0.04$ OD) on 7-dpi ($P < 0.05$). On 15-dpi, the ceruloplasmin activity in challenged L. rohita in all treatment groups became almost normal as that of unchallenged control and the differences were insignificant ($P > 0.05$). The highest serum ceruloplasmin activity was recorded in the vitamin-E diet group during the feeding and post-challenge regimen ($P < 0.05$).

### 3.4.2. Serum anti-protease activity

The anti-protease activity of the control diet-fed L. rohita was $77.53 \pm 4.17\%$. The serum anti-protease activities following 15- and 30-dpf with vitamin-E diet were $81.72 \pm 0.66\%$ and $81.89 \pm 1.01\%$, respectively. In the probiotic diet group, the respective levels were $80.50 \pm 0.69\%$ and $80.68 \pm 1.04\%$ (Fig. 3a). Though the anti-protease activities increased with days of feeding, no significant differences were observed among the treatment groups and days of feeding ($P > 0.05$). The observed serum anti-protease levels of control diet-fed and challenged L. rohita on 6-hpi and 15-dpi were observed to be $81.33 \pm 0.97\%$ and $81.91 \pm 1.50\%$, respectively (Fig. 3b). Serum anti-protease activity of vitamin-E diet-fed and challenged L. rohita on 6-hpi and 15-dpi were $80.50 \pm 0.69\%$ and $80.68 \pm 1.04\%$, respectively (Fig. 3b). Serum anti-protease activity of probiotic diet-fed and challenged L. rohita decreased slightly to $77.21 \pm 1.77\%$ on 6-hpi. On 15-dpi, an activity of $81.71 \pm 1.42\%$ was observed. There existed significant differences ($P < 0.05$) in serum anti-protease activities between vitamin-E and probiotic diet groups on 6-hpi and 7-dpi in challenged L. rohita, with the highest activities in the vitamin-E diet group.

### 3.4.3. Serum myeloperoxidase (MPO) activity

The serum MPO activity of control diet-fed L. rohita was $0.13 \pm 0.01$ OD. Following 15- and 30-dpf with vitamin-E diet, the serum MPO activities were $0.16 \pm 0.02$ and $0.25 \pm 0.007$ OD, respectively. In probiotic diet-fed L. rohita, the MPO activities following 15- and 30-dpf were $0.11 \pm 0.01$ and $0.23 \pm 0.05$ OD, respectively (Fig. 4a). Statistically, significant differences in MPO activities were observed among the treatment groups and days of feeding ($P < 0.05$). However, the difference in serum MPO activities of vitamin-E and probiotic diets during the days of feeding was insignificant ($P > 0.05$). The observed MPO levels of control diet-fed and challenged L. rohita on 6-hpi and 15-dpi were observed to be $0.13 \pm 0.01$ and $0.17 \pm 0.03$ OD, respectively (Fig. 4b). A significant reduction in MPO activity ($P < 0.05$) from $0.25 \pm 0.007$ to $0.15 \pm 0.009$ OD was recorded in vitamin-E diet-fed and challenged L. rohita on 6-hpi. This was followed by a significant increase ($P < 0.05$) to $0.31 \pm 0.04$ OD on 15-dpi (Fig. 4b). In probiotic diet-fed and challenged L. rohita, the MPO activity was significantly decreased ($P < 0.05$) from $0.23 \pm 0.05$ to $0.19 \pm 0.04$ OD on 6-hpi followed by a significant increase ($P < 0.05$) on 15-dpi ($0.22 \pm 0.02$ OD). On 15-dpi, the MPO activity in challenged L. rohita in all treatment groups became almost normal and the differences were insignificant ($P > 0.05$). There existed significant differences in serum MPO activities between the challenged vitamin-E and probiotic diet groups, with the highest levels in the vitamin-E diet group ($P < 0.05$).
3.4.4. Respiratory oxidative burst (ROB) activity

