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Enhancing Nile Tilapia Health and Survival: The Impact of Vitamin Premix Dietary Supplementation Against *Aeromonas sobria* Infection

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Abstract

Diseases epidemics associated with high mortality rates produce high economic fatalities in the aquaculture industry. Vitamin premix (VP) supplementation may bolster the immune response in Nile tilapia. Accordingly, we aim to find optimal feeding strategies to enhance farmed fish health and survival. Our study performed several biochemical, immunological, and histopathological analyses to assess the effect of VP supplementation in promoting growth performance-related parameters. The results revealed that different levels of VP could significantly enhance Nile tilapia growth, feed utilization, and survival. Moreover, the findings provided a comprehensive understanding of the temporal and treatment-dependent changes in metabolites and lysozyme activity, shedding light on the intricate physiological responses of Nile tilapia to varying VP levels in their diets. Our study offers innovative approaches to improve the well-being and longevity of cultivated Nile tilapia by providing a sustainable method for reducing the adverse effects of bacterial problems in aquaculture environments.

Keywords: Nile tilapia; Vitamin premix; Dietary supplementation; *Aeromonas sobria*

Introduction

Aquaculture is a vital pillar in meeting the escalating global demand for high-quality protein, with the Nile tilapia (*Oreochromis niloticus*) being a cornerstone of freshwater fish farming (Abd El-Hack *et al*. 2022). However, the industry grapples with multifaceted challenges, among which the threat of bacterial infections looms large, leading to considerable economic repercussions (Dadgostar 2019). *Aeromonas sobria*, a recognized pathogenic agent, has been implicated in severe infections, contributing to a substantial burden on the Nile tilapia farming sector (Nicholson *et al*. 2020).

Researchers have been actively exploring innovative strategies to fortify the immune response and bolster disease resistance in aquaculture species in response to these challenges. One avenue of increasing interest involves the dietary supplementation of vitamin premixes (VPs), recognized for their pivotal roles in immune function, growth, and overall health (Alagawany *et al*. 2021). Despite the growing body of literature exploring nutritional interventions, a discernible gap exists in our understanding of the specific impact of VP supplementation on Nile tilapia resistance against *Aeromonas sobria* infection.

Vitamins, essential micronutrients, play key roles in various physiological processes, including immune response modulation (Gombart *et al*. 2020). Nevertheless, the specific dynamics of how VPs influence Nile tilapia resistance to *Aeromonas sobria* infection remain underexplored. Our study aims to address this knowledge gap by systematically examining the effects of different VP dietary supplementation levels over 60 days. The central focus is to evaluate the capacity of these interventions to enhance the innate defenses of Nile tilapia and, consequently, mitigate the impact of *Aeromonas sobria* infection. By elucidating the potential of VP supplementation to bolster the immune response in Nile tilapia, this study seeks to inform strategies for optimizing dietary approaches.

Material and method

VP supplementation

Rearing conditions of Nile tilapia

Apparently healthy Nile tilapia (*Oreochromis niloticus*; n = 120; 14.5 ± 5 g) were randomly collected from the earthen ponds of Abbassa Fish Farm. The fish were allocated in a random manner across 12 glass aquaria. These aquaria were filled with de-chlorinated tap water and provided with sufficient aeration and underwater internal power filters. The fish were observed for a period of two weeks to allow them to acclimate to their new environment before the experimental diet was introduced. Weekly, 30% of the water was replaced to

ensure optimal water quality. The mean water temperature recorded during the experiment was 28 ˚C, while the pH ranged from 6.5 to 7.5. The study was performed at the Central Laboratory for Aquaculture Research (CLAR), which is part of the Agriculture Research Center, the Ministry of Agriculture in Alabassa, Abo-Hamad, Sharkia, Egypt. The study was conducted with the approval number ZU-IACUC/1/F/406/2022.

Experimental design, diets, and feeding trial

The fish were assigned randomly to four groups, with each group consisting of 30 fish. The fish were then placed evenly throughout three glass aquaria, each measuring 70 × 60 × 50 cm. Within each group, there were three replicates, with each group containing 10 fish. The initial group (control T1) was provided with the basic diet without any additional supplements (CON). The T2, T3, and T4 groups were provided with basal meals enriched with 1%, 0,1% ,0,01% respectively , for a duration of 60 days as in blood sampling. The fish were provided with the formulated diet (**Table 1**) twice a day, at a quantity equivalent to 3% of their body weight.

Table 1 presents a detailed breakdown of the formulated diets (CON, VP0.001, VP0.01, and VP1.0) used in the experiment, elucidating the composition of each treatment. This formulation ensures a standardized nutritional baseline across all treatments and lays the groundwork for a controlled experimental environment where observed effects can be attributed to variations in VP supplementation.

Table 1. Ingredients and proximate chemical analysis of the formulated with 1.5% VP

Ingredients	**Treatments**			
	CON	**VP0.01%**	**VP0.1%**	**VP1.0%**
Fish meal (60%)	6	6	6	6
Soybean meal (48%)	43.8	43.8	43.8	43.8
Ground corn	21.3	21.3	21.3	21.3
Wheat bran	19.4	19.4	19.4	19.4
Cod fish oil	2.65	2.65	2.65	2.65
Corn oil	1.35	1.35	1.35	1.35
Vitamins premix	1.5	+0.01%	+0.1%	+1.0%
Minerals	1.5	1.5	1.5	1.5
Starch	2.5	2.5	2.5	2.5
Chemical analysis (%)				
Dry matter	91.01	91.01	91.01	91.01
Crude protein	30.21	30.21	30.21	30.21
Crude fat	3.48	3.48	3.48	3.48
Ash	8.65	8.65	8.65	8.65
Fiber	5.10	5.10	5.10	5.10
Nitrogen-free extract	52.56	52.56	52.56	52.56
Gross energy (Kcal/100g)	419.06	419.06	419.06	419.06
Protein-to-energy ratio	72.08	72.08	72.08	72.08

Evaluating growth performance-related parameters

The growth performance of Nile tilapia was conducted by specific growth rate (SGR) (%/day) = 100 (final body weight (g) – initial body weight (g))/experimental period (day) as well as feed conversion ratio (FCR) = Feed intake (g) /weight gain (g).

