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Research Article

Enhancing Nile tilapia health status and immunity against *Aeromonas hydrophila* with a combination of probiotics and immunostimulants (Vimolert®)

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Abstract

This study explores the potential of the probiotics and immunostimulants combination (Vimolert®) to enhance the blood biochemistry profile, histopathology, and immune response of Nile Tilapia against *Aeromonas hydrophila* infection. A total of 150 Nile tilapia were used and divided into five groups, including a negative control group, a positive control group, and three groups fed with Vimolert®-supplemented diets at different doses for four weeks. After this period, groups 2 to 5 were infected with *A. hydrophila*. The biochemistry parameters including alanine aminotransferase, Aspartate aminotransferase, alkaline phosphatase, urea, and creatinine significantly increased in the positive control group compared to the treated groups. The total protein and albumin levels were significantly lower in the positive control group than in the treated groups, while the globulin level was higher in the treated groups than in the positive control group. Regarding the immune response, which includes nitroblue tetrazolium and lysozyme activity, it was observed that the groups receiving Vimolert® experienced significant increases in the fourth and sixth weeks compared to the positive control group. The histopathological examination unveiled that the positive control group exhibited pronounced pathological alterations, including degeneration and necrosis in the liver tissue, necrosis, and a reduction in hematopoietic cells in the kidney tissue, as well as edema, degeneration, and necrosis in the muscle tissue. Furthermore, there were infiltrations of mononuclear cells and melanomacrophages between the necrotic muscle fibers. In contrast, the treated group displayed milder to moderately altered tissue conditions. Group 4 had the maximum protection after infection compared to the other treated groups. In conclusion, this study underscores the potential of Vimolert® (a mixture of probiotics and immunostimulants) in improving the immune response and resistance of Nile tilapia against *A. hydrophila* infection. This finding holds significant promise for enhancing the health status and survival of Nile Tilapia in the aquaculture industry, thereby contributing to the sustainability and productivity of this industry.

Keywords: Nile tilapia, Preventive measures, *Aeromonas hydrophila* infection, Biochemistry profile, Histopathological changes, Immune response

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Introduction

Aquatic creatures, especially fish, are prone to a wide range of diseases. Bacteria contribute significantly to fish losses in aquaculture, accounting for more than 50% of these losses (Miccoli *et al*., 2021). Inadequate management practices such as high stocking density, overfeeding, and poor water quality substantially increase the susceptibility of aquatic animals to various diseases (Banerjee *et al*., 2017). Tropical countries have a disproportionate burden of disease outbreaks, resulting in substantial economic losses (Robar *et al*., 2010).

Among these diseases, *Aeromonas hydrophila* stands out as a major cause of outbreaks in freshwater fish, characterized by severe symptoms (Plumb, 1999; Nicholson *et al*., 2020). This disease is known by several synonyms, including Aeromonad septicemia and Red Pest (Yardimci and Aydin, 2011). Fish infected with *A. hydrophila* naturally exhibit significantly elevated levels of the enzyme GOT and bilirubin compound in their blood serum, along with higher levels of LDH, BUN, and creatinine. Additionally, a decrease in glucose concentration has been observed (Aydin and Ciltas, 2004).

When *Oreochromis spp.* were infected with 1×10^7 CFU/ml of viable *A. hydrophila* in 0.1 ml serum ALT, protein, albumin, and globulin levels in the blood decreased, as reported by Fayza *et al*. (2011). Das *et al*. (2011) also documented a significant decline in plasma glucose levels following an intraperitoneal challenge with *A. hydrophila* (2.24×10^7 CFU/fish). In pacu fish challenged with 1×10^8 CFU of *A. hydrophila*, total serum protein and globulin content increased one week after the bacterial infection, indicating the elevation in defense proteins, as reported by Biller-Takahashi *et al*. (2013).

In fish infected with *A. hydrophila*, serum alkaline phosphatase, total serum protein, albumin, and bilirubin levels significantly increased following IP injection at a concentration of (3×10^7 CFU/ml) (Pal *et al*., 2015). Supplementing *O. niloticus* and *Mugil cephalus* with 0.1 or 0.2% Biogen® led to a significant increase in serum albumin and globulin levels and a decrease in serum cholesterol and glucose levels compared to the control group (Elam, 2004). Indian major Carp pre-challenged with *A. hydrophila* and treated with *B. subtilis* exhibited increased serum protein and globulin levels, and a declined A/G ratio, with higher AST and ALT activity during *A. hydrophila* challenge (Kumar *et al*., 2006). Nile tilapia supplemented with Biogen® at levels of 1, 2, 3, and 4 g/kg feed showed lower levels of transaminase enzymes ALT and AST in the serum (Soltan *et al*., 2016). *B. subtilis* treatment improved alkaline phosphatase (ALP) and reduced serum Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in Nile tilapia compared to the control group (Tang *et al*., 2017). Additionally, Nile tilapia that were fed diets containing commercial immunostimulants

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(probiotics) for 8 weeks demonstrated a significant increase in serum protein, albumin, globulin, and A/G ratio (Kord *et al*., 2021).

Despite the significant economic losses and health risks posed by *Aeromonas hydrophila* in aquaculture, there is currently a lack of comprehensive research on the potential benefits of combining probiotics and immunostimulants in preventing this infection. Our study aims to address this gap by investigating the effects of such a combination on the biochemistry profile, histopathology, and immune response of Nile tilapia.

In light of this research gap, our study seeks to determine whether the combination of probiotics and immunostimulants (Vimolert®) can enhance the biochemistry profile, histopathology, and immune response of Nile tilapia, ultimately aiming to prevent *Aeromonas hydrophila* infection.