The ROB activity of control diet-fed L. rohita was 0.43±0.02 OD. The ROB activities following 15- and 30-dpf with vitamin-E diet were 0.55±0.001 and 0.64±0.11 OD, respectively. In probiotic diet-fed L. rohita, the respective ROB activities were 0.51±0.02 and 0.82±0.06 OD (Fig. 5a). Statistically significant differences in ROB activities were observed among the treatment groups and days of feeding (P<0.05). The probiotic diet-fed L. rohita recorded the highest ROB activities (P<0.05). In control diet-fed and challenged L. rohita, the significant reduction in ROB activity to 0.31±0.07 OD was observed on 6-h followed by a significant increase to 0.44±0.07 OD (P<0.05) on 15-dpi (Fig. 5b). The reduction in ROB activity from 0.64±0.11 to 0.39±0.12 OD in vitamin-E diet-fed and challenged L. rohita on 6-hpi was significant (P<0.05). On 15-dpi, a significant increase (P<0.05) to 0.65±0.13 OD was noted (Fig. 5b). In probiotic diet-fed and challenged L. rohita, the ROB activity decreased significantly to 0.28±0.03 OD on 6-hpi followed by a significant increase (0.64±0.09 OD) on 15-dpi (P<0.05). The ROB activities in challenged L. rohita of all treatment groups became almost normal on 15-dpi and the differences were insignificant (P>0.05). However, the decrease in ROB activities was the least in the vitamin-E diet group. Insignificant differences existed in ROB activities of challenged groups of vitamin-E and probiotic diets fed L. rohita (P>0.05).

3.4.5. Serum phagocytic activity

The serum phagocytic activity of control diet-fed L. rohita was 54.65±0.37%. The phagocytic activities following 15- and 30-dpf with vitamin E diet were 65.25±5.59% and 76.76±1.72%, respectively. In the probiotic diet group, the respective phagocytic activities were 57.47±3.61% and 63.83±4.85% (Fig. 6a). Statistically significant differences in phagocytic activities were observed among the and days of feeding (P<0.05). In control diet-fed and challenged L. rohita, the serum phagocytic activities were 44.36±5.74% and 56.10±4.10% on 6-hpi and 1-dpi, respectively (Fig. 5b). In vitamin-E diet-fed and challenged L. rohita, a significant reduction in phagocytic activity from 65.25±5.59 to 50.05±5.71% was observed on 6-hpi. This was followed by a significant increase to 76.00±2.00% on 15-dpi (P<0.05). In probiotic diet-fed and challenged L. rohita, the phagocytic activity was decreased significantly to 45.11±4.58% on 6-hpi and increased significantly to 67.48±3.25% on 15-dpi (P<0.05). The phagocytic activity of challenged L. rohita in all treatment groups became nearly normal and the differences were insignificant on 15-dpi (P>0.05). There existed significant differences in the phagocytic activities of challenged L. rohita of vitamin-E and probiotic diets groups on 7-dpi and 15-dpi (P<0.05), with the highest in the vitamin-E diet group.

4. Discussion

The fish health management measures are critical factors to prevent fish diseases. When faced with disease problems, the common response has been to turn to antimicrobial drugs, which may lead to the development of drug-resistant bacteria and transfer of resistant genes among bacteria [28], the accumulation of residual antibiotics in aquaculture products [29], environmental pollution [30] and detrimental effect on the microbial biodiversity [31]. It also has resulted in trade restrictions
in export markets [32]. The drawbacks of using antibiotics evoked a keen interest in immunomodulators [33, 34] and probiotics [35, 36] in aquaculture. Stimulation of non-specific host defense mechanisms using immunostimulants enhances the disease resistance and growth of the hosts [37]. As carps are the major cultivable species in Indian aquaculture, the present study evaluated and compared the immunomodulatory effects of vitamin-E (100 mg/kg dry feed) and probiotic (5 g/kg dry feed) diets on the innate immune responses of _L. rohita_ during the 30 days feeding regime and the resistance _A. hydrophila_ N10P infection.

The SGR is one of the important growth parameters used as an amenable and reliable endpoint to envisage fish growth [23]. The dietary supplementation of vitamin-E and commercial probiotics caused a significant increase in SGR; while the FCR decreased significantly compared to control. The results of the present study corroborate the findings of Sau et al. [38] in _L. rohita_, Paul et al. [39] in _C. catla_, _L. rohita_ and _C. mrigala_, when fed with vitamin-E diet. Also, our results were, more or less, comparable to the findings of Mohapatra et al. [40], who recorded better SGR and FCR values in the probiotic diet-fed group than the control. The high SGR observed in the vitamin-E diet-fed group may be attributed to the decreased metabolic energy demand of fish. These results validated the fact that vitamin-E and probiotic diets have growth-promoting effects on _L. rohita_. Though both groups had a better growth rate compared to the control, the vitamin-E diet-fed group fared better than the probiotic diet-fed _L. rohita_.