Sampling and analysis techniques

Blood sampling

At zero, 30, and 60 days of the experimental period, blood samples were obtained from the caudal blood vessels of each fish in all the experimental groups. The blood samples were obtained using aseptic syringes without anticoagulant, kept at ambient temperature for 6 h, and then subjected to centrifugation at 1000 rpm for 5 min to isolate the serum (Soivio *et al.* 1975). Subsequently, the serum was employed to quantify the immunological and biochemical markers.

Lysozyme activity

The lysozyme activity was quantified using a Fig electric colorimeter equipped with a turbidity measurement adapter. A dilution series was created to generate the calibration curve by combining the standard lysozyme from hen egg-white (Fluka, Switzerland) with a suspension of *Micrococcus lysodeikticus* (ATCC No. 1698 Sigma) after dilution. Afterward, 10 mL of a standard solution or serum was combined with 200 mL of a *Micrococcus* suspension consisting of 35 mg of *Micrococcus* dry powder dissolved in 95 mL of a 1/15 M phosphate buffer, along with 5.0 mL of a NaCl solution. The extinction changes were quantified at a wavelength of 546 nm by measuring the extinction immediately after adding the lysozyme-containing solution (beginning of the reaction) and after a 20-min incubation of the sample at 40˚C (end of the reaction) using an ELISA reader (Bio TEC, ELX800G, USA).

Tissue processing

The gills, liver, and spleen tissue specimens, which had been kept in formalin, were treated using an automated tissue processor. The preparation involved an initial fixation and dehydration phase comprising two steps. The fixation process involved immersing the tissue in a solution of 10% buffered formalin for 48 h, and then removing the fixative by rinsing it with distilled water for 30 min. Dehydration was achieved by sequentially immersing the tissues in a series of alcohol solutions with increasing concentrations (70%, 90%, and 100%). The tissue was first subjected to 70% alcohol for a duration of 120 min, followed by 90% alcohol for 90 min, and after that, underwent two cycles of 100% alcohol, with each cycle lasting 1 h. The dehydration process was performed by immersing the samples in a mixture of 50% alcohol and 50% xylene for 1 hour, followed by pure xylene for 90 min. The samples were saturated with liquefied paraffin wax, incorporated, and obstructed. The paraffin slices, which were 4-5 um thick, underwent staining with Hematoxylin and Eosin according to the method described by Suvarna *et al.* (2012). The stained sections were analyzed for circulatory disruptions, inflammation, degenerations, apoptosis, necrosis, and any other abnormal alterations in the studied tissues.

Challenge test

Upon completion of the feeding experiment, the fish from each group were gathered and placed into tanks at a density of 10 fish per 100 L, with each tank containing duplicates. The challenge test was performed utilizing *A. hydrophila*. A preliminary challenge experiment was conducted to ascertain the LD_{50} (lethal dose) of the pathogenic bacteria. Next, the fish were exposed to pathogenic *Aeromonas sobria*. The bacteria were cultivated in nutrient broth at 30 °C for 24 h in an incubator. Afterward, the bacterial cells were collected by centrifuging the broth at 3,000 g for 30 min. The resulting pellets were then suspended in 1.0 mL of 0.1% peptone water. The fish were injected with a sub-lethal dose of 0.1 mL of the bacterial suspension. The suspension was obtained from virulent *A. hydrophila* broth that had been cultured for 24 h and had a concentration of 5×10^5 CFU/mL. The fish group received an intraperitoneal injection of 0.1 mL of saline solution, serving as the negative control. All fish groups were also intraperitoneally injected and thereafter monitored for ten days to document any aberrant clinical symptoms and daily fish mortality. *Aeromonas sobria* was isolated from the gills, liver, kidneys, and spleen of the fish that were in a dying or recently deceased state. The relative percent of fish survival (RPS) at day ten was determined using the formula: RPS = 1 – (% mortality in the test group/% mortality in the control group) × 100.

Statistical analyses

The statistical analyses were conducted using the SPSS program V.10 (SPSS, Richmond, USA). The collected data underwent one-way ANOVA. Mean differences were assessed using Duncan's new multiple-range test at a significance level of 5%.

Results

Growth performance

Table 2 provides a comprehensive overview of the growth performance, feed intake, feed conversion ratio (FCR), and survival rates of Nile tilapia *Oreochromis niloticus* over 60 days. Regarding growth parameters, the initial fish weights across treatments were similar. However, significant differences emerged in the final weights, where CON, VP0.01, and VP0.1 exhibited comparable values, while VP1.0 demonstrated a significantly higher final weight. A similar trend was observed in weight gain, with CON, VP0.01, and VP0.1 treatments showing comparable values and VP1.0 presenting a slightly lower weight gain. The SGR reflected a significant difference, with VP1.0 exhibiting a higher SGR than the other treatments.

Regarding feed utilization, feed intake displayed significant differences, with VP1.0 having a significantly higher intake than CON, VP0.01, and VP0.1. The FCR exhibited distinctions, control group showing significant increase than, VP0.01, VP0.01 and VP1.0.also, the first groups VP0.01showing higher than , VP0.01 and VP1.0 groups . Fish survival rates varied significantly, with CON and VP0.01 treatments having comparable rates, VP0.1 showing a higher survival rate, and VP1.0 demonstrating the highest survival rate.

Table 2. Growth performance, feed intake, feed conversion ratio, and survival of Nile tilapia fed diets containing different vitamin premix levels.

Item[1]	Treatments[2]			
	CON	VP0.01	VP0.1	VP1.0
Initial weight (g)	14.2 ± 0.418	14.1 ± 0.205	14.03 ± 0.094	14.14 ± 0.309
Final weight (g)	32^{a} ± 0.199	32^{a} ± 0.34	32^{a} ± 0.35	34^{b} ± 0.35
Weight gain (g)	16.6^{a} ± 1.32	17.6^{a} ± 0.90	17.5^{a} ± 0.31	17.1^{b} ± 0.43
SGR (%g/day)	39^{a} ± 0.135	339^{a} ± 0.329	339^{a} ± 0.45	345^{b} ± 0.12
Feed intake (g feed/fish)	32.83^{b} ± 8.50	30.56^{a} ± 7.96	31.58^{a} ± 7.22	27.79^{a} ± 5.89
FCR	1.73^{a} ± 0.22	1.50^{a} ± 0.17	1.54^{a} ± 0.27	1.37^{b} ± 0.20
Fish survival (%)	90^{a} ± 0.22	90^{a} ± 0.39	93^{a} ± 0.36	100^{b} ± 0.22

[1]SGR: specific growth rate; FCR: feed conversion ratio

[2] Fish-fed basal diets containing 0, 0.01, 0.1, and 1.0 g/kg of vitamins premix,1%, 0.1%, and 0.01%, respectively.