Materials and methods

Experimental design

The study involved 150 Nile tilapia (*Oreochromis niloticus*) with an initial average body weight of 55.00±2.00g and an average total length of 10±3cm, sourced from the Central Laboratory for Aquaculture Researches (CLAR) in Abou Hammad City, Sharkia Governorate. It's crucial to note that these fish were carefully selected for their initial good health and absence of visible external lesions. To ensure proper acclimation to the experimental conditions, all fish underwent a two-week acclimation period in glass aquaria. During this phase, close monitoring confirmed their adaptation to the laboratory environment. The allocation of fish into experimental groups was carried out meticulously to ensure statistical robustness. Specifically, the 150 fish were divided into 5 groups, each comprising three replicates, with 10 fish in each replicate. This design aimed to minimize bias and to provide a solid foundation for drawing statistically sound conclusions from our experimental data. By clearly specifying the number of fish in each group, the organization into replicates, and the rationale behind this design, we aim to enhance the transparency and comprehensibility of our experimental setup (Table 1). The handling of fish and all experiments adhered to the regulations set by the Animal Ethics Review Committee of Suez Canal University (AERC-SCU), Egypt

Diets

Basal diet

The basal diet utilized in this study was thoughtfully chosen to meet the nutritional requirements of Nile tilapia. It consisted of 30% crude protein and 3000 kcal/kg metabolizable energy, sourced from the Fish Research Unit at the Faculty of Veterinary Medicine at Zagazig University. The diet consisted of fish meal (75 g/kg diet), meat meal (150 g/kg diet), corn (350 g/kg diet), soybean (200 g/kg diet), flour (100 g/kg diet), bran (73.5 g/kg diet), oil (50 g/kg diet), vitamins (0.75 g/kg diet), and minerals (0.75 g/kg diet).

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Experimental diet

The experimental diet, a crucial aspect of this study, was supplemented with Vimolert®, which is a commercial product that contains a unique combination of probiotics and immunostimulants. Vimolert® is a soluble powder manufactured by Cairo-Bio-Pharm Factory located in Block 5, Industrial Zone 6A South Aljumekih Extention-10th of Ramadan Alsharqia Egypt. It falls under the pharmacological category of enhancing cellular and non-cellular defense. The composition of Vimolert® includes thymol crystals (at least 5000 mg), ginseng (at least 3000 mg), nano zinc (at least 4000 mg), bifidobacteria (at least 1×10^8 CFU), bee pollen (at least 1000 mg), Sodium butyrate (at least 20000 mg), *Enterococcus spp*. (at least 1×10^8 CFU, Mannan oligosaccharides (at least 100000 mg), Beta-glucan blend (at least 100000 mg), and Lysozyme enzyme (at least 10000 IU). Vimolert® was integrated into the preventive diet at concentrations of 2.5 g/100g, 3 g/100g, and 3.5 g/100g of feed (Table 1).

Table 1: The plan for conducting the experiment.

	Group	No. of Fish	Treatments (type, dose and duration)	
			Vimolert for 4 weeks	**Intramuscular injection of *A.hydrophila* bacteria After 4 weeks**
Control Negative	1	30	Not given	Not given
Control Positive	2	30	Not given	0.5 mL (1×10^7 CFU/mL)
Experimental groups	3	30	2.5 g/100g of feed	0.5 mL (1×10^7 CFU/mL)
	4	30	3 g/100g of feed	0.5 mL (1×10^7 CFU/mL)
	5	30	3.5 g/100g of feed	0.5 mL (1×10^7 CFU/mL)

Experimental infection

Upon completing the 4-week feeding trial, the response of groups (2-5) to the pathogenic strain *A. hydrophila* was assessed through experimental infection. The pathogenic strain used in the experiment was obtained from a previous study isolated from Nile tilapia and can be found in the GenBank databases under the accession numbers of OQ253432 (Salah *et al*., 2023). The bacterial inoculum was prepared by culturing on TSB (Oxoid) for 24 hours at 37°C, harvesting through centrifugation, washing twice, resuspending in sterile 0.85 % saline, and counting via the McFarland standard technique. The fish were then administered an intraperitoneal injection of 0.5 mL of *A. hydrophila* suspension, containing 10^7 bacteria/mL, following the protocol described by Fayza *et al*. (2011). To determine the relative level of protection (RLP) among the challenged fish, the mortality (%) of the treated group and control group was measured using the formula as outlined by Ruangpan *et al*. (1986):

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RLP (%)= (1 − (mortality (%) of treated group/mortality (%) of the control group) × 100

Blood samples

The blood samples were collected from the caudal vessels of each fish group at 4 and 6 weeks after the beginning of the experiment. The blood was drawn into sterile and labeled test tubes without anticoagulant to separate the serum for further biochemical analysis, following the method described by Coles (1986).

The levels of hepatic function

Serum ALT activity was determined using the calorimetric method of Reitman and Frankel (1957) and expressed in IU/l. Serum AST activity was determined using the kinetic method of Tietz (1976) and expressed in IU/l. Serum ALP activity was also determined using the kinetic method of Tietz (1976) and expressed in IU/l The concentrations of serum total and direct bilirubin were determined colorimetrically according to the method of Tietz (1995), and the indirect bilirubin concentration was calculated mathematically by subtracting direct bilirubin from total bilirubin. Bilirubin concentrations were expressed in the serum as mg/dl.

Serum protein profiles and A/G ratio

The concentrations of serum total protein and albumin were measured using the method described by Vassault *et al*. (1986) and Doumas and Biggs (1976), respectively. The concentrations were expressed in grams per deciliter (g/dl). The concentration of serum globulins was calculated by subtracting the albumin value from the total protein value and expressed in g/dl.

The levels of kidney function test

Serum urea and creatinine concentrations were measured using the method described by Vassault *et al.* (1986) and Henry (1974), respectively. The concentrations of urea and creatinine were expressed as mg/dl in the serum.

Immunological parameters

NBT levels and Lysozyme activity were chosen in this study because they offer valuable insights into the fish's immune function and overall health. These parameters are particularly relevant when studying the immune response of fish to pathogens like *A. hydrophila*, as they help assess the fish's ability to mount an effective defense against bacterial infections.

Nitroblue tetrazolium (NBT) levels

Half a milliliter of whole blood was collected from the caudal vein using syringes containing heparin. The homogenized blood was utilized to measure Nitroblue tetrazolium (NBT) levels, following the protocol described by Studnicka *et al*. (1985).

The levels of Lysozyme activity

Lysozyme activity was assessed using a turbidity assay with chicken egg lysozyme (Sigma) as a standard and lyophilized Micrococcus lysodeikticus in 0.04 M sodium phosphate buffer

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(pH=5.75) as the substrate. To carry out the assay, 50 ml of serum was added to 2 ml of the bacterial suspension, and the reduction in absorbance at 540 nm was measured after incubation for 0.5 and 4.5 min at 22ºC, following the method described by Parry *et al*. (1965).

Histopathological examination

Specimens of liver, kidneys, and muscle were removed from the experimental fish and prepared following the methods described by Bancroft *et al*. (1996).

