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Effect of Probiotic and Immunostimulant Complex on Immune Response in the Nile Tilapia Challenged with *Aeromonas hydrophila*: A Prophylactic Approach

Osama A. Abd Allah[1], Salah M. Aly[2*], Mohamed E. Abo El-Atta[3], Taghrid M N abdelhakim[3], Enas H. Mohamed[1], Mohamed Fathi[4]

[1]Department of Clinical Pathology, Faculty of Veterinary Medicine, Suez Canal University, Egypt
[2]Department of Pathology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, 41522, Egypt
[3]Central Laboratory for Aquaculture Research, Agriculture Research Center, Egypt
[4]National Institute of Oceanography and Fisheries (NIOF), Egypt

[*]**Corresponding Author: aquavet@hotmail.com**

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ABSTRACT

This study delved into the impact of the probiotic and immunostimulant complex, Vimolert®, on the immune response of the Nile tilapia fish challenged with *Aeromonas hydrophila*. The investigation encompasses an in-depth analysis of hematological and immune parameters within the context of fish diets enriched with Vimolert®. The experimental setup entailed the involvement of 150 Nile tilapia fish, thoughtfully partitioned into five equitable groups. Among these, groups 1 and 2 functioned as control-negative and control-positive entities, respectively. Meanwhile, groups 3, 4, and 5 were subjected to varying doses of Vimolert® (2.5, 3, and 3.5g/ 100g of feed, respectively) as a prophylactic measure. At the culmination of the four-week feeding regimen, the fish in groups 2- 5 encountered a challenge with *A. hydrophila*. In the initial four weeks of the experiment, groups 3, 4, and 5 witnessed a noteworthy surge in red blood cell (RBC) count and hemoglobin (Hb) concentration, while other hematological parameters like hematocrit (Ht) value, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) remained unchanged. Concurrently, there were substantial elevations in total leukocyte and neutrophil counts across all groups, underscoring a strengthened immune response. Notably, phagocytic percent and phagocytic index exhibited significant enhancements in groups 3, 4, and 5 compared to the control group. After a six week interval, a slight decrement was observed in RBC count, Hb concentration, and Ht, while MCV exhibited significant augmentation in groups 2, 3, 4, and 5. Lymphocyte and monocyte counts experienced modest increases, while eosinophil and basophil counts remained unaltered. Nevertheless, the six week period witnessed a highly significant augmentation in all leukocyte subtypes across all groups, with group 4 demonstrating the most substantial increase and group 2 the most restrained. Group 2 displayed a significant decline in phagocytic percent and phagocytic index in comparison to the control group, whereas groups 3, 4, and 5 demonstrated remarkable improvements in these parameters relative to group 2. In summation, this study culminates in the assertion that the probiotic and immunostimulant complex, Vimolert®, at a dosage of 3g/ 100g of feed, stands as an efficacious prophylactic agent against *A. hydrophila* infection in the Nile tilapia fish. These findings contribute to a deeper understanding of the potential of Vimolert® in fortifying the immune responses of fish and, by extension, elevating their resilience against pathogenic challenges, offering promising implications for aquaculture practices.

INTRODUCTION

The Nile tilapia (*Oreochromis niloticus*) stands as a prominent and extensively cultivated species in the world of tilapia aquaculture, catering to commercial demands. This industry has witnessed remarkable global expansion, with tilapia emerging as the second most vital fish in terms of production volume, trailing only behind carp **(FAO, 2012)**. A discernible trend towards intensification and commercialization is taking root in tilapia aquaculture (**Goncalves *et al.*, 2011)**. The ubiquity of fish farming has grown significantly, driven by the supply of proteins, vitamins, minerals, and oils, with a principal aim of optimizing fish yield per unit of space, volume, or through precise management practices.