[a–b] Means having different letters in the same row indicate significant difference at $p < 0.05$.

Figs. 1A–B depict the effects of different VP supplementation levels in the Nile tilapia on mortality and survival percentages, respectively, after infection with *Aeromonas sobria.* These visuals represent the outcomes of the infection challenge, highlighting the potential protective effects of VP supplementation on the overall survival of Nile tilapia.

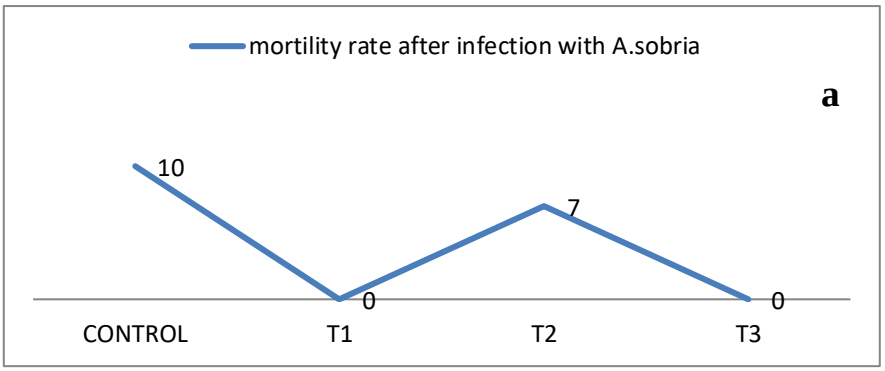

Fig. 1. Effects of various levels of vitamin premix supplemented with dietary for Nile tilapia on mortality (**a**) and survival percentages (**b**) after infection with *Aeromonas sobria*.

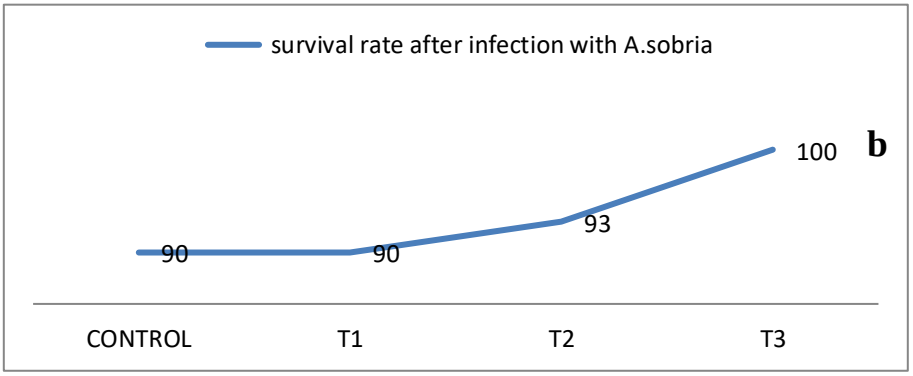

Fig. 2 presents the RPS of Nile tilapia under different dietary conditions. The treatments (T1 to T4) represent varying VP levels in the basal diet. Notably, dietary supplementation with VP for 45 days (T2, T3, and T4) significantly improved the resistance of Nile tilapia against *Aeromonas sobria* infection compared to the control group (T1), which exhibited the lowest fish survival. A synergistic effect was particularly observed in the T2 group, demonstrating the highest fish survival after intraperitoneal infection, resulting in maximum RPS values. The clinical signs of fish mortality included exophthalmia, ascites, tail and fin rots, and scalelessness, accompanied by external hemorrhage. Postmortem findings revealed septicaemic lesions in the internal organs, providing crucial insights into the pathological consequences of the infection challenge.

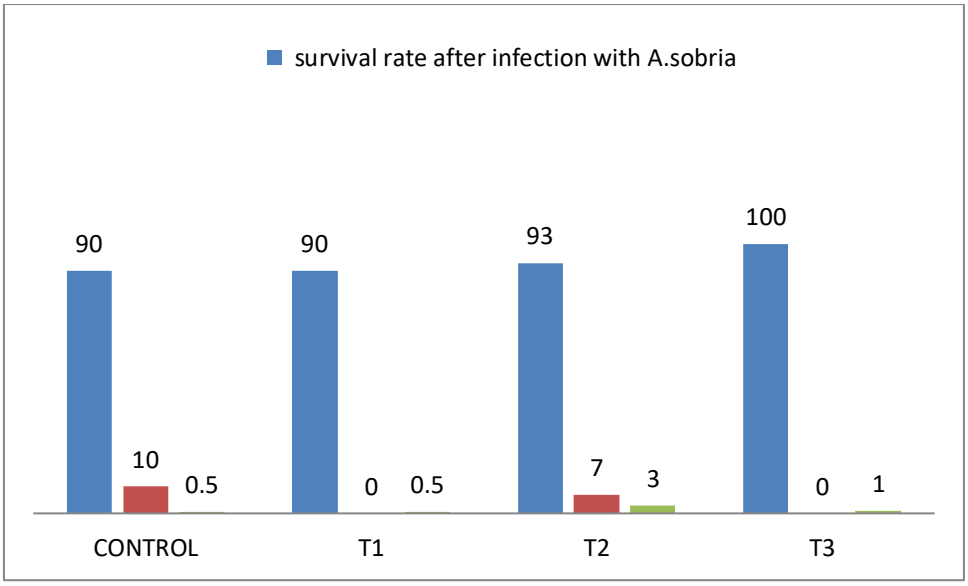

Fig. 2. Relative percent of survival of Nile tilapia.

Biochemical and immunological analys

Creatinine (mg/dL)

Over the assessed time points, significant variations in creatinine levels were observed. At 15 days, CON and VP0.01 treatments exhibited comparable levels, while VP1.0 demonstrated a slightly higher level. At 30 days, significant differences emerged, with VP1.0 showing a significantly elevated creatinine level. By y 60

Total Protein (mg/dL), Throughout the evaluation periods, total protein concentrations varied among treatments. At 30 and 60 days, CON showed lower protein levels compared to other treatments. However, by day 60, a reversal occurred, with CON displaying a higher protein concentration. These findings underscore the temporal shifts in total protein influenced by VP supplementation.