Statistical analysis

The data collected from the experiment were analyzed using the statistical software, SPSS 27.0 (SPSS Inc., USA). A significance level of $p \leq 0.05$ was employed, and the analysis was conducted using one-way analysis of variance (ANOVA). The results were presented as mean±standard error (SE). Post hoc tests are a series of pairwise comparisons that help identify which specific groups differ significantly from each other after a significant ANOVA result. Different letters were used to represent significantly different means within the same column. The letter "a" was assigned to the highest mean value. The statistical method for determining significant differences between means was based on the approach described by Tamhane and Dunlop (2000).

Results

This study investigates the potential of a probiotics and immunostimulants combination (Vimolert®) to enhance the biochemistry profile, histopathology, and immune response of Nile tilapia against *Aeromonas hydrophila* infection.

The impact of administrating this combination of probiotics and immunostimulants on the survival and relative level of protection against A. hydrophila exposure

In the control negative group (group 1), no mortality occurred, resulting in a 100 % survival rate. In contrast, the control positive group showed a 50 % mortality rate. Group 3, which received 2.5 g Vimolert® per 100g of feed (group 3) exhibited 10 % mortality rate, and an 80% relative level of protection against *A. hydrophila*. In Group 4, where 3 grams of Vimolert® per 100 grams of feed (group 4) were administered, the mortality rate was 3.33%, indicating a high survival rate of 96.67% and a relative level of protection of 93.43%. In contrast, Group 5, which received 3.5 grams of Vimolert® per 100 grams of feed (group 5), had a lower mortality rate of 6.67%, a survival rate of 93.33%, and a relative level of protection of 86.66%. (Table 2).

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Table 2: Total mortality, survivability percent and relative level of protection of infected Nile tilapia with *A. hydrophila*.

Treatment/Groups	Total mortality	Survivability (%)	Relative level of protection
	NO.	Percent	Percentage
Group 1	0	100	-
Group 2	15	50.00	-
Group 3	3	90.00	80
Group 4	1	96.67	93.34
Group 5	2	93.33	86.66

Group.(1): control negative, Group.(2): infected with *A. hydrophila* 0.5 mL (1×10^7 CFU/mL), Group.(3): vimolert 2.5 g/100g of feed then *A. hydrophila* 0.5 mL (1×10^7 CFU/mL), Group.(4): vimolert 3 g/100g of feed then *A. hydrophila* 0.5 mL (1×10^7 CFU/mL), and Group.(5): vimolert 3.5 g/100g of feed then *A. hydrophila* 0.5 mL (1×10^7 CFU/mL).

The levels of hepatic function tests

During the fourth week of the experiment, no significant changes were observed in the levels of serum ALT, AST, ALP activities, and bilirubin concentrations (including total, direct, and indirect) in all groups compared to the control group (group 1), as indicated in Table 3. However, by the sixth week of the experiment, a highly significant increase ($p\leq0.01$) in the serum ALT, AST, ALP activities, and bilirubin (total, direct, and indirect) concentration was observed in group 2, with a slight increase in groups 3, 4, and 5. The smallest increase in these parameters was observed in groups 3 and 4. These changes were also compared to the control group (Group 1).

Table 3: Serum liver function concentration (mean values ±SE) of Nile tilapia in Groups (1-5) at 4th and 6th weeks of the experiment ($p\leq0.05$).

Sample groups	Parameters											
	ALT (IU/L)		AST (IU/L)		ALP (IU/L)		Total bilirubin (mg/dl)		Direct bilirubin (mg/dl)		Indirect bilirubin (mg/dl)	
	4th w	6th w	4th w	6th w	4th w	6th w	4th w	6th w	4th w	6th w	4th w	6th w
Group 1	24.79^{a} ± 0.26	24.19^{d} ± 0.05	110.28^{a} ± 4.88	114.27^{d} ± 3.19	25.16^{a} ± 0.25	25.06^{c} ± 0.42	0.44^{a} ± 0.01	0.44^{d} ± 0.01	0.16^{a} ± 0.01	0.17^{d} ± 0.01	0.28^{a} ± 0.01	0.27^{d} ± 0.01
Group 2	24.65^{a} ± 0.22	45.73^{a} ± 1.03	110.04^{a} ± 4.91	199.63^{a} ± 3.99	24.98^{a} ± 0.18	85.25^{a} ± 3.41	0.43^{a} ± 0.02	0.99^{a} ± 0.02	0.15^{a} ± 0.02	0.36^{a} ± 0.01	0.28^{a} ± 0.03	0.63^{a} ± 0.03
Group 3	24.31^{a} ± 0.32	27.96^{c} ± 1.31	105.24^{a} ± 3.23	159.83^{b} ± 0.64	24.88^{a} ± 0.25	30.00^{c} ± 0.77	0.42^{a} ± 0.01	0.53^{c} ± 0.00	0.15^{a} ± 0.01	0.18^{c} ± 0.02	0.27^{a} ± 0.01	0.34^{c} ± 0.02
Group 4	24.12^{a} ± 0.43	25.16^{d} ± 0.48	106.47^{a} ± 3.02	116.40^{cd} ± 1.80	24.89^{a} ± 0.27	29.16^{c} ± 0.58	0.41^{a} ± 0.00	0.58^{c} ± 0.02	0.15^{a} ± 0.01	0.20^{c} ± 0.01	0.27^{a} ± 0.01	0.38^{bc} ± 0.03
Group 5	24.32^{a} ± 0.19	31.21^{b} ± 0.44	108.50^{a} ± 3.23	124.57^{c} ± 3.71	24.88^{a} ± 0.45	39.02^{b} ± 0.65	0.42^{a} ± 0.01	0.70^{b} ± 0.02	0.17^{a} ± 0.01	0.28^{b} ± 0.02	0.26^{a} ± 0.01	0.42^{b} ± 0.03

Means at the same column followed by different letters were significantly different at $p\leq0.05$ and the highest value was represented with the letter a.

Group (1):control negative, **Group** (2):infected with *A. hydrophila* 0.5 mL (1×10^7 CFU/mL), **Group** (3):vimolert 2.5 g/100g of feed then *A. hydrophila* 0.5 mL (1×10^7 CFU/mL), Group (4):vimolert 3 g/100g of feed then *A. hydrophila* 0.5 mL (1×10^7 CFU/mL), Group (5):vimolert 3.5 g/100g of feed then *A. hydrophila* 0.5 mL (1×10^7 CFU/mL). Infection by *A. hydrophila* was done by the end of 4th week of experiment. W: week.