The successful cultivation of tilapia necessitates a meticulous consideration of factors encompassing carrying capacity, nutrition, yield, and the quality of water **(Solomon & Taruwa, 2011)**. In this dynamic context, one of the key challenges facing the aquaculture industry is the proliferation of *Aeromonas hydrophila*, a notorious pathogen responsible for epizootic ulcerative septicemia and motile *Aeromonas* septicemia in the Nile tilapia. This opportunistic pathogen has wreaked havoc in fish farms, precipitating substantial economic losses **(Fang *et al.*, 2004)**. The virulence of such bacteria, often found in pond and river waters, hinges on the physicochemical attributes of the aquatic environment. While these bacteria are Gram-negative and widely dispersed, they still pose a threat to freshwater fish, exemplified by *A. hydrophila* **(Bailone *et al.*, 2010)**. Notably, the key trigger for disease outbreaks associated with *A. hydrophila* in intensive farming conditions is the stress experienced by fish, even though it is part of the normal microbiota of healthy fish **(Cipriano, 2001)**.

Numerous studies have explored the consequences of *A. hydrophila* infection on the blood parameters of diverse fish species; these investigations have revealed that infections can lead to a decline in serum ALT, protein, albumin, globulin levels, and a substantial reduction in plasma glucose levels **(Fayza *et al.*, 2011)**. However, it is worth noting that in certain species, infection can elevate the total serum protein and globulin content, indicating an increase in defense proteins **(Das *et al.*, 2011; Biller-Takahashi *et al.*, 2013; Pal *et al.*, 2015)**. Additionally, the supplementation of certain fish species with Biogen® has been shown to raise serum albumin and globulin levels, while reducing serum cholesterol and glucose levels **(Elam, 2004)**.

The utilization of medicinal plant-derived immunostimulants has emerged as a promising strategy to bolster immune responses and disease resistance in aquatic animals, offering an environmentally friendly and sustainable approach to controlling aquaculture diseases **(Munaeni *et al.*, 2020)**. The deployment of medicinal plants not only provides a cost-effective solution, but also ensures environmental compatibility over prolonged usage **(Citarasu, 2010)**. Concurrently, probiotics, encompassing live, dead, or microbial

cell components, have found their place in aquaculture by enhancing host growth, immunity, and overall health, thereby fortifying the resistance against a spectrum of infections **(Aly, 2008; Aly *et al.*, 2016; Dawood *et al.*, 2019a)**. Probiotics function by restoring the natural microbial equilibrium within the host's gut and surrounding environment, leading to improved water quality and more efficient feed utilization **(Martínez *et al.*, 2012)**. These probiotics also stimulate the secretion of intestinal mucus, promote microvilli growth, and establish robust barriers against invading pathogens **(Standen *et al.*, 2016; Dawood *et al.*, 2019b)**.

The study introduced Vimolert®, a soluble powder manufactured by Cairo-Bio-Pharm Factory in Egypt, which belongs to the pharmacological category aimed at enhancing both cellular and non-cellular defense. This complex comprises a blend of specific ingredients in precise quantities, including thymol crystal (≥ 5000mg), ginseng (≥ 3000mg), nano zinc (≥ 4000mg), bifidobacterium (≥ 108CFU), bee pollen (≥ 1000mg), sodium butyrate (≥ 20000mg), *Enterococcus* spp. (≥ 108CFU), mannan oligosaccharides (≥ 100000mg), beta-glucan blend (≥ 100000mg), and lysozyme enzyme (≥ 10000IU).

Against this backdrop, the primary objective of our study was to assess the impact of the probiotic and immunostimulant complex, Vimolert®, on the immune response of the Nile tilapia when faced with a challenge from *Aeromonas hydrophila*. Specifically, we aimed to investigate how Vimolert® supplementation influenceed hematological and immune parameters in the Nile tilapia, shedding light on its potential immunostimulatory effects in the face of this pathogenic threat.

To realize these objectives, we undertook an extensive analysis of diverse immune parameters in the Nile tilapia, which were fed diets enriched with Vimolert®. Through a comprehensive evaluation of hematological parameters and cellular immune responses, we seeked to gain insight into the immunostimulatory potential of Vimolert® and its impact on the fish's immune system when confronting *Aeromonas hydrophila*.