Albumin (mg/dL)

Albumin levels exhibited fluctuations across treatments and time points. At 15 days, significant differences were observed, with CON having higher albumin levels than VP0.01 and VP1.0. By day 30, the trend reversed, indicating the intricate dynamics of albumin concentrations in response to VP levels. By day 60, disparities persisted, suggesting a sustained influence on albumin levels.

Table 3 details the metabolic changes, including creatinine levels, total protein, albumin, globulin concentrations, and lysozyme activity in Nile tilapia subjected to diets with varying VP levels over 60 days.

Item	Time of assessment (days after treatment)	Treatments			
		CON	**VP0.01**	**VP0.1**	**VP1.0**
Creatinine (mg/dL)	15 days	$0.167^{c} \pm 0.023$	$0.207^{bc} \pm 0.021$	$0.19^{c} \pm 0.006$	$0.206^{bc} \pm 0.003$
	30 days	$0.15^{c} \pm 0.029$	$0.16^{c} \pm 0.021$	$0.133^{c} \pm 0.008$	$0.35^{a} \pm 0.057$
	60 days	$0.08^{cd} \pm 0.005$	$0.1767^{c} \pm 0.008$	$0.1767^{c} \pm 0.008$	$0.27^{b} \pm 0.005$
Total protein (mg/dL)	15 days	$1.08^{de} \pm 0.005$	$1.2^{bcd} \pm 0.057$	$1.067^{e} \pm 0.012$	$1.02^{e} \pm 0.006$
	30 days	$1.05^{e} \pm 0.02$	$1.14^{cde} \pm 0.005$	$1.05^{e} \pm 0.012$	$1.22^{abc} \pm 0.063$
	60 days	$1.31^{ab} \pm 0.095$	$1.23^{abc} \pm 0.035$	$1.33^{a} \pm 0.015$	$1.28^{ab} \pm 0.003$
Albumin (mg/dL)	15 days	$0.91^{ab} \pm 0.015$	$0.96^{a} \pm 0.033$	$0.77^{c} \pm 0.063$	$0.86^{abc} \pm 0.006$
	30 days	$0.64^{d} \pm 0.018$	$0.9^{abc} \pm 0.057$	$0.81^{bc} \pm 0.057$	$0.82^{bc} \pm 0.057$
	60 days	$0.97^{a} \pm 0.014$	$0.9^{abc} \pm 0.057$	$0.78^{bc} \pm 0.003$	$0.77^{c} \pm 0.014$
Globulin (mg/dL)	15 days	$0.15^{f} \pm 0.017$	$0.2^{ef} \pm 0.0577$	$0.39^{bc} \pm 0.057$	$0.166^{f} \pm 0.008$
	30 days	$0.49^{ab} \pm 0.04$	$0.26^{def} \pm 0.04$	$0.273^{def} \pm 0.01$	$0.36^{cd} \pm 0.01$
	60 days	$0.26^{def} \pm 0.02$	$0.29^{cde} \pm 0.06$	$0.59^{a} \pm 0.04$	$0.55^{a} \pm 0.01$
Albumin / Globulin (%)	15 days	$6.17^{b} \pm 0.01$	$10^{a} \pm 0.58$	$2.77^{e} \pm 0.06$	$5.71^{b} \pm 0.04$
	30 days	$1.49^{f} \pm 0.03$	$3.90^{c} \pm 0.012$	$3^{de} \pm 0.58$	$1.82^{f} \pm 0.01$
	60 days	$3.53^{cd} \pm 0.06$	$3.2^{cde} \pm 0.06$	$1.45^{f} \pm 0.02$	$1.42^{f} \pm 0.03$
Lysozyme activity	15 days	$0.077^{a} \pm 0.005$	$0.0543^{ab} \pm 0.008$	$0.068^{ab} \pm 0.0005$	$0.076^{a} \pm 0.001$
	30 days	$0.051^{ab} \pm 0.039$	$0.033^{bc} \pm 0.005$	$0.063^{ab} \pm 0.0005$	$0.068^{ab} \pm 0.003$
	60 days	$0.001^{c} \pm 0.0033$	$0.034^{bc} \pm 0.001$	$0.005^{c} \pm 0.001$	$0.052^{ab} \pm 0.005$

Globulin (mg/dL)

Globulin concentrations demonstrated significant variations in treatment groups. At15 days, CON had lower globulin levels than VP0.1, while VP1.0 exhibited the highest concentration. At subsequent time points, these differences remained, highlighting the sustained impact of VP levels on globulin dynamics.

Albumin/Globulin (%)

The albumin-to-globulin ratio showed significant fluctuations, indicating the overall protein balance. At 15 days, CON displayed a higher ratio than VP0.01

and VP1.0. By day 30, significant differences emerged, with VP0.01 exhibiting the highest ratio. At day 60, these disparities persisted, emphasizing the intricate interplay between albumin and globulin influenced by VP levels.

Lysozyme Activity

Lysozyme activity exhibited significant changes over time and among treatments. At 15 days, CON demonstrated the highest activity, while VP1.0 exhibited slightly lower levels. By day 30, differences were more pronounced, with VP0.01 displaying the highest lysozyme activity. By day 60, variations persisted, underscoring the enduring impact of VP levels on lysozyme activity in Nile tilapia.

Table 3. Metabolites changes and lysozyme activity of *Oreochromis. niloticus* fed diets containing various levels of vitamin premix.

[1] Fish-fed basal diets containing 0, 0.01, 0.1, and 1.0 g/kg of vitamins premix, CON, VP0.01, VP0.1 and VP1.0, respectively. [a–f] Means having different letters in the same row indicates a significant difference at $p < 0.05$.

Histological examinations

The microscopic evaluations provide detailed insights into the morphological changes in gill, liver, and spleen tissues in response to premix treatment and bacterial challenge, shedding light on the potential impacts of these interventions on the examined organ histopathology.