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Serum protein profiles and A/G ratio

In the fourth week of the experiment, significant increases ($p \leq 0.05$) were observed in the serum levels of total protein, albumin, and globulin in Groups 2, 3, 4, and 5 when compared to the control group (group 1), as indicated in Table 4. Group 2 exhibited the smallest increase in these parameters, while no significant changes were observed in the A/G ratio. However, in the sixth week of the experiment, a significant decrease was observed in the serum levels of total protein, albumin, and A/G ratio in groups 2, 3, 4, and 5 when compared to the control group (group 1), as indicated in Table 5. Group 2 had the lowest values for these parameters. Furthermore, a significant increase was observed in the serum levels of globulin in groups 2, 3, 4, and 5, with the highest levels observed in group 4.

Table 4: Serum protein profile and A/G ratio (mean values±SE) of Nile tilapia in Groups (1-5) at 4th and 6th weeks of the experiment ($p \leq 0.05$).

Sample groups	Parameters							
	Total proteins (g/dl)		Albumin (g/dl)		Globulins (g/dl)		A/G Ratio	
	4th w	6th w	4th w	6th w	4th w	6th w	4th w	6th w
Group 1	2.99^{b}±0.03	3.13^{ab}±0.05	2.04^{b}±0.03	2.15^{a}±0.07	0.95^{c}±0.02	0.98^{b}±0.05	2.16^{a}±0.05	2.22^{a}±0.16
Group 2	3.01^{b}±0.07	1.50^{c}±0.15	2.02^{b}±0.02	0.49^{d}±0.07	0.99^{bc}±0.07	1.02^{b}±0.20	2.08^{a}±0.16	0.56^{b}±0.16
Group 3	3.81^{a}±0.20	2.91^{b}±0.02	2.65^{a}±0.12	0.98^{c}±0.04	1.16^{a}±0.10	1.93^{a}±0.03	2.31^{a}±0.09	0.51^{b}±0.03
Group 4	3.73^{a}±0.19	3.06^{ab}±0.07	2.67^{a}±0.19	1.05^{c}±0.06	1.08^{abc}±0.03	2.02^{a}±0.07	2.47^{a}±0.16	0.52^{b}±0.04
Group 5	3.72^{a}±0.22	3.25^{a}±0.11	2.59^{a}±0.18	1.28^{b}±0.12	1.14^{ab}±0.05	1.97^{a}±0.05	2.28^{a}±0.12	0.65^{b}±0.07

Means at the same column followed by different letters were significantly different at $P \leq 0.05$ and the highest value was represented with the letter a.

Group (1):control negative, **Group** (2):infected with *A. hydrophila* 0.5 mL (1×10^7 CFU/mL), **Group** (3):vimolert 2.5 g/100g of feed then *A. hydrophila* 0.5 mL (1×10^7 CFU/mL), **Group** (4):vimolert 3 g/100g of feed then *A. hydrophila* 0.5 mL (1×10^7 CFU/mL), **Group** (5):vimolert 3.5 g/100g of feed then *A. hydrophila* 0.5 mL (1×10^7 CFU/mL). Infection by *A. hydrophila* was done by the end of 4th week of experiment. W: week.

Table 5: Some kidney function tests (mean values±SE) of Nile tilapia in the groups received Vimolert® at 4th and 6th weeks of the experiment ($p \leq 0.05$).

Sample Groups	Parameters			
	Urea (mgdl)		Creatinine (mg/dl)	
	4th w	6th w	4th w	6th w
Group 1	6.06^{a} ±0.15	5.87^{b}±0.34	0.54^{a}±0.02	0.55^{b}±0.03
Group 2	5.82^{ab}±0.30	8.81^{a}±0.19	0.55^{a}±0.03	1.02^{a}±0.03
Group 3	5.26^{bc}±0.07	5.51^{bc}±0.09	0.36^{c}±0.02	0.40^{c}±0.03
Group 4	4.62^{c}±0.29	4.98^{c}±0.32	0.34^{c}±0.01	0.38^{c}±0.00
Group 5	5.04^{bc}±0.34	5.53^{bc}±0.16	0.44^{b}±	0.45^{c}±0.03

Means at the same column followed by different letters were significantly different at $p \leq 0.05$ and the highest value was represented with the letter a. **Group** (1):control negative, **Group** (2):infected with *A. hydrophila* 0.5 mL (1×10^7 CFU/mL), **Group** (3):vimolert 2.5 g/100g of feed then *A. hydrophila* 0.5 mL (1×10^7 CFU/mL), **Group** (4):vimolert 3 g/100g of feed then *A. hydrophila* 0.5 mL (1×10^7 CFU/mL), **Group** (5):vimolert 3.5 g/100g of feed then *A. hydrophila* 0.5 mL (1×10^7 CFU/mL). Infection by *A. hydrophila* was done by the end of 4th week of experiment. W: week.

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Kidney function test levels

During the fourth week of the experiment, there was a slight decline in the serum levels of urea and creatinine across all groups, relative to the control group (group 1), with concentrations nearly within the normal range. In contrast, group 2 exhibited a significant increase in serum concentrations of urea and creatinine during the sixth week of the experiment when compared to the control group (group 1), as depicted in Table 5. On the other hand, groups 3, 4, and 5 showed a remarkable decline in the serum concentrations of urea and creatinine when compared to the control group.

Immunological parameters

During the fourth week of the experiment, significant increases in the NBT values and lysozyme activity were observed in groups 3, 4, and 5 when compared to the control group (group 1), as shown in Table 6. However, no significant changes were observed in group 2. In the sixth week of the experiment, there was a significant decrease in NBT values and lysozyme activity in group 2 when compared to the control group (group 1). In contrast, groups 3, 4, and 5 exhibited a significant increase in the NBT and lysozyme activity compared to group 2 (Table 6).

Table 6: Nitroblue tetrazolium (NBT) values and lysozyme activity (mean values±SE) of Nile tilapia in Groups (1-5) at 4th and 6th weeks of the experiment ($p \leq 0.05$).