The ceruloplasmin activities in _L. rohita_ fed with vitamin-E and probiotic diets were increased significantly on 30-dpf, but the quantum of increase in ceruloplasmin activity was the maximum in vitamin-E diet-fed _L. rohita_. The vitamin-E and probiotic diets fed _L. rohita_ elicited 1.22 and 1.04 folds higher ceruloplasmin activity than the control on 30-dpf. Ceruloplasmin transports copper to the target tissue, which plays an important role in the defense of the organism against stress [41]. Hence, the increase in ceruloplasmin levels in fish following immunostimulation, strengthen the immune system to fight against invading bacteria [42]. Exposure to pathogens or tissue injury also elicited changes in the levels of several plasma proteins including ceruloplasmin [43]. Upon cessation of vitamin-E/probiotic feeding and _A. hydrophila_ N10P challenge, a significant increase in ceruloplasmin activities was recorded in _L. rohita_ on 7-dpi, which became normal as that of unchallenged control on 15-dpi in all groups. Likewise, Sahoo et al. [26] noted a significant and highest upregulation of ceruloplasmin in _A. hydrophila_ infected _L. rohita_ on 3-dpi, which, however, came back to the initial level on 15-dpi in the survivors. While, in our study, the higher ceruloplasmin activities were determined on 7-dpi with _A. hydrophila_ in vitamin-E or probiotic diet-fed _L. rohita_, indicating better immune modulation. Our results also corroborate the observations of increased ceruloplasmin levels in _A. hydrophila_ infected _L. rohita_.

Fig. 3. Serum antiprotease activity of _Labeo rohita_ fed with [a] control, vitamin-E and probiotic diets, and [b] followed by challenge with _Aeromonas hydrophila_ N10P. hpi: hours post-injection; dpi: days of post-injection; a-b: Bars of the vitamin-E feed group sharing common alphabets differed significantly (P<0.05); 1-3: Bars of the probiotic feed group sharing common numerical differed significantly (P<0.05); *: The pairs of bars with an asterisk (*) symbol differed significantly (P<0.05).
This increase in ceruloplasmin levels during the bacterial challenge probably contributed to the efforts to regain homeostasis [44].

The dietary supplementation of vitamin-E or probiotics did not affect the anti-protease activities, as observed from the insignificant differences in their activities among the treatment groups and days of feeding. Even after the challenge with *Aeromonas hydrophila* N10P, only a slight but insignificant increment in anti-protease activities was noticed. It seemed that the activity of anti-proteases of *L. rohita* was unaffected during vitamin-E or probiotic feeding and *A. hydrophila* infection, which corroborate the findings of Sahoo et al. [26] and Magnadóttir [45]. Yet, some contradictions were noticed in some other reports [41, 46].

The serum MPO activities in *L. rohita* fed with vitamin-E and probiotic diets were increased significantly on 30-dpf, but the quantum of increase in the MPO activity was maximum in the vitamin-E diet-fed *L. rohita*. Essentially, the increased MPO activity may help to produce hypohalous acids central to the microbicidal activity of neutrophils [47] and offer protective effects against the invading pathogens. Likewise, several earlier studies reported enhanced MPO activities in fish after immunostimulant [16, 46] and probiotics [48] administration. Further, the *A. hydrophila* N10P challenge increased the MPO activities on 7-dpi in vitamin-E and probiotic diets fed *L. rohita* compared to control, with the highest increment in the vitamin-E group. These results suggested that the dietary supplementation of vitamin-E, in particular, or the commercial probiotic, to some extent, may offer protective effects against the *A. hydrophila* infection. The protective effect lasted longer in the vitamin-E group as observed by the MPO levels on 15-dpi; while in the probiotic group, the MPO levels became normal as that of the control. Our results corroborate the observations of Kumar et al. [48], who recorded enhanced resistance of probiotic-fed *L. rohita* fingerlings to *A. hydrophila* infection by significantly increasing the MPO activity compared to control.