Microscopic evaluation of gill tissues

In the control group, fish revealed normal histo-morphologic structures, including the gill rockers with their epithelial lining that comprises mucus cells, chloride cells, and the subepithelial stromal cells (eosinophilic granular cells and some lymphocytes). The gill arch showed normal fibro-muscular elements, osteocartilaginous terminals, and stromal structures comprising some eosinophilic granular cells. The primary and secondary gill filaments were apparently normal: lamellar epithelial cells, pillar cells, mucus-secreting cells (goblet cells), and chloride cells. Gill sections of the 1% VP treatment group depicted comparatively normal histo-morphological structures in most of the examined cases. However, a few cases revealed focal changes at the tip of the gill filament represented by congested capillaries, focal denudation of the secondary filament structures (epithelial lifting and necrosis and capillary-pillar cells disappearance), round cell infiltration and chloride cells proliferation. Gill sections of the 0.1% VP group denoted minor changes, as the tips of a few filaments revealed congestion epithelial lifting and stromal round cell infiltration. Gill sections of the 0.01% VP treatment group demonstrated comparatively somewhat more prominent morpho-pathological changes than the other groups. The tips of some filaments appeared focally denuded, fused, thick, and enlarged by severely congested capillaries,

epithelial proliferation beside round cells, and eosinophilic granular cell infiltration **(Fig. 3).**

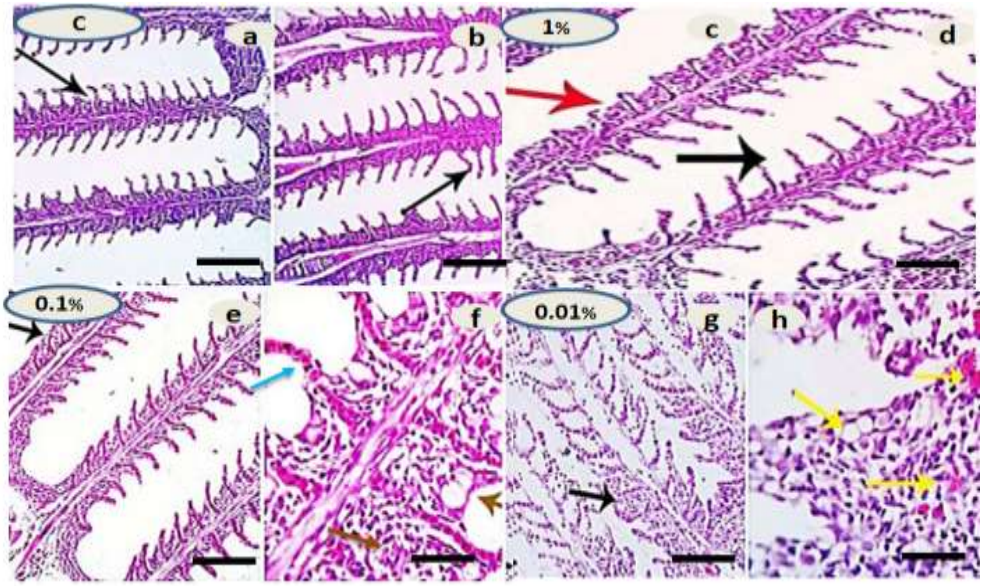

Fig. 3. Photo-micrograph of control (C) and VP treated gills at (1%, 0.1%, and 0.01%) showing normal micromorphological structures of the preserved gill filament structures (black arrows) in control. Almost normal histo-morphological structures in some parts (black arrow) and focal epithelial denudation in other parts (red arrow) in 1% treatment. Tip of the gill filament showing congested, telangiectatic capillaries (blue arrow), focal denudation of the secondary filament structure (black arrow), and chloride cell proliferation (brown arrows) in 0.1% treatment. In 0.01% of treatments, signs observed in the tips of some filaments appear focally denuded, fused, thick (black arrow) beside round cells, and eosinophilic granular cells infiltration (yellow arrows). Scale bars (a) and (d) = 100 µm, and (b), (c), and (e) = 50 µm.

Microscopic evaluation of liver tissues

The control group had normally organized hepatic lobules with well-district boundaries and radially arranged hepatic cords around well-formed, thin-walled central veins engorged with nucleated erythrocytes. Elongated and branched hepatic sinusoids were observed among the hepatic cords. The large hepatocytes showed cytoplasmic ribosomal basophilia and mild to moderate metabolically induced vacuolation (glycogen and fat globules deposition), with a centrally located moderately enlarged nucleus, sometimes double nucleation, and enlarged nucleoli. The hepato-porta area and the hepatic-pancreatic structures were well-developed, and neither sometimes completely missed. Liver sections of 1% VP exhibited histo-morphological changes

comparable to that of control fish with normally organized hepatic lobules, well-district boundaries, and radially arranged hepatic cords around well-formed thin-walled central veins engorged with nucleated erythrocytes. The hepato-portal blood vessels were mildly dilated in the liver sections of 0.1% VP-treated fish. The large hepatocytes showed cytoplasmic ribosomal basophilia and mild to moderate metabolically induced vacuolation. Neither melanomacrophage nor pigmentary deposits were observed in liver sections of 0.01% VP-treated fish (**(Fig. 4)**.

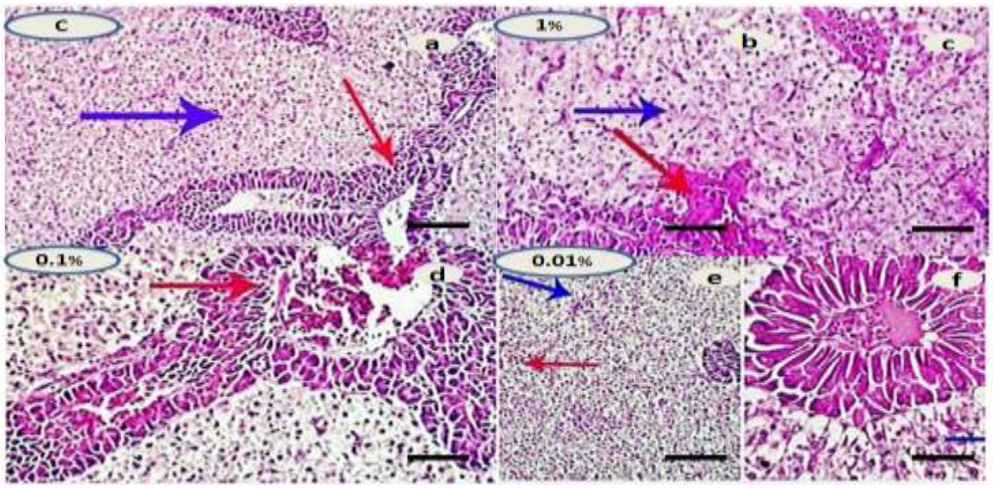

Fig. 4. Photo-micrograph of control (C) and VP treated liver at (1%, 0.1%, and 0.01%) showing normally arranged hepatic cords (blue arrow) and normal hepatic-pancreas (red arrow) in control. Showing normally organized hepatic lobules, well-district boundaries, and radially arranged hepatic cords (blue arrow) around well-formed thin-walled central veins engorged with nucleated erythrocytes (red arrow) in 1% VP. The hepatic-portal blood vessels appear mildly dilated in the liver (red arrow) in 0.1% VP. The hepatocytes appear large, with cytoplasmic ribosomal basophilic and centrally located moderately enlarged nuclei (red arrow), double nucleation, and enlarged nucleoli (red arrow) in 0.01% VP. Scale bars (a) and (e) = 100 µm, and (b–d) and (f) = 50 µm.