Sample groups	Parameters			
	NBT Values (mg mL^{-1})		Lysozyme activity (ng/mL)	
	4th w	6th w	4th w	6th w
Group 1	0.02^{b}±0.001	0.02^{b}±0.004	1.56^{c}±0.01	1.56^{b}±0.01
Group 2	0.06^{b}±0.07	$0.0.05^{b}$±0.001	1.57^{c}±0.01	0.93^{c}±0.01
Group 3	0.21^{a}±0.002	0.18^{a}±0.002	2.00^{b}±0.04	1.84^{a}±0.05
Group 4	0.21^{a}±0.001	0.18^{a}±0.003	2.75^{a}±0.02	1.82^{a}±0.08
Group 5	0.23^{a}±0.01	0.19^{a}±0.004	2.74^{a}±0.02	1.85^{a}±0.07

Means at the same column followed by different letters were significantly different at $p \leq 0.05$ and the highest value was represented with the letter a.

Group (1):control negative, **Group** (2):infected with *A. hydrophila* 0.5 mL (1×10^{7} CFU/mL), **Group** (3):vimolert 2.5 g/100g of feed then *A. hydrophila* 0.5 mL (1×10^{7} CFU/mL), **Group** (4):vimolert 3 g/100g of feed then *A. hydrophila* 0.5 mL (1×10^{7} CFU/mL), **Group** (5):vimolert 3.5 g/100g of feed then *A. hydrophila* 0.5 mL (1 v 10^{7} CFU/mL). Infection by *A. hydrophila* was done by the end of 4th week of experiment. W: week.

Histopathological examination

During the sixth week of the experiment, the histopathological analysis of the control negative group (group 1) revealed typical hepatic and renal parenchyma in Nile tilapia liver and kidney tissue sections. Additionally, muscle tissue sections showed a regular tissue architecture (Fig. 1).

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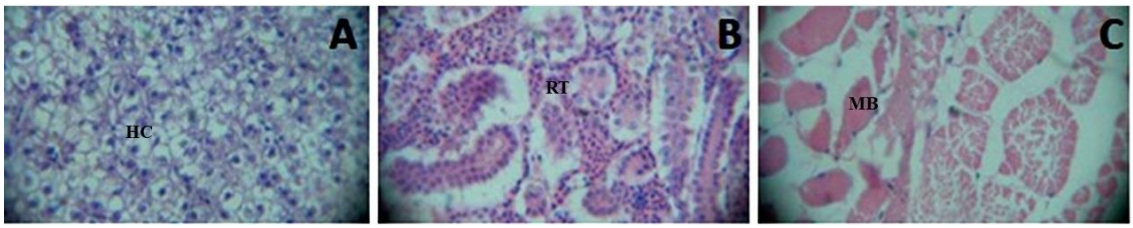


Figure 1: Photomicrograph from Nile tilapia in control negative group at 6th week Post-Experiment (PE), (A) Liver showing apparently normal hepatic parenchyma, HC; Hepatocytes, (B) Kidney showing apparently normal renal parenchyma, RT: Renal tubules. (C) Muscle showing apparently normal tissue architecture, MB: Muscle bundles (H&E, 100 X).

In contrast, the Control positive group (group 2) exhibited significant histopathological changes. including vacuolar degeneration and coagulative necrosis in liver tissue, focal necrosis, depletion of hematopoietic cells, activation of melanomacrophage, tubular nephrosis in kidney tissue, intramuscular edema, hyaline degeneration, and Zenker's necrosis in the muscle tissue (Fig. 2).

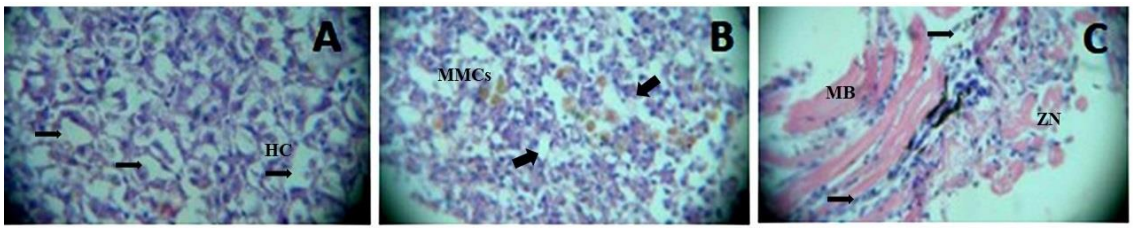


Figure 2: Photomicrograph from Nile tilapia in control positive group at 6th week PE (A). Liver showing vacuolar degeneration (arrowed), coagulative necrosis in the majority of hepatocytes, HC, (B). Kidney showing focal necrosis, hematopoietic cells and focal activation of Melanomacrophages, MMCs together with marked tubular nephrosis (arrowed), (C). Muscle showing intramuscular edema, focal hyaline degeneration and Zenker's necrosis, ZN in the muscle bundles, focal mononuclear cell infiltration (arrowed) and MMCs were evident in between the necrotic muscles, MB: Muscle bundles (H&E X 100).

Histopathological analysis of the group that received 2.5 g Vimolert® per 100g of feed (group 3), showed focal vacuolar degeneration of hepatopancreatic cells along with focal proliferation of MMCs in liver tissue, tubular nephrosis, characterized mainly by vacuolar degeneration with some melanomacrophage and mononuclear cell infiltration in kidney tissue, mild edema and focal hyaline degeneration in the muscle bundles (Fig. 3).

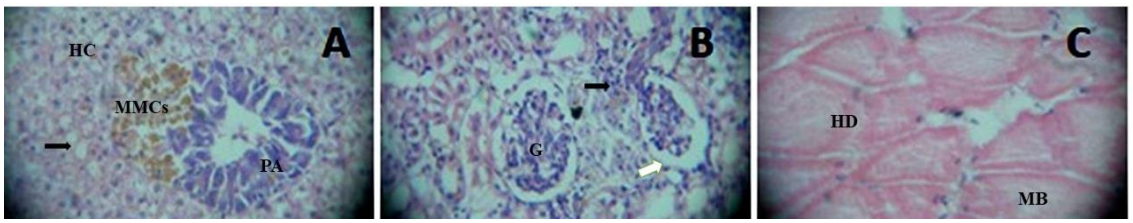


Figure 3: Photomicrograph from Nile tilapia supplemented with 2.5 g/100g Vimolert® at 6th week PE (A). Liver showing focal vacuolar degeneration (arrowed) of hepatopancreatic cells with focal proliferation of MMCs, HC; Hepatocytes, PA: Pancreatic acinar cells, (B). Kidney showing tubular nephrosis of renal epithelium of mainly vacuolar degeneration with some melanomacrophage and mononuclear cells infiltration (black arrow), with expansion of bowman's space, G: Glomeruli, (C). Muscle showing mild edema and focal hyaline degeneration, HD, in the muscle bundles, MB (H&E, 100 X).