MATERIALS AND METHODS

Experimental design

A total of 150 Nile tilapia were collected in this study and were systematically organized into 5 groups, each consisting of 30 fish. Within each group, a further division was made into 3 replicates. The first group served as the control (Group 1) and received no treatment. The second group, acting as the positive control (Group 2), did not receive any feed additives. Groups 3 to 5 were administered varying dosages of the probiotic and immunostimulant complex, Vimolert at rates of 2.5/ 100, 3/ 100, and 3.5g/ 100g, as a prophylactic regimen spanning a period of 4 weeks. At the culmination of this 4 week interval, bacterial infection was induced in groups 2 to 5. The inoculum, consisting of

0.5ml (1* 10^7 CFU/ ml) of *A. hydrophila*, was administered to these groups, a dosage derived from a precedent study by **Fayza *et al.* (2011)**. Conversely, the control negative group (Group 1) was intraperitoneally injected with sterilized saline.

Experimental diet

The Nile tilapia in groups 3 to 5 were fed a basal diet, which was enriched with Vimolert®, containing probiotics and immune stimulant substances at varying concentrations (2.5/ 100, 3/ 100, and 3.5g/ 100g). The basal diet was sourced from the Fish Research Unit, Faculty of Veterinary Medicine, Zagazig University, and was meticulously formulated to provide a crude protein content of 30% and a metabolizable energy of 3000kcal/ kg. The ingredients of the diet included fish meal (75g/ kg diet), meat meal (150g/ kg diet), corn (350g/ kg diet), soybean (200g/ kg diet), flour (100g/ kg diet), bran (73.5g/ kg diet), oil (50g/ kg diet), vitamins (0.75g/ kg diet), and minerals (0.75g/ kg diet). The diet was prepared in pellet form, air-dried at room temperature for 24 hours, and stored in a refrigerator at 4°C to ensure daily freshness and optimal nutritional quality. Feeding was conducted twice a week.

Experimental infection

Following the 4 week feeding trial, the response of groups 2- 5 to the pathogenic strain *A. hydrophila* subsp. *hydrophila* was evaluated. The control negative group (Group 1) received an intraperitoneal injection of sterile saline (0.85% sodium chloride, NaCl). The inoculum for group 2- 5 was prepared by culturing *A. hydrophila* on TSB (oxoid) for 24 hours at 37°C, followed by centrifugation at 6100xg for 30 minutes, washing, resuspension in sterile saline solution, and enumeration using the McFarland standard technique. The fish were intraperitoneally injected with 0.5ml of *A. hydrophila* suspension, containing 10^7 bacteria /L **(Fayza *et al.*, 2011)**. The pathogenic strain employed in the study was isolated from the Nile tilapia and can be cross-referenced in the GenBank databases under the accession numbers of OQ253432 **(Aly *et al.*, 2023)**.

Blood samples

Blood samples were collected from the caudal vessels of fish in each group at both week 4 and week 6. These samples were collected in K2-EDTA tubes and were subsequently utilized for a spectrum of hematological tests **(Coles, 1986)**.

Hematological studies

To evaluate the hematological profile of the fish, various parameters were assessed, including red blood cell (RBC) count, hemoglobin (Hb) concentration, hematocrit (Ht) value, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total and differential leukocytic counts. Automated cell counters were used for these evaluations, as applied by **Feldman *et al.* (2000)**.

Phagocytosis and phagocytic index

To measure phagocytosis and phagocytic index, the following steps were executed, following the protocols outlined by **Wilkinson (1977)** and **Lucy and Larry (1982)**: (a) *E. coli* was cultured on MacConkey agar medium at 37°C for 24 hours, counted using an improved Neubauer counting chamber, and suspended in buffer peptone water to a concentration of 1.5 x 10^8 CFU/ml, matching with the 0.5 McFarland standard. (b) A leukocytic suspension was prepared using sterile techniques. A 3ml venous blood sample was collected on heparin and mixed with dextran to separate the red blood cells from the leukocytes. The leukocyte-rich plasma was removed, and the deposited cells were washed and suspended in RPMI media containing 1% fetal calf serum. The neutrophil cell count was adjusted to 2 x 10^6 cells/ ml in phosphate-buffered saline.