Microscopic evaluation of spleen tissues

The results revealed splenic cords as a mesh of fibroblast-like cells with foci of various blood cells. White pulp, consisting mainly of lymphoid cells, typically surrounds arterial vessels, sometimes assuming a nodular pattern. The characteristic immune melanomacrophage centers (MMC) form small parenchyma clusters. The splenic sinusoids were mild to moderately congested and occupied a large area of the tissue, constituting the red pulp of the spleen. Characteristic proliferative aggregations of melanomacrophages, both perivascular and interstitial. The 1%, 0.1%, and 0.01 % VP groups'

spleens revealed histo-morphological structures comparable to those of the control group. However, the lymphoid elements were more prevalent, particularly around the small-size blood vessels forming aggregations or well-distinct nodular arrangement and splenic cords, especially in the 1% VP. The MMCs were mild to moderately activated in the 0.1% VP **(Fig. 5)**.

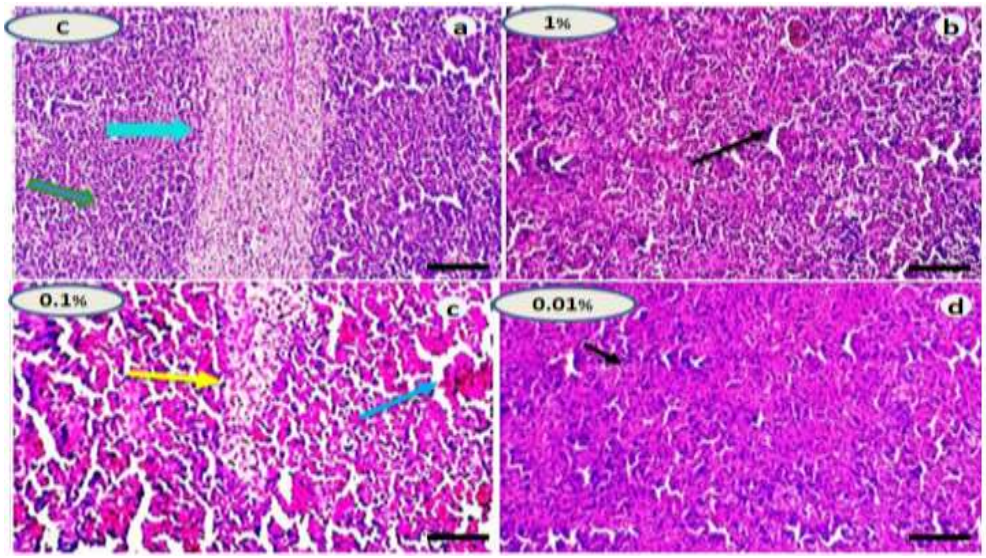

Fig. 5. Photo-micrograph of control (C) and VP-treated spleen at (1%, 0.1 %, and 0.01%) showing apparently normal splenic lymphoid elements (green arrow) and mildly activated melanomacrophage centers (Light blue arrow) in control. A distinct nodular arrangement and splenic cords exist, especially in 1% and 0.1% VP (black arrows), showing prevalent lymphoid elements around the small-sized blood vessels (blue arrow), melanomacrophage centers appear mildly to activate (yellow arrow) in 0.01% VP. Scale bars (a–b) and (d) = 100 µm, and (c) = 50 µm.

Microscopic evaluation of gill tissues after bacterial challenge

The examined sections from the gills of the control group showed focal epithelial proliferative changes with partial replacement of the secondary filaments. A focal increase of goblet cells was observed near the lesion. Gill arches and rockers showed marked hyperemia, edema, and eosinophilic granular cell infiltration. Some of the gill filaments revealed focal and or total epithelial necrosis. The base of some filaments showed complete necrosis of the epithelial lining. Chloride cells gain a "vacuolated" aspect due to liquid retention and increased size in the gill filaments of fish treated with 1% VP, revealing focal filament capillary congestion, focal epithelial denudation, and necrosis beside goblet cells proliferation, which was marked at the basal parts of gill filaments. Some of the primary filaments showed round cell infiltration.

The gill arch showed mild vascular dilatation, edema, and eosinophil's granular cell infiltration. Gills of the 0.1 and 0.01% VP-treated groups showed focal denudation of the lining epithelium, thickening, and adhesion due to chronic inflammatory cell infiltration. Some filaments showed complete necrosis of their epithelial, marked goblet cells metaplasia, and infiltration of round cells and eosinophilia granular cells. Gill rockers showed the same pathological events. Lymphocytes extensively infiltrated the gill arches and bases of gill filaments, macrophages, and eosinophilia granular cells **(Fig. 6).**