The ROB activities of vitamin-E and probiotic diets fed *L. rohita* were increased significantly on 30-dpf. The quantum of increase in ROB activity was, however, the maximum in probiotic diet-fed *L. rohita* on 30-dpf. The vitamin-E and probiotic diets fed *L. rohita* elucidated 1.48 and 1.90 folds higher ROB activity, respectively than the control on 30-dpf. The results indicated that both vitamin-E and commercial probiotics have the ability to prime the innate immunity of *L. rohita*. In contrast, Nayak et al. [18] recorded that the ROB activity was significantly higher in the vitamin-C group than the probiotic fed *L. rohita*. Likewise, the stimulation of ROB activity after dietary probiotic supplementation involving other feeding regimes and feeding durations have been previously reported in various fish [19, 49] or diets containing vitamin-E and other immunostimulants [46, 34, 50]. Upon *A. hydrophila* challenge, a significant reduction in ROB activities was recorded in all groups on 6-hpi and 7-dpi. However, the reduction was the least in the vitamin-E group compared to other groups, which suggested that the vitamin-E have the better ability to modulate the immune responses. On 15-dpi, the ROB activities in *A. hydrophila* N10P challenged *L. rohita* of all treatment groups became normal and the differences were insignificant compared to unchallenged control. Likewise, several earlier studies...
recorded significantly increased ROB activities in probiotic [51, 52] or vitamin fed fish [16]. The significant reduction in ROB activity during the post-challenge was probably due to the exhaustion of ROB activity of the phagocytes following *A. hydrophila* infection. Our results indicated that both vitamin-E and probiotic diets enhance the immunity of *L. rohita* to overcome the stress caused by the *A. hydrophila* challenge.

The serum phagocytic activities of vitamin-E and probiotic diets fed *L. rohita* were significantly increased on 30-dpf. Nevertheless, the quantum of the increase was the maximum in vitamin-E diet-fed *L. rohita*. The vitamin-E and probiotic diets fed *L. rohita* elucidated 1.40 and 1.16 folds higher phagocytic activity than the control on 30-dpf. The results indicated that vitamin-E was able to enhance the phagocytic activities of *L. rohita* better when compared to the commercial probiotic or control diets. Likewise, earlier studies on the immunomodulatory effect of dietary vitamin-C [53, 54] and immunostimulants [55] on fish showed, more or less, similar effects. In the present study, the *A. hydrophila* N10P challenge caused a significant increase in serum phagocytic activities on 7-dpi, with the maximum in the vitamin-E group. It suggested that vitamin-E have a better ability to modulate the phagocytic activities of challenged fish. However, the serum phagocytic activities became normal as that of unchallenged control on 15-dpi in all treatment groups. Alike the present study, the fish fed with immunostimulants [16, 53] or probiotics [56] followed by *A. hydrophila* challenge exhibited increased phagocytic activities compared to control. The ROB and MPO activities are the major functional indicators of phagocytic cells in the system [57] and both these activities were higher in the vitamin-E diet-fed *L. rohita* of this study. Hence, the phagocytic system might be playing a major role in rendering resistance to aeromoniasis.

From the data generated on growth indices and non-specific immune responses, it was observed that the vitamin-E diet-fed *L. rohita* gave markedly better results in terms of SGR, FCR, ceruloplasmin, antiprotease, myeloperoxidase and phagocytic activities. While the ROB activity was better in probiotic diet-fed *L. rohita* only during the feeding regimen. These findings indicated that dietary supplementation vitamin-E may improve the growth indices and non-specific immune responses of *L. rohita* better against the pathogenic *A. hydrophila* and enhance the overall health status than the commercial probiotics.

5. Conclusion

In general, the dietary supplementation of vitamin-E at 100 mg/kg feed and commercial probiotics (Rhodomax™) at 5 g/kg feed caused a significant increase in non-specific immune parameters compared to control, which may serve as an important management tool in carp aquaculture. Nevertheless, the results suggested that vitamin-E feeding might be priming the immune system better in such a way that by the time of infection, the fish might mount effective defensive responses, thereby, protecting itself from the pathogen. Overall, the vitamin-E at 100 mg/kg feed could be supplemented in diets for the positive immune response.
responses in \textit{L. rohita}. However, the cost-effectiveness, ease of availability, mode of application at appropriate doses, etc. are to be considered for economic benefits.