Statistical analysis

Data collected was subjected to analysis using SPSS software. Significance was assessed using one-way analysis of variance (ANOVA), followed by post hoc test (Duncan's method) with a significance level set at $P \leq 0.05$, following the method of **Tamhane and Dunlop (2000)**. Data were presented in the form of mean ±SE, with different letters signifying means in the same column that were statistically significant, where the highest value was represented by the letter (a).

RESULTS

Clinical observations

Fish of group (2) that was infected with *A. hydrophila* without receiving the probiotic and immunostimulant complex, Vimolert®, exhibited pronounced clinical signs. These included a loss of appetite, abnormal swimming patterns, stagnation, skin ulcers, and petechial hemorrhages on both the skin and gills. These signs referred to bacteremia or septicemia and were indicative of *Aeromonas* bacterial infection. Upon death, the postmortem examination revealed evident signs such as scale loss, gill congestion, petechial bleeding on the body and fins, shallow to deep ulcers on various parts of the body surface, a pale yellowish liver with a friable consistency, yellowish ascitic fluid, and congested, swollen and pale kidneys.

In stark contrast, the negative control group (group 1) and groups 3- 5, which were administered Vimolert at varying doses (2.5/ 100, 3/ 100, and 3.5g/ 100g) as a preventive measure for a duration of 4 weeks, did not manifest clear clinical symptoms or postmortem lesions throughout the study period.

Hematological results

Changes in erythrogram

In comparison to the control group (Group 1), significant increases in red blood cell (RBC) count and hemoglobin (Hb) concentration were observed in groups 3, 4 and 5 after the fourth week. Group 4 exhibited the most substantial rise in these parameters, indicating a positive impact. However, there were no significant changes in hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) values during this period. Group 2 did not display significant alterations in any of these parameters.

Upon reaching the sixth week, a non-significant change in RBC count and Hb concentration was noted across treated groups compared to the control group (Group 1). Groups 2, 3, 4 and 5 exhibited a significant decrease in Ht, with group 4 experiencing the most substantial decline. Meanwhile, MCV showed a significant increase in groups 2, 3, 4, and 5, with group 2 displaying the most significant rise. No significant changes were observed in MCH and MCHC values in groups 3, 4 and 5 (Table 1).

Changes in leukogram

Following the fourth week of the experiment, the leukogram data were compared to those of the control group (Group 1). Notably, all groups displayed a significant increase in both total leukocyte and neutrophil counts. Group 4 exhibited the most substantial increase, while group 2 displayed the lowest increase in these counts. However, lymphocyte and monocyte counts showed only slight increases, with no significant changes observed in eosinophil and basophil counts across all groups during this period.

As the experiment progressed to the sixth week, another evaluation of the total leukocyte, neutrophil, eosinophil, basophil, lymphocyte, and monocyte counts was conducted in comparison to the control group (Group 1). All groups (Groups 2, 3, 4, and 5) displayed highly significant increases in these counts. Once again, group 4 exhibited the most substantial increase, while group 2 showed the lowest increase (Table 2).