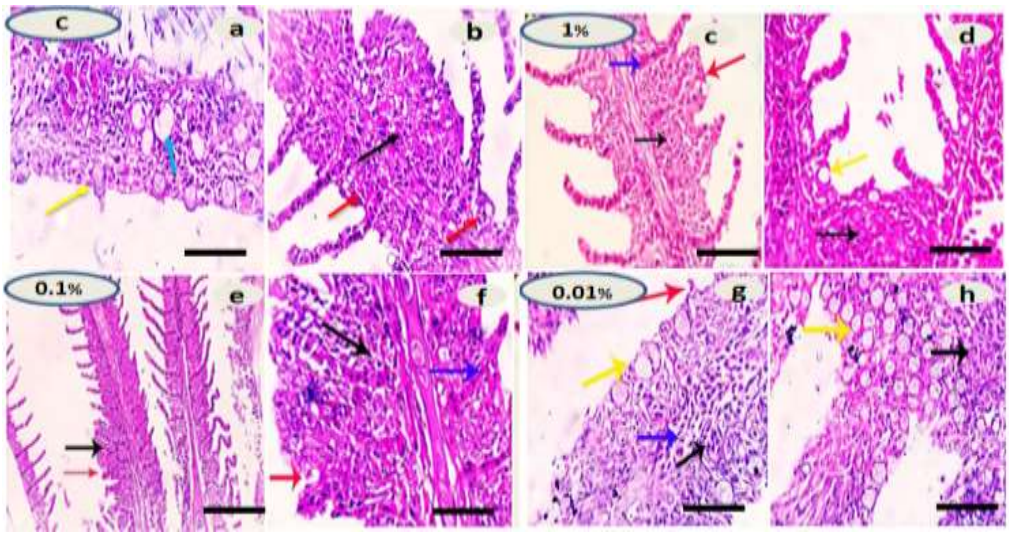

Fig. 6. Photo-micrograph of control (C) and VP treated gills at (1%, 0.1%, and 0.01%) after bacterial challenge showing necrosis in the epithelial cells (yellow arrow), diffuse hypertrophy of chloride cells (blue arrow), degeneration of cartilaginous bar (black arrows) and increase number of mucous cells (red arrows) in control. Focal denudation of the lining epithelium (red arrow) marked goblet cells metaplasia (yellow arrows), infiltration of round cells (black arrows), and severe congestion of blood lacunae (blue arrow) in 1% VP. Fusion of the secondary lamellae (red arrows), the proliferation of the gill epithelium (black arrows), and severe congestion (blue arrow) in 0.1% VP. Focal denudation of the lining epithelium (red arrow), inflammatory cell infiltration (black arrows), congestion of blood lacunae (blue arrow), and chloride cells increase in size by 0.01% treatment. Scale bars (a–d) and (f–h) = 100 µm, and (e) = 50 µm.

Microscopic evaluation of liver tissues after bacterial challenge

The examined liver sections of the control group revealed distinct histopathologic changes represented by focal hepatocellular degenerative and early necrotic changes, focal vascular dilatation, hepato-pancreatic degenerative, apoptotic, and necrotic changes in focal or total patterns beside focal aggregations and infiltration of melanomacrophages replaced by diffuse.

After exposure to a bacterial challenge, certain liver sections treated with 1% VP exhibited focal hepato-pancreatic dissociation with mild eosinophilia granular cell infiltration. Other sections revealed apparently normal hepatic parenchyma and vascular structures. Meanwhile, some liver sections of 0.1% and 0.01% VP demonstrated moderate hepato-portal congestion, degenerative and focal necrotic changes in the hepato-pancreatic acini, infiltration of melanomacrophages in addition to hepatocellular degeneration, mainly hydropic degeneration, and macrosteatosis. Other liver sections were apparently normal **(Fig. 7).**

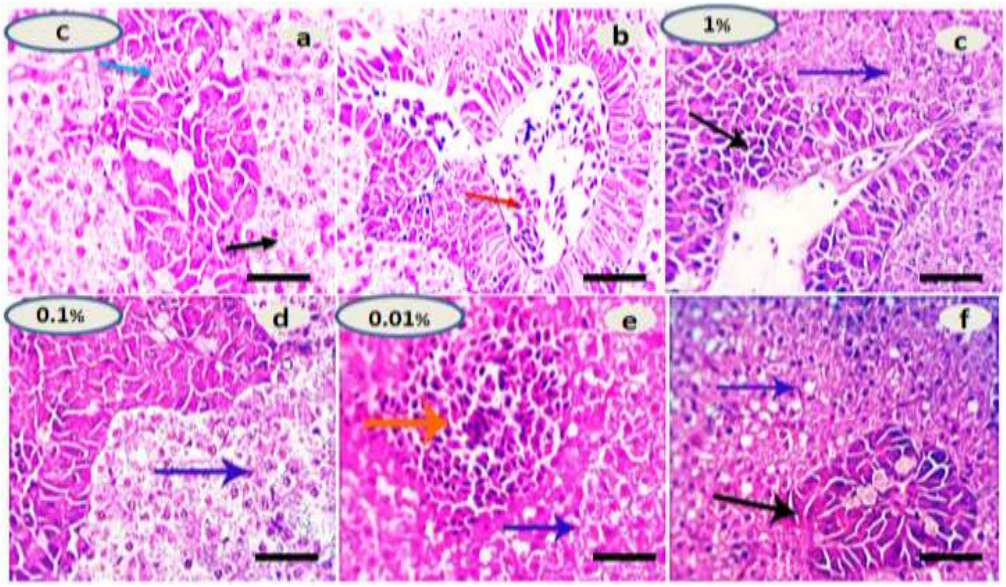

Fig. 7. Photo-micrograph of control (C) and VP treated liver at (1%, 0.1%, and 0.01%) after bacterial challenge showing early necrotic changes (black arrow), focal vascular dilatation (red arrow), and aggregations of mild stain melanomacrophages (blue arrows) in control. Apparently, normal hepatic parenchyma and vascular structures. (Black and blue arrows) in 1% VP. Focal apoptotic changes in the hepato-pancreatic acini (orange arrow), infiltration of melanomacrophages (black arrow), and hydropic degeneration (blue arrows) in 0.1 % and 0.01% VP. Scale bars (a–f) = 50 µm.

Microscopic evaluation of spleen tissues after bacterial challenge

The control group exhibited splenic lesions characterized by diffuse lymphoid depletion, vascular dilatation, localized hemorrhages, and significant activation of the melanomacrophage centers. Upon examination of spleen specimens from fish subjected to a 1% VP and subsequently exposed to bacterial challenge, certain sections displayed lymphoid elements that appeared in a normal state. Additionally, activated melanomacrophage centers were observed, which also contained lymphoid elements that appeared to be

normal. Nevertheless, the splenic sections in most other treatments (0.1 and 0.01%) exhibited significantly stimulated proliferating melanomacrophage centers and congested splenic sinusoids. A slight reduction in the number of lymphoid cells was seen in several areas (**Fig. 8**).