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The liver tissue sections of Nile tilapia that received 3 g Vimolert® per 100g of feed (group 4) revealed vacuolar degeneration and coagulative necrosis in most hepatocytes with some infiltration of MMCs. Furthermore, the kidney tissue sections demonstrated lobular nephrosis with a significant proliferation of mononuclear cells and MMCs. Additionally, the muscle tissue sections exhibited notable edema, focal hyaline degeneration, and Zenker's necrosis, as illustrated in Figure 4.

Histopathological analysis, the liver tissue sections of Nile tilapia from group 5, which received 3.5 g Vimolert® per 100g of feed, showed vacuolar degeneration and coagulative necrosis and hepatocyte infiltration by melanomacrophages. Kidney tissue sections from this group displayed tubular nephrosis with significant necrosis of the renal epithelium and proliferation of mononuclear and melanomacrophage cells. Muscle tissue sections exhibited mild intramuscular edema and focal necrosis (Fig. 5).

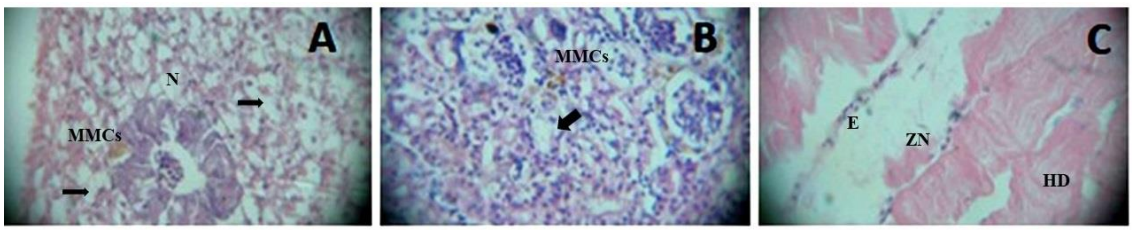


Figure 4: Photomicrograph from Nile tilapia supplemented with 3 g/100g Vimolert® at 6th week PE (A). Liver showing vacuolar degeneration (arrowed) and coagulative necrosis (N) of most hepatocyte, (B). Kidney showing tubular nephrosis (arrowed) with numerous proliferation of mononuclear cells and MMCs, (C). Muscle showing marked edema, E, focal hyaline degeneration, HD, as well as Zenker's necrosis, ZN (H&E, 100 X).

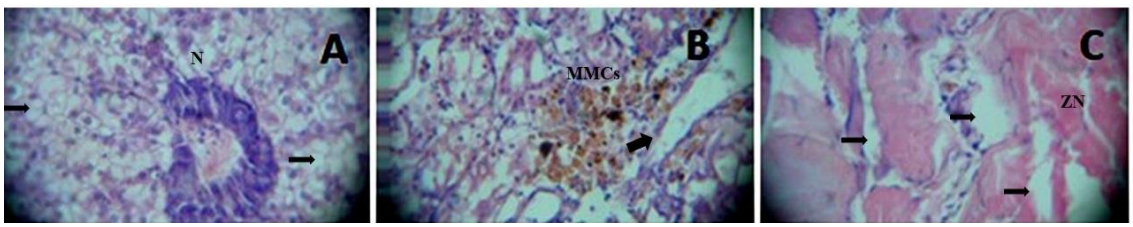


Figure 5: Photomicrograph from Nile tilapia supplemented with 3.5 g/100g Vimolert® 6th week PE (A). Liver showing vacuolar degeneration (arrowed) of hepatocyte with coagulative necrosis, N, (B). Kidney showing marked tubular nephrosis (arrowed) with marked necrosis of renal epithelium and proliferation mononuclear and melanomacrophage, MMCs cells, (C). Muscle showing mild intramuscular edema (arrowed) and focal necrosis, Zenker's necrosis, (ZN) (H&E, 100 X).

Discussion

Following experimental infection, the control positive group (group 2) experienced a substantial mortality rate of 50%, whereas groups 3, 4, and 5 displayed significantly lower mortality rates of 10%, 3.33%, and 6.67%, respectively. These groups were administered the probiotic and immunostimulants supplement (Vimolert®), which contains *Bifidobacterium* and *Enterococcus spp*.,

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resulting in the enhanced resistance to the bacteria through the activation of both cellular and humoral immune responses. Vimolert® also incorporates lysozyme, known for its efficacy against certain Gram-negative bacteria. However, it remains unclear whether this efficacy stems from its enzymatic activity or other bacteriolytic properties. Lysozyme appears to act as a biological response modifier by interacting with intestinal bacteria and pathogens, thereby stimulating immune cell activation through the release of specific peptidoglycans.

The mortality rates and relative level of protection (RLP) significantly improved in groups that received a combination of probiotics and immunostimulants compared to the control group. This finding aligns with prior research conducted by Aly *et al*. (2008), who indicated that Nile tilapia supplemented with *B. subtilis* and *Lactobacillus* exhibited increased RLP against *P. fluorescens* and *A. hydrophila*. Similar studies have shown that dietary supplementation with probiotics, such as *P. fluorescens* AH2 and *B. subtilis* AB1, and yeast products like GrostBioticR-A, can confer protection against various fish pathogens including *V. anguillarum, mycobacteria*, and *Aeromonas*. However, it's worth noting that Gram *et al*. (1999) didn't observe a significant effect of *P. fluorescens* AH2 on furunculosis-related mortality in salmon challenged with *A. salmonicida*. In contrast, El-Asely *et al.* (2014) reported substantial protection against *A. hydrophila* in fish fed with 2.5% (w/v) honey bee pollen (HBP) for 20 and 30 days, with the highest protection (93%) observed in this group.

After monitoring liver enzymes and bilirubin concentrations in the initial sample collected in the fourth week following administration of the probiotics and immunostimulant (Vimolert®), no significant changes were observed in ALT, AST, ALP, total bilirubin, direct bilirubin, or indirect bilirubin levels in all groups (2, 3, 4, and 5) when compared to the control group. This lack of significant changes can be attributed to the absence of harmful substances in Vimolert® that could adversely affect liver function capacity. These results were consistent with the histopathological findings of the liver, which revealed normal cellular details and tissue architecture in groups 1. Groups 3, 4, and 5 showed mild parenchymal edema and focal vacuolar degeneration of hepatocytes, but without any pathological infiltration.