Table 1. Erythrogram (mean values ±SE) of Nile tilapia in groups (1- 5) at 4th and 6th week of the experiment

Parameters	RBCs (×10⁶/µl)		Hb (g/dl)		Ht (%)		MCV (fl)		MCH (pg)		MCHC (%)	
sample / groups	4th w	6th w	4th w	6th w	4th w	6th w	4th w	6th w	4th w	6th w	4th w	6th w
Gp. 1	2.28^{b} ± 0.13	2.31^{a} ± 0.12	7.66^{b} ± 0.12	7.55^{a} ± 0.08	22.50^{a} ± 0.19	22.48^{a} ± 0.17	99.61^{ab} ± 4.95	97.87^{b} ± 4.40	33.82^{a} ± 1.47	31.38^{b} ± 1.20	34.02^{a} ± 0.28	33.59^{a} ± 0.39
Gp. 2	2.23^{b} ± 0.16	1.08^{b} ± 0.09	7.68^{b} ± 0.06	5.02^{b} ± 0.06	22.50^{a} ± 0.19	15.65^{b} ± 0.36	102.31^{a} ± 6.45	147.05^{a} ± 9.03	34.87^{a} ± 2.17	47.61^{a} ± 3.96	34.12^{a} ± 0.19	32.10^{a} ± 0.83
Gp. 3	2.63^{a} ± 0.03	2.13^{a} ± 0.08	8.18^{ab} ± 0.13	7.54^{a} ± 0.26	23.37^{a} ± 0.55	22.50^{a} ± 0.23	88.86^{ab} ± 1.86	105.85^{b} ± 3.01	31.15^{a} ± 0.56	35.37^{b} ± 0.44	35.08^{a} ± 1.07	33.48^{a} ± 0.84
Gp. 4	2.68^{a} ± 0.05	2.30^{a} ± 0.10	8.60^{a} ± 0.41	7.76^{a} ± 0.13	23.65^{a} ± 0.51	22.87^{a} ± 0.02	88.20^{b} ± 2.02	100.17^{b} ± 4.13	32.17^{a} ± 2.07	34.01^{b} ± 1.57	36.55^{a} ± 2.37	33.94^{a} ± 0.57
Gp. 5	2.65^{a} ± 0.06	2.18^{a} ± 0.16	8.48^{ab} ± 0.37	7.20^{a} ± 0.40	23.84^{a} ± 0.64	21.96^{a} ± 0.70	90.35^{ab} ± 3.83	102.05^{b} ± 5.04	32.17^{a} ± 1.91	33.32^{b} ± 1.36	35.53^{a} ± 0.66	32.73^{a} ± 0.89

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.
Gp.(1): Control negative, Gp.(2): Infected with *A. hydrophila* 0.5ml (1 x 10^{7}CFU/ ml), Gp.(3): Vimolert 2.5g/ 100g of feed then *A. hydrophila* 0.5ml (1 x 10^{7}CFU/ ml), Gp.(4): Vimolert 3g/ 100g of feed then *A. hydrophila* 0.5ml (1 x 10^{7}CFU/ ml), Gp.(5): Vimolert 3.5g/ 100g of feed then *A. hydrophila* 0.5ml (1 x10^{7}CFU/ ml.

Table 2. Leukogram ($\times 10^3$ / µl) (mean values ±SE) of Nile tilapia in groups (1- 5) at 4th and 6th weeks of the experiment