Compliance with ethical standards

All of the methods, animal care, and experimental protocols used in this study followed the relevant guidelines and regulations of the Government of India. All the experimental ethical protocols with \textit{Labeo rohita} as an experimental animal were approved (Ref. No. FFS/Adm-6/2012/March 2012) by the University.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the Vice-Chancellor, West Bengal University of Animal and Fishery Sciences, Kolkata for providing the necessary infrastructure facilities to carry out the work. The research work was supported by the Indian Council of Agricultural Research, Government of India, New Delhi under the Niche Area of Excellence program vide Grant F. 10(12)/2012-EPD dated 23.03.2012.

References

[1] V.P. Saini, M.L. Ojha, M.C. Gupta, P. Nair, A. Sharma, V. Luhar, Effect of dietary probiotic on growth performance and disease resistance in \textit{Labeo rohita} (Ham.) fingerlings, Int. J. Fish. Aquat. Stud. 1 (6) (2014) 07–11.

[2] P. Jayasankar, Present status of freshwater aquaculture in India - A review, Indian J. Fish. 65 (4) (2018) 157–165, https://doi.org/10.21077/ijf.2018.65.4.81300-20.

[3] M.G. Bondad-Reantaso, R.P. Subasinghe, J.R. Arthur, K. Ogawa, S. Chinabut, R. Adlard, Z. Tan, M. Shariff, Disease and health management in Asian aquaculture, Vet. Parasitol. 132 (3–4) (2005) 249–272, https://doi.org/10.1016/j.vetpar.2005.07.005.

[4] A.V. Joseph, R.S. Sasidharan, H.P. Nair, S.G. Bhat, Occurrence of potential pathogenic \textit{Aeromonas} species in tropical seafood, aquafarms and mangroves off Cochin coast in South India, Vet. World. 6 (6) (2013) 300–306, https://doi.org/10.5455/vetworld.2013.300-306.

[5] T.J. Abraham, S. Sarker, G. Dash, A. Patra, H. Adikesavalu, \textit{Chryseobacterium} sp. PLJ\textsubscript{2} and \textit{Aeromonas hydrophila} co-infection in pacu, \textit{Piabactus brachypomus} (Cuvier, 1817) fries cultured in West Bengal, India, Aquaculture 473 (2017) 223–227, https://doi.org/10.1016/j.aquaculture.2017.02.016.

[6] S. Magnoed, P. Singh, M.H. Saimoon, K. Munir, Emerging role of immunostimulants in combating the disease outbreak in aquaculture, Int. Aquat. Res. 3 (2011) 3.

[7] E. Vallejos-Vidal, F. Reyes-López, M. Teles, S. MacKenzie, The response of fish to immunostimulant diets, Fish Shellfish Immunol 56 (2016) 34–69, https://doi.org/10.1016/j.fsi.2016.06.028.

[8] R. HariKrishnahan, C. Balasundaram, M.S. Heo, Fish health aspects in grouper aquaculture, Aquaculture 320 (1–2) (2011) 1–21, https://doi.org/10.1016/j.aquaculture.2011.07.022.

[9] R. Castro, C. Tafalla, Overview of fish immunity. Mucosal Health in Aquaculture, Academic Press, Spain, 2015, pp. 3–54, https://doi.org/10.1016/B978-0-12-417186-2.00002-9.

[10] A.E.A. Bekhit, J.D. Morton, C.O. Dawson, J.H. Zhao, H.Y. Lee, Impact of maturity on the physicochemical and biochemical properties of Chinook salmon roe, Food Chem 117 (2) (2009) 318–325, https://doi.org/10.1016/j.foodchem.2009.04.009.
L. Priyadarsani et al.
Fish and Shellfish Immunology Reports 2 (2021) 100013

[1] L.G. Wang, E.C. Li, J.G. Qin, Z.Y. Du, N. Yu, Y.Q. Kong, D.X. Feng, L.Q. Chen, Effect of oxidized fish oil and α-tocopherol on growth, antioxidation status, serum immune enzyme activity and resistance to Aeromonas hydrophila challenge of Chinese mitten crab Eriochir sinensis, Aquac. Nutr. 21 (4) (2015) 414–424, https://doi.org/10.1111/ana.12171.

[2] J.H. Pan, L. Feng, W.D. Jiang, P. Wu, S.Y. Kuang, L. Tang, Y.A. Zhang, X.Q. Zhou, Y. Liu, Vitamin E deficiency depressed fish growth, fish immunity and structural integrity of immune organs in grass carp (Ctenopharyngodon idella): referring to NF-κB, TOR and Nrf2 signaling, Fish Shellfish Immunol. 60 (2017) 219–236, https://doi.org/10.1016/j/fsi.2016.11.044.