Conversely, in the 2nd sample collected in the 6th week of the experiment, significant increases were observed in the levels of AST, ALT, ALP, total bilirubin, direct bilirubin, and indirect bilirubin in group 2, which was injected with *A. hydrophila* without receiving the immunestimulants and probiotics as prophylaxis. These liver enzymes are commonly used as indicators of liver function and integrity, and the significant elevation of their levels in the blood suggests liver damage or tissue necrosis (Soltan *et al*., 2008). The increase in ALT activity is associated with hepatocyte membrane

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damage (Rehulka, 2002). These results may be attributed to the effect of bacterial endotoxins, which cause damage to hepatocytes and the extensive release of liver enzymes, leading to their increase in the bloodstream. The histopathological examination of the liver confirmed these results, where vacuolar degeneration and coagulative necrosis were evident in the majority of hepatocytes. These findings may be attributed to the toxins, hemolysin, and proteases produced by *A. hydrophila*, which cause liver damage. These results align with the findings of Aly *et al*. (2008).

It appears that the immunostimulants and probiotics (Vimolert®) given as a prophylactic before injecting *A. hydrophila* have a protective effect on liver enzymes and histopathological findings in groups (3, 4, and 5). The decrease in ALT, AST, and ALP levels in these groups compared to the infected group (2) could be attributed to the presence of *Bifidobacterium*, *Enterococcus* spp., Beta-glucan, Nanozinc, and thymol crystals in the immune stimulant. Thymol crystal has been reported to have antibacterial properties and has been found to decrease ALT levels in Nile tilapia fingerling fish when added to their food in certain quantities (Shehata *et al*., 2013). Biogen® supplementation has also been found to decrease the levels of ALT and AST in Nile tilapia (Soltan *et al*., 2016). Histopathological analysis showed normal hepatocyte architecture in the probiotic-fed groups, while the infected group with *Streptococcus iniae* showed severe diffused necrosis, mononuclear cell infiltration, and loss of normal architecture (Moustafa *et al*., 2021).

Additionally, the presence of *Bifidobacterium* and *Enterococcus spp*. in Vimolert® may have contributed to the decrease in bilirubin levels. *Bifidobacterium* and *Enterococcus spp*. have been reported to have a protective effect on the liver by improving liver function and reducing liver damage (Wang *et al*., 2019). Beta-glucan, another component of Vimolert®, has been reported to have immunomodulatory effects that can help reduce inflammation and liver damage (Yan *et al*., 2017). Finally, nanozinc, also present in Vimolert®, has been reported to have hepatoprotective effects and can help reduce liver damage caused by oxidative stress (Zheng *et al*., 2016). Therefore, the combined effects of these components in Vimolert® may have contributed to the decrease in bilirubin levels observed in groups (3, 4, and 5) in the 2nd sample.

In the 2nd sample, there was a significant decrease or near-normal level of albumin, total protein, and globulin in group 2 that was injected with *A. hydrophila* without any prophylactic treatment. This could be attributed to the liver damage caused by bacterial endotoxins and toxins, which affects liver function and decreases the synthesis of albumin and globulin. In groups (3, 4, and 5) that were given Vimolert® as a prophylactic, there was a significant increase or near-normal levels of albumin, total protein, and

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globulin compared to the infected group (2). This result could be attributed to the presence of beta-glucan and ginseng, which have immunomodulatory effects and can stimulate liver function to produce albumin and globulin. The probiotics in Vimolert® may also contribute to the improvement of liver function as reported by Elam (2004). The A/G ratio, which represents the ratio of albumin to globulin, was significantly increased in the 1st sample in groups (3, 4, and 5) due to the increase in albumin and globulin levels. This ratio is used as an indicator of liver function, and an increase in the A/G ratio is indicative of improved liver function.

It is important to note that the decrease in total protein and albumin levels in the 2nd sample could be attributed to the bacterial infection and subsequent liver damage, as the liver is responsible for the synthesis of many plasma proteins, including albumin. The decrease in albumin levels could lead to edema and fluid accumulation in the tissues, which may contribute to the development of ascites, a common complication in liver disease (Berzigotti *et al*., 2018). The observed degenerative changes in the liver and kidney in the histopathological examination further support the negative impact of the bacterial infection on these organs.

The second sample from groups 3, 4, and 5 exhibited a noteworthy rise in total protein, albumin, and globulin levels, as well as a considerable decrease in the A/G ratio when compared to the infected group (2). These findings were attributed to the presence of immune stimulants, such as enterococcus, Beta-glucan, mannan oligosaccharides, and lysozyme enzyme in (Vimolert®), which improved liver function and enhanced non-specific immune response resulting in an increase in serum protein. These results are in accordance with Kord *et al.* (2021) who reported a significant increase in serum total protein, albumin, and globulin in Nile tilapia that were fed commercial immune stimulants (probiotics) for eight weeks. Furthermore, the decrease in the A/G ratio was due to an increase in immune protein (globulin). These findings align with Kumar *et al*. (2006), who reported that Indian major carp treated with *B. subtilis* and post-challenged with *A. hydrophila* showed increased serum protein and globulin levels, while the A/G ratio decreased during the challenge with *A. hydrophila*.

Regarding the kidney function test results, the 1st sample showed a slight decrease in urea and creatinine levels, which were close to normal in groups (3, 4, and 5). This suggests that (Vimolert®) does not have any harmful effects on kidney tissue and does not disturb renal function. These results were consistent with the histopathological findings of the kidney, which appeared normal in renal parenchyma and tissue architecture, with mild lobular nephritis, massive hematopoietic cell infiltration, and MMCs proliferation.

Conversely, the 2nd sample showed a highly significant increase in urea and creatinine levels in group 2 due to the action of bacterial toxins on the kidney (glomeruli and tubules). This result is in

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agreement with El-Alem *et al*. (2017), who reported that intraperitoneal injection of *A. hydrophila* (2.5×10^8 CFU/mL) increased urea and creatinine levels compared to the control in the first ten days after injection due to disturbance of renal function. The increase in BUN level was probably associated with an increased protein catabolism that occurs during fasting, infection, and blood loss. A greater increase in BUN and creatinine may indicate prerenal uremia caused by hypovolemia, a condition in which glomerular filtration decreases, and BUN absorption increases, leading to an increase in BUN presence in the plasma (Rehulka, 2002). The histopathological findings of the kidney in group 2 showed focal necrosis of hematopoietic cells, renal epithelium, focal activation of melanomacrophage, and marked tubular nephrosis. These results were consistent with Ghaly *et al*. (2022), who noted that naturally renal fish infection with Aeromonas exhibited albumin dystrophy symptoms and hyaline droplet formation in the proximal tubule epithelium.