Parameters	T.L.C.		Neutrophils		Eosinophils		Basophils		Lymphocytes		Monocytes	
sample / groups	4th w	6th w	4th w	6th w	4th w	6th w	4th w	6th w	4th w	6th w	4th w	6th w
Gp.1	$17.93^{c} \pm 0.36$	$18.41^{c} \pm 0.13$	$10.26^{b} \pm 0.42$	$9.94^{b} \pm 0.44$	$0.40^{a} \pm 0.05$	$0.40^{b} \pm 0.07$	$0.21^{a} \pm 0.01$	$0.22^{b} \pm 0.01$	$6.20^{ab} \pm 0.39$	$6.69^{c} \pm 0.52$	$0.96^{ab} \pm 0.02$	$0.97^{c} \pm 0.06$
Gp.2	$17.89^{c} \pm 0.07$	$25.01^{b} \pm 0.30$	$10.38^{b} \pm 0.35$	$10.43^{b} \pm 0.16$	$0.24^{a} \pm 0.05$	$0.43^{b} \pm 0.08$	$0.18^{a} \pm 0.02$	$0.52^{a} \pm 0.01$	$5.99^{ab} \pm 0.23$	$12.24^{b} \pm 0.30$	$0.99^{a} \pm 0.00$	$1.40^{b} \pm 0.06$
Gp.3	$19.73^{b} \pm 0.17$	$38.18^{a} \pm 0.47$	$12.61^{a} \pm 0.15$	$13.88^{a} \pm 0.27$	$0.43^{a} \pm 0.05$	$0.81^{a} \pm 0.06$	$0.17^{a} \pm 0.03$	$0.52^{a} \pm 0.01$	$5.57^{b} \pm 0.07$	$20.97^{a} \pm 0.33$	$0.93^{bc} \pm 0.02$	$1.79^{a} \pm 0.05$
Gp.4	$20.93^{a} \pm 0.46$	$38.48^{a} \pm 1.16$	$13.24^{a} \pm 0.59$	$14.06^{a} \pm 0.39$	$0.40^{a} \pm 0.08$	$0.62^{ab} \pm 0.08$	$0.22^{a} \pm 0.00$	$0.52^{a} \pm 0.01$	$6.14^{ab} \pm 0.15$	$22.13^{a} \pm 0.73$	$0.91^{c} \pm 0.00$	$1.83^{a} \pm 0.03$
Gp.5	$20.19^{ab} \pm 0.35$	$37.89^{a} \pm 0.44$	$12.49^{a} \pm 0.37$	$13.16^{a} \pm 0.29$	$0.27^{a} \pm 0.09$	$0.75^{a} \pm 0.09$	$0.18^{a} \pm 0.02$	$0.53^{a} \pm 0.01$	$6.33^{a} \pm 0.05$	$21.79^{a} \pm 0.20$	$0.93^{bc} \pm 0.01$	$1.67^{a} \pm 0.06$

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.
Gp.(1): Control negative, Gp.(2): Infected with *A. hydrophila* 0.5ml (1 x 10^7CFU/ ml), Gp.(3): Vimolert 2.5g/ 100g of feed then *A. hydrophila* 0.5ml (1 x 10^7CFU/ ml), Gp.(4): Vimolert 3g/ 100g of feed then *A. hydrophila* 0.5ml (1 x 10^7CFU/ ml), Gp.(5): Vimolert 3.5g/ 100g of feed then *A. hydrophila* 0.5ml (1 x 10^7CFU/ ml.

Changes in phagocytic percent and phagocytic index

Following the fourth week of the experiment, a notable shift in phagocytic parameters was observed in the different experimental groups compared to the control group (Group 1). Groups 3, 4 and 5 exhibited a significant increase in both phagocytic percent and phagocytic index, indicating an enhanced phagocytic response. However, group 2 showed no significant change in these parameters during this period.

Upon completing the sixth week of the experiment, a further assessment of the phagocytic percent and phagocytic index was performed in comparison to the control group (Group 1). Interestingly, group 2 displayed a significant decrease in these parameters compared to the control group, suggesting a decline in the phagocytic response. In contrast, groups 3, 4 and 5 displayed a significant increase in both phagocytic percent and phagocytic index compared to group 2 that signified a renewed and strengthened phagocytic response (Table 3).

Table 3. The mean values (± SE) of phagocytic percent and phagocytic index of the Nile tilapia in groups 1- 5 during the 4th and 6th weeks of the experiment

Parameters / Groups	Phagocytic percent		Phagocytic index	
Time	**4th w**	**6th w**	**4th w**	**6th w**
Gp. 1	72.65^{b} ± 1.74	73.87^{a} ± 1.48	0.77^{bc} ± 0.03	0.81^{a} ± 0.02
Gp. 2	74.76^{ab} ± 3.09	55.67^{b} ± 1.85	0.76^{c} ± 0.03	0.55^{d} ± 0.01
Gp. 3	80.37^{a} ± 1.48	70.22^{a} ± 2.08	0.83^{ab} ± 0.01	0.71^{c} ± 0.01
Gp. 4	81.42^{a} ± 3.72	74.00^{a} ± 0.96	0.85^{a} ± 0.01	0.77^{ab} ± 0.02
Gp. 5	81.27^{a} ± 0.99	72.16^{a} ± 0.70	0.83^{a} ± 0.01	0.72^{bc} ± 0.01