[3] N.V. H. the use of probiotics in aquaculture, J. Appl. Microbiol. 119 (4) (2015) 917–935, https://doi.org/10.1111/jam.12886.

[4] M. Anzhalah, H.A. Gohad, H.A. Moobudda, H.A. Abo-State, Effect of probiotics on performance and nutrients digestibility of Nile tilapia (Oreochromis niloticus) fed low protein diets, Nature Sci. 8 (5) (2010) 46–53.

[5] V. Selvaraj, T. Sampath, V. Sekar, Administration of yeast glucan enhances survival and non-specific immune parameters in carp (Cyprinus carpio) infected with Aeromonas hydrophila, Fish Shellfish Immunol. 19 (6) (2005) 293–306, https://doi.org/10.1016/j/fsi.2005.01.001.

[6] A. Tewary, B.C. Patra, Use of Vitamin C as an immunostimulant-effect on growth, nutritional quality, and immune response of Labeo rohita (Ham.), Fish Physiol. Biochem. 34 (2008) 251–259, https://doi.org/10.1007/s10698-007-9184-z.

[7] S. Sahu, B.K. Das, B.K. Mishra, J. Pradhan, N. Sarangi, Effect of Magnifera indica kernel as a feed additive on immunity and resistance to Aeromonas hydrophila in Labeo rohita fingerlings, Fish Shellfish Immunol. 23 (2007) 109–118, https://doi.org/10.1016/j/fsi.2006.09.009.

[8] S.K. Nayak, P. Swain, S.C. Mukherjee, Effect of dietary supplementation of probiotic and vitamin C on the immune response of Indian major carp, Labeo rohita (Hamilton), Fish Shellfish Immunol. 23 (4) (2007) 892–896, https://doi.org/10.1016/j/fsi.2007.02.008.

[9] S.S. Giri, S.S. Sen, V. Sukumaran, Effects of dietary supplementation of potential probiotic Pseudomonas aeruginosa VSG-2 on the innate immunity and disease resistance of tropical minor fish, Labeo rohita, Fish Shellfish Immunol. 22 (6) (2012) 1135–1140, https://doi.org/10.1016/j/fsi.2012.03.019.

[10] J. Ortuno, M.A. Esteban, J. Meseguer, High dietary intake of α-tocopherol acetate non-specific immune response and growth of Sparus aurata L., Fish Shellfish Immunol. 10 (2000) 285–297, https://doi.org/10.1016/S0921-8109(99)00038-8.

[11] W.E. Ricker, Computation and interpretation of biological statistics of fish populations, Bull. Fish. Res. Bd. Can. 191 (1975) 1–382.

[12] S. Maqsood, P. Singh, M.H. Samoon, A.K. Prusty, P. Das, K. Paniprasad, K.N. Mohanta, S. Kumar, Effect of dietary supplemented andrographolide on growth, non-specific immune parameters and resistance against Aeromonas hydrophila in Labeo rohita (Hamilton), Fish Shellfish Immunol. 35 (5) (2013) 1433–1441, https://doi.org/10.1016/j/fsi.2013.08.002.

[13] R.C. Bull, Trace minerals and immunity. Beef Cattle Handbook, E-publishing Inc. BCH-3543, Wisconsin, USA, 1999, pp. 1–5.

[14] S. Jain, V. Guatam, S. Nanda, S. Kapil, A. Prasad, BFE920 and BCH-5454, as diagnostic tool, J Pharm Biomed Anal 31 (2001) 118–127, https://doi.org/10.1016/S0731-7085(00)00385-6.

[15] N. Wu, Y.L. Song, B. Wang, X.Y. Zhang, X.J. Zhang, Y.L. Wang, Y.Y. Cheng, D. Chen, X.Q. Xia, Y.S. Lu, Y.A. Zhang, Fish gut-liver immunity during homeostasis or immune response regulation by the integrative transcriptome and proteome studies, Sci. Rep. 6 (2016) 1–17, https://doi.org/10.1038/srep36048.

[16] B. Magnadottir, Innate immunity of fish (overview), Fish Shellfish Immunol. 20 (2) (2006) 137–151, https://doi.org/10.1016/j.fsi.2005.09.006.