In the 2nd sample of groups (3, 4, and 5), there was a significant decrease in urea and creatinine levels compared to the control and Group 2. These results were attributed to the immune stimulant (Vimolert®), which contains the lysozyme enzyme with a bacteriocidal effect as bacterial cell lysis occurs, leading to improved renal function and excretion. These results were consistent with Nasr *et al*. (2019), who reported that dietary supplementation with commercial or gut-isolated probiotic bacteria significantly reduced creatinine and urea levels in *A. hydrophila*-infected fish. The reduction may be attributed to their role in improving kidney histology, which was also consistent with Kamgar *et al.* (2013). The histopathological findings of the kidney in groups (3, 4, and 5) showed tubular nephrosis of the renal epithelium mainly with vacuolar degeneration, melanomacrophage, and mononuclear cell infiltration.

The results of the study showed that there were significant increases in NBT values and lysozyme activity in groups 3, 4, and 5 when compared to groups 1 and 2. These increases were attributed to the combination of probiotics and immunostimulants (Vimolert®) which contains probiotics, bee pollen, beta-glucan, and lysozyme. Lysozyme, which has bactericidal activity, acts as an opsonin that activates the complement system and phagocytes. The lysozyme activity was significantly higher in all groups given probiotic-supplemented diets when compared to the untreated control group. The injection of beta-glucan induced significantly elevated lysozyme activity, which is in line with the findings of Misra *et al*. (2006). Moreover, the results of the study were consistent with El-Asely *et al*. (2014), who reported that fish fed with 2.5% (w/v) honey bee pollen for 20 and 30 days exhibited significant protection against challenge with *A. hydrophila* and had a significant increase in phagocytic activity and serum bactericidal activity.

In the 2nd sample, there was a significant increase in group 2 due to the

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injection of bacteria and the immune response. There was also a highly significant increase in groups 3, 4, and 5, which can be attributed to the presence of lysozyme enzyme in the commercial combination of probiotics and immunostimulants (Vimolert®), which aids in destroying the bacterial cell wall and engulfment by phagocytes. The results of the study were in agreement with Kord *et al*. (2021), who reported that the presence of lysozyme aids in the degradation of the peptidoglycan layer of bacterial cell walls and can serve as an opsonin to stimulate phagocytosis or polymorphonuclear leukocytes.

Several fish species, including *O. niloticus*, have been stimulated through probiotic supplementation either in the inviable or inactivated form, leading to enhanced phagocytic activity, as evident in lactobacillus acidophilus supplementation (Rutherfurd-Markwick and Gill, 2004). The results of the study were consistent with Amphan *et al*. (2019), who reported that phagocytosis percentage was increased by beta-glucan feeding. In addition to innate immunity, beta-glucan-fed fish demonstrated enhanced disease resistance against *A. hydrophila* challenge at the end of the fourth week of the trial.

The results of the study were also in agreement with Salinas *et al*. (2005) and Panigrahi *et al.* (2007), who found that probiotics could enhance the natural complement activity of fish. Many probiotics in dietary and water treatments have been reported to stimulate the complement components. Lysozyme is a basic humoral immune defense factor that is typically developed in aquatic animals when it targets bacterial peptidoglycans, often Gram-positive bacteria, in the cell membrane, culminating in the stimulation of bacterial phagocytosis by phagocytic cells. These findings agreed with Aly *et al*. (2008), who discovered that fish fed with diets supplemented with Bacillus spp. had substantially higher lysozyme activity than that in fish fed a control diet for four weeks.

Regarding the histopathological findings after *A. hydrophila* injection, liver tissues in group 2 showed vacuolar degeneration and coagulative necrosis in the majority of hepatocytes. Kidneys exhibited focal necrosis, proliferation of hematopoietic cells, focal activation of melanomacrophage, marked tubular nephrosis, and mild degenerative changes. Muscle tissues showed intramuscular edema, focal hyaline degeneration, and Zenker's necrosis in muscle bundles, focal mononuclear cell infiltration, and melanomacrophage cells. These results were consistent with the study conducted by Aly *et al*. (2020), which also reported edema and focal coagulative necrosis in the skin and underlying musculature. Liver tissues showed vacuolar degeneration of hepatocytes, coagulative necrosis, and congestion in hepatic and pancreatic ducts. Kidneys exhibited mild tubular nephrosis of the renal epithelium and focal interstitial hemorrhage. Groups (3, 4, and 5) exhibited fewer degenerative alterations compared to group (2) due to commercial combinations of probiotics and immunostimulants (Vimolert®).

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They showed focal vacuolar degeneration of hepatopancreatic cells with focal proliferation of melanomacrophage cells, tubular nephrosis of renal epithelium with mainly vacuolar degeneration and some melanomacrophage and mononuclear cells infiltration in the kidneys, and mild edema and focal hyaline degeneration in muscle bundles.

In this study, histopathological examination of various organs in experimental tilapia groups receiving different combinations of probiotics and immunostimulants showed significant proliferation of melanomacrophage cells and hyperplasia of hematopoietic tissue compared to the control group. These findings were similar to those of Ngamkala *et al*. (2010), who observed no pathological abnormalities in non-challenged fish.

It could be concluded that the probiotic supplement Vimolert®, improved resistance to the bacteria by activating both cellular and humoral immune responses. Vimolert® also contains lysozyme, which has been shown to be effective against some Gram-negative bacteria, and consequently, the mortality rates and relative level of protection (RLP) of *A. hydrophila* infected tilapia were significantly improved in groups that received a combination of probiotics and immunostimulants compared to the control positive group. Moreover, a significant decrease in the concentrations of ALT, AST, ALP, urea, and creatinine and an increase in globulin together with a highly significant increase of NBT and lysozyme enzymes as well as improvement of the histopathology were evident in Vimolert® treated group in the comparison with the *A. hydrophila* infected group. The researchers concluded that the mixture of probiotics and immunostimulants improved the health status, immune response, and resistance of Nile Tilapia against *A. hydrophila* infection.

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