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. Gp.(1): Control negative, Gp.(2): Infected with *A. hydrophila* 0.5ml (1 x 10^7CFU/ ml), Gp.(3): Vimolert 2.5g/ 100g of feed then *A. hydrophila* 0.5ml (1 x 10^7CFU/ ml), Gp.(4): Vimolert 3g/ 100g of feed then *A. hydrophila* 0.5ml (1 x 10^7CFU/ ml), Gp.(5): Vimolert 3.5g/ 100g of feed then *A. hydrophila* 0.5ml (1 x 10^7 CFU/ ml).

DISCUSSION

The findings of this study underscore the promising potential of the probiotic and immunostimulant complex, Vimolert®, in bolstering the immune response of the Nile tilapia against *A. hydrophila* infection, shedding light on its applicability in aquaculture for promoting fish health and resilience.

The clinical manifestations observed in group 2, consisting of fish infected with *A. hydrophila* but not receiving Vimolert®, closely resembled the symptoms typically associated with *Aeromonas* infections. These symptoms encompassed reduced appetite, erratic swimming patterns, stasis and skin ulceration, aligning with previous reports **(Cipriano, 2001)**. Particularly noteworthy were the petechial hemorrhages on the skin and gills, indicative of bacteremia or septicemia, severe systemic infections commonly associated with aromonas. Notably, the high mortality rates in group 2, reaching up to 70%, further underscore the virulence of *A. hydrophila*. Postmortem examinations revealed multiple pathological lesions, including scale loss, gill congestion, petechial bleeding and various skin ulcerations. Furthermore, the recorded alterations in the liver, reflected the severity of the systemic infection. Kidney examination revealed congestion, enlargement, and swelling, further confirming the systemic impact of the infection.

In stark contrast, groups 3- 5, which received Vimolert® prophylactically, displayed no discernible clinical symptoms or postmortem lesions throughout the study. This preventive effect can be attributed to the probiotic components within Vimolert®, notably Bifidobacterium and Enterococcus spp., recognized for their ability to enhance fish resistance to bacterial infections by activating both cellular and humoral immune responses **(Nouh *et al.*, 2009)**. It is crucial to emphasize that these findings pertain specifically to the Nile tilapia, and generalizing these results to other fish species or aquaculture systems should be approached with caution. Future research encompassing a broader range of fish species and diverse bacterial pathogens will contribute to a more comprehensive understanding of Vimolert's potential applications across different aquaculture contexts.

Changes in erythrogram

The results from the erythrogram analysis conducted after the fourth week of Vimolert® administration revealed distinct patterns. Group 2, which did not receive the immune stimulant component, exhibited no significant changes compared to the control group, which is in line with expectations. In contrast, groups 3, 4 and 5 displayed a substantial increase in the red blood cell (RBC) count and hemoglobin (Hb) concentration, implying a positive impact. This augmentation can be attributed to the presence of probiotics and immune stimulant substances within Vimolert®, including Bifidobacterium, Enterococcus spp., thymol crystals, betaglucan and nanozinc. These

components potentially enhance erythropoietin production and promote erythrocyte stability.

These findings are consistent with studies conducted by **Soltan *et al.* (2016)** and **Kord *et al.* (2021)** in which fish fed with probiotics exhibited elevated hemoglobin levels. This is possibly due to increased iron absorption facilitated by enhanced acid production in the gut **(Mohapatra *et al.*, 2014)**.

Conversely, the significant decline in RBCs, Hb and Ht observed in group 2 during the second sample at the sixth week, following *A. hydrophila* injection, can be attributed to the hemolytic activity of β-hemolysin produced by *A. hydrophila*. This hemolysin has the potential to induce RBC lysis, leading to anemia, as previously observed **by Pal *et al.* (2015)**. Additionally, septicemia induced by the infection may result in RBC lysis due to the action of bacterial enterotoxins.