[17] J.K. Pandya, M. DeBonee, M.G. Corradini, M.E. Camire, D.J. McClements, A. Ottir, Innate immunity of fish (overview), Fish Shellfish Immunol. 20 (2) (2006) 137–151, https://doi.org/10.1016/j.fsi.2005.09.006.

[18] J.K. Jena, Pathophysiology of experimental Aeromonas hydrophila infection in Puntius sarana: early changes in blood and aspects of the innate immune-related gene expression in survivors, Vet. Immunol. Immunopathol. 142 (2) (2011) 207–218, https://doi.org/10.1016/j.vetimm.2011.05.017.

[19] A. Das, P.K. Sahoo, B.R. Mohanty, J.K. Jena, Pathophysiology of experimental Aeromonas hydrophila infection in Puntius sarana: early changes in blood and aspects of the innate immune-related gene expression in survivors, Vet. Immunol. Immunopathol. 142 (2) (2011) 207–218, https://doi.org/10.1016/j.vetimm.2011.05.017.

[20] R.C. Bull, Trace minerals and immunity. Beef Cattle Handbook, E-publishing Inc. BCH-3543, Wisconsin, USA, 1999, pp. 1–5.

[21] R.P. Wilkie-Grant, N.J.M. Magon, D.T. Harwood, A.J. Kettle, M.C. Visser, C. Winterbourne, M.B. Hampton, Myeloperoxidase-dependent lipid peroxidation promotes the oxidative modification of cytosolic proteins in phagocytic neutrophils, J. Biol. Chem. 290 (15) (2015) 9966–9965, https://doi.org/10.1074/jbc.M114.514302.

[22] P. Kumar, V.I. Kaur, A. Tyagi, S. Nayar, Probiotic potential of putative lactic acid bacteria isolated from the fish gut: immune modulation in Labeo rohita (Ham), in: P. Jithendran, R. Sarawathy, C.P. Balasubramanian, K.P. Kumarranagur Vasagan, V. Jayasankar, R. Raghavan (Eds.), BRAQCON 2019: World Brackishwater Aquaculture Conference, J. Coast. Res. 86, 2019, pp. 119–127, https://doi.org/10.2112/JCOASTRES.86.3.119.

[23] B.R. Beck, D. Kim, J. Jeon, S.M. Lee, H.K. Kim, O.J. Kim, The effects of combined dietary probiotics Lactococcus lactis BFE920 and Lactobacillus plantarum FGL0001 on innate immunity and disease resistance in olive flounder (Paralichthys olivaceus), Fish Shellfish Immunol. 42 (2) (2016) 177–185, https://doi.org/10.1016/j.fsi.2015.10.035.

[24] J.K. Pandya, M. DeBonee, M.G. Corradini, M.E. Camire, D.J. McClements, A. Ottir, Innate immunity of fish (overview), Fish Shellfish Immunol. 20 (2) (2006) 137–151, https://doi.org/10.1016/j.fsi.2005.09.006.

[25] K.P. Sahoo, R.P. Ramas, K.P. Prasad, K. Kumar, E. Nilavan, S. Kumar, Effect of dietary supplemented andrographolide on growth, non-specific immune parameters and resistance against Aeromonas hydrophila in Labeo rohita (Hamilton), Fish Shellfish Immunol. 35 (5) (2013) 1433–1441, https://doi.org/10.1016/j.fs...
against *Aeromonas hydrophila*, *Aquaculture* 275 (1-4) (2008) 26–33, https://doi.org/10.1016/j.aquaculture.2007.12.022.

[56] R. Kumar, S.C. Mukherjee, R. Ranjan, S.K. Nayak, Enhanced innate immune parameters in *Labeorohita* (Ham.) following oral administration of *Bacillus subtilis*, *Fish Shellfish Immunol* 24 (2008) 168–172, https://doi.org/10.1016/j.fsi.2007.10.008.

[57] M. Reyes-Becerril, T. López-Medina, F. Ascencio-Valle, M.A. Esteban, Immune response of gilthead seabream (*Sparus aurata*) following experimental infection with *Aeromonas hydrophila*, *Fish Shellfish Immunol.* 31 (4) (2011) 564–570, https://doi.org/10.1016/j.fsi.2011.07.006.