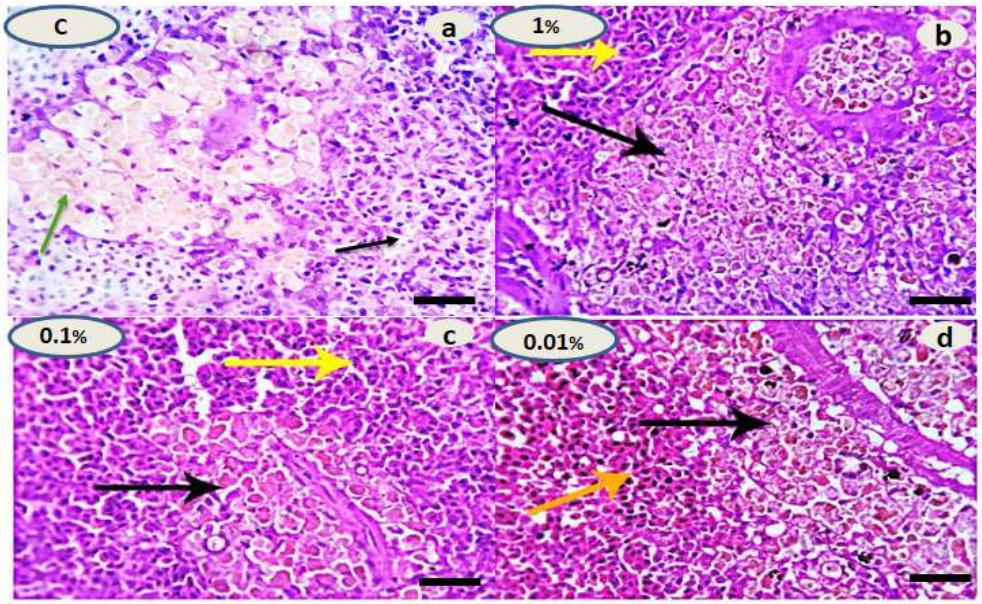

Fig. 8. Photo-micrograph of control (C) and VP treated spleen at (1%, 0.1%, and 0.01%) after bacterial challenge showing lymphoid depletion (black arrows) and marked activation of the MMCs. (Green arrow) in control. Apparently, normal lymphoid elements (yellow arrows) are in 1% and 0.1% VP. Mildly congested splenic sinusoids (orange arrow) in 0.01% VP. Scale bars (a–d) = 50 µm.

Discussion

The comprehensive investigation conducted in this study delves into the intricate relations between VP supplementation, immune response modulation, histopathological alterations, growth performance, and metabolic dynamics in Nile tilapia under the challenge of *Aeromonas sobria* infection. The amalgamation of various analytical approaches, including microscopic evaluations, growth parameter assessments, and biochemical analyses, provides a nuanced understanding of the multifaceted implications of vitamin levels in the aquaculture setting.

The microscopic evaluations of gill tissues revealed distinct histo-morphological changes and a potential correlation between these changes and VP supplementation dosage. The differential responses observed, such as focal denudation, congestion, and cellular infiltration, underscore the nuanced nature of the immune response and highlight the potential of VP to influence

gill health (Wintergerst *et al*. 2007). The liver and spleen assessments further contribute to this narrative, illustrating systemic alterations that could be attributed to varying vitamin concentrations (Barrea *et al*. 2018). The liver response, marked by changes in blood vessels and hepatocyte abnormalities, suggests a potential influence of vitamin levels on hepatic functions (Licata *et al*. 2021). Similarly, splenic alterations, particularly changes in nodular arrangements and lymphoid elements, provide crucial insights into the immunomodulatory effects of VP.

The microscopic evaluation after bacterial challenge sheds light on the protective effects of VP supplementation against *Aeromonas sobria* infection. The improvements, including reduced denudation and inflammatory cell infiltration, align with the enhanced survival rates observed in the corresponding figures. Histopathological changes in the liver and spleen after bacterial challenge further emphasize the protective role of VP, with significant reductions in necrotic changes and lymphoid depletion in treated groups. These findings collectively support the hypothesis that vitamin supplementation contributes to the mitigation of bacterial-induced histopathological alterations, which is crucial for the overall health and survival of Nile tilapia (Almarri *et al.* 2023).

The growth performance and feed utilization data present intriguing patterns requiring careful consideration; meanwhile, vitamin supplementation results in nonsignificant differences in initial weights, final weights, and weight gain. The significant increase in SGR in the VP1.0 group suggests a potential growth-promoting effect associated with higher vitamin concentrations. Feed intake variations among groups indicate a dose-dependent response, with the VP1.0 group exhibiting higher feed intake. The FCR differences underscore the nuanced relation between vitamin levels and feed efficiency. Importantly, the highest survival rate in the VP1.0 group indicates a potential positive correlation between vitamin concentration, growth, and survival. These findings collectively advocate for carefully considering vitamin concentrations in aquaculture diets, aiming for an optimal balance that maximizes growth and survival.

Analyzing metabolic profiles provides valuable insights into the physiological effects of VP supplementation (Nasreldin *et al.* 2023). Creatinine levels, indicating renal function, displayed significant variations, highlighting the intricate balance influenced by vitamin concentrations. Total protein levels exhibited temporal fluctuations, underscoring the dynamic nature of nutrient metabolism in response to varying vitamin levels. Albumin and globulin concentrations and the albumin/globulin ratio suggest intricate interactions between vitamin levels and protein synthesis, reflecting the systemic impact of VP on the overall metabolic health of Nile tilapia. The temporal variations

in lysozyme activity, a crucial component of the innate immune system, demonstrate the potential role of VP in modulating immune responses over time.

Our findings have substantial implications for the field of aquaculture. The nuanced effects highlighted the need for precise nutrient management strategies to optimize the health and performance of aquatic organisms. The dose-dependent responses underscore the importance of tailored vitamin supplementation for optimal growth, immune response, and overall survival. Meanwhile, higher vitamin concentrations demonstrated positive effects on growth and survival. Further research is warranted to fine-tune these levels, ensuring maximum benefits without potential drawbacks.

Conclusion

This study contributes significantly to understanding the multifaceted effects of VP supplementation in Nile tilapia. The integrated approach, combining histopathological evaluations, growth parameters, and metabolic profiling, enriches our comprehension of the intricate interactions between vitamins, immune responses, and overall health in aquaculture. Further research is strongly encouraged to unravel the underlying molecular mechanisms and to establish refined recommendations for optimal vitamin levels, fostering sustainable and efficient aquaculture practices.

Ethics declarations

Conflict of interest

The authors declare that there are no conflicts of interest.

Ethical treatment of animals:

The experimental procedures conducted in this study were reviewed and approved by the ZU-IACUC (Zagazig University Institutional Animal Care and Use Committee) in accordance with ethical guidelines for animal research. The approval number for this study is ZU-IACUC/1/F/408/2022. Approval was granted for the period from December 29, 2022, to December 29, 2024, ensuring compliance with ethical standards throughout the duration of the study.

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