The observed changes in mean corpuscular volume (MCV) are indicative of erythrocyte swelling, reflecting macrocytic anemia. The increase in MCV may be attributed to erythrocyte swelling due to hypoxic conditions, impaired water balance, or osmotic stress, aligning with the findings of **Haniffa and Abdul Kader (2011)**. Furthermore, the reduction in mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) levels suggests a lower hemoglobin concentration in RBCs, indicative of an anemic condition. The decrease in MCHC, a reliable indicator of RBC swelling unaffected by blood volume or cell count, following *A. hydrophila* infection, may be attributed to RBC swelling or a decline in hemoglobin synthesis, as previously explained by **Haniffa and Abdul Kader (2011)**. The increase in MCV may be attributed to more significant erythrocyte swelling in group 2 compared to groups 3, 4 and 5. These results are consistent with those of **Pal *et al.* (2015)**.

Groups 3, 4 and 5, receiving Vimolert® as a prophylactic measure, displayed either a slight decrease or nearly normal erythrogram parameters compared to the control group. This outcome could be attributed to the action of Bifidobacterium and Enterococcus spp. found in Vimolert®, which produce inhibitory compounds that competitively exclude pathogenic bacteria, in agreement with **Velmurugan and Rajagopalm (2009)**.

Changes in leukogram

Following the fourth week of Vimolert® administration, the total leukocyte and neutrophil counts exhibited significant increases in groups 3, 4 and 5 compared to the control group. This enhancement can be attributed to the immunostimulant component, which augments both cellular and non-cellular immune responses. In contrast, lymphocyte and monocyte counts showed only slight increases, with no significant changes in eosinophil and basophil counts across all groups during this period.

Following the progression of the experiment to the sixth week, all groups (Groups 2, 3, 4, and 5) displayed highly significant increases in total leukocyte, neutrophil, eosinophil, basophil, lymphocyte and monocyte counts. The immune response was particularly robust in group 4, demonstrating the most substantial increase, while group 2 exhibited the least increase. These observations highlight the effectiveness of Vimolert® in bolstering the immune response to *A. hydrophila* infection. The pronounced increase in leukocyte and neutrophil counts is indicative of enhanced innate immune defenses in groups 3, 4, and 5, a finding in line with the work of **Amphan *et al.* (2019)** emphasizing the role of β-glucan in promoting phagocytosis and disease resistance against *A. hydrophila*.

Changes in phagocytic percent and phagocytic index

The administration of Vimolert® after the fourth week brought about a significant increase in phagocytic percent and phagocytic index in groups 3, 4 and 5, showcasing the immunostimulant effects of Vimolert®. This substantial boost in phagocytic activity highlights the potential of Vimolert® in strengthening the innate immune response.

Conversely, group 2 exhibited a significant increase in these parameters after the sixth week of infection. However, groups 3, 4 and 5 displayed a highly significant increase compared to group 2. This difference can be ascribed to the presence of lysozyme enzymes in Vimolert®, which facilitate the destruction of bacterial cell walls and enhance phagocytosis by phagocytes **(Kord *et al.*, 2021)**.

CONCLUSION

This study provided compelling evidence of Vimolert's ability to mitigate the impact of *A. hydrophila* infection in the Nile tilapia. The combined probiotic and immunostimulant approach demonstrated remarkable efficacy in preventing clinical symptoms, reducing mortality, and enhancing various hematological and immune parameters. These findings presented valuable insights for the aquaculture industry, highlighting Vimolert® as a potential tool to bolster fish health and disease resistance. However, it is essential to consider the species-specific effects and the need for further research to elucidate the precise mechanisms underlying Vimolert's immunomodulatory effects. This study primarily focused on the Nile tilapia, and the translation of these results to other fish species and aquaculture conditions should be investigated in future research.

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