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Effects of Dietary Riboflavin on the Immune Status of the Nile Tilapia (*Oreochromis niloticus*)

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ABSTRACT

The prospective effect of riboflavin (vitamin B2) to enhance the immune status of the Nile tilapia (*Oreochromis niloticus*) was investigated. Fish were divided into 4 groups before feeding for 2 weeks with 0, 10, 15, and 20mg/ kg of riboflavin. Some humoral immune parameters such as antiproteases, lysozyme, bactericidal, IgM, IL-12, total protein, and globulin, as well as hematological parameters (red and white blood cell counts) were estimated. The results revealed enhancement in all the humoral immune parameters of fish groups fed diets supplemented with riboflavin compared to the control fish, especially with the 10mg/ kg dose which recorded the highest significant increase ($P< 0.05$) in some parameters like antiproteases, lysozyme, bactericidal, and IgM compared to the control. In addition, the group that received 10mg/ kg of riboflavin recorded the highest significant number of the red blood cells ($P< 0.05$). A significant increase in number of white blood cells ($P< 0.05$) was observed in 10 and 20mg/ kg treated groups. The current study suggests adding riboflavin to the diet, particularly at a 10mg/ kg dose, since it may be an effective food additive for enhancing the haematological and immune status of the Nile tilapia and increasing its resistance to disease.

INTRODUCTION

Fish production is considered a global evergrowing industry. Its expansion relies on addressing significant challenges, including enhancing and optimizing nutrition for each fish species and managing or preventing disease outbreaks. Creating natural commercial diets for fish that do not compromise their growth and health is an important goal in nutrition, thereby ensuring high-quality products for consumers **(Cruz-Garcia *et al.*, 2011)**. On the other hand, the rapid and intensive development of aquaculture subject the

fish to stressful conditions that weaken their immune system and elevation susceptibility to disease causing notable economic losses **(Kumar *et al.*, 2012)**. Traditional strategies used in fish farms to control or prevent the disease have typically centered around the utilization of antibiotics and chemotherapeutic **(Santos & Ramos, 2016)**. Although antibiotics are effective in treating diseases, there are concerns regarding the accumulation of antibiotic residues in the environment or in fish tissues, as well as the emergence of antimicrobial-resistant strains. These factors discourage their use, particularly due to potential implications for human health **(Muñoz de la Peña & Espinosa-Mansilla, 2009; Santos & Ramos, 2016)**. Thus, using immunostimulant like natural products, either as extracts or compounds, can optimize nutrition and monitor diseases, providing a convenient solution for both fish farmers and consumers **(Awad & Awaad, 2017)**. Vitamins and minerals are natural compounds essentially added to fish diets to promote their growth and overall health **(Misra *et al.*, 2007)**.

Riboflavin or vitamin B2 is a water-soluble molecule and is a fundamental component of the vitamin B complex, which is essential for fish nutrition **(Deng & Wilson, 2003)**. Riboflavin comprises two crucial cofactors, flavin mononucleotide and flavin adenine dinucleotide, which play essential roles in various oxidative-reduction processes and energy metabolism pathways. It can suppress or inactivate the growth of different microorganisms such as bacteria, fungi, viruses, and parasites, highlighting the potential role of riboflavin as an antimicrobial agent **(Farah *et al.*, 2022)**. Riboflavin deficiency induced a reduction in the growth performance of the juvenile Jian carp **(Li *et al.*, 2010)** and a decrease in the growth and gill immunity of the young grass carp **(Chen *et al.*, 2015)**. Riboflavin reduced the harmful effect of arsenic pollution and high temperature on the growth performance, and immune status of the striped catfish **(Kumar, 2021)** and enhanced the growth of the *Channa punctatus* fingerlings **(Zehra & Khan, 2018)**.

The Nile tilapia is the most favorite and common fish in Egypt. Due to the increase in human population, Egypt increased the production of the tilapia aquaculture and currently became the third-largest tilapia producer in the world after China and Indonesia **(FAO, 2020)**. The nutritional needs of fish can change depending on fish species, their growth stage, and various factors. For example, studies carried out on the rainbow trout, revealed that the amount of riboflavin required during the fingerling stage (initial weight 2g) is twice as much as that needed after this stage (initial weight 60g) **(Amezaga & Knox, 1990)**. To our knowledge, there are little or even no literature about the effect of riboflavin either on immune, or growth, or general fish health of the Nile tilapia.

Therefore, this study focused on the influence of one of the essential vitamins, riboflavin (vitamin B2) on the Nile tilapia's immune response to resist disease infection, and some hematological parameters (white and red blood cells). Furthermore, this

research was concerned with determining the optimal dose required for the Nile tilapia to stimulate general fish health.

MATERIALS AND METHODS

1. Preparation of experimental diet

The commercial fish diet (floating 35% protein) was crushed and mixed with 0, 10, 15, 20mg/ kg of riboflavin (Modern lab, Cairo, Egypt). The mixture was moistened with water and reshaped into pellets before being left to dry in the air.

2. Experimental design

Approximately 84 Nile tilapia (*Oreochromis niloticus*), each weighing an average of 30 ± 5g, were obtained from a commercial fish farm located in Cairo, Egypt. The fish were allowed to acclimatize for two weeks in 50L aquaria filled with de-chlorinated tap water with a constant aeration at 27 ± 2°C. During the acclimatization period, the fish were fed a commercial diet twice a day, amounting to 3% of their body weight. Fish were divided into four groups; each with 21 fish (7 fish per replicate) before feeding for 2 weeks with the four diets prepared before.

3. Sampling

After 2 weeks, blood samples were drawn from the fish caudal vein of each group using a 3ml syringe and transferred to Vacuettes without heparin. Blood samples were left to clot at 4˚C for two hours before centrifuging at 1600g for 25min and then stored at - 20˚C until use.

4. Innate humoral immune parameters

4.1. Antiproteases activity

The serum anti-trypsin activity was determined following the protocol by **Lange (2001)**. Briefly, 20µl of a trypsin solution (5mg/ ml) and 20µl of serum were incubated for 10 minutes at 22˚C. Then, 200µl of 0.1 M PBS and 250µl of a 2% azocasein solution (20mg/ ml PBS) were added to the mixture, then incubated for one hour at 22˚C. The reaction was stopped with 500µl of 10% (v/v) TCA, followed by a 30-minute incubation. The mixture was centrifuged at 6000 x g for 5 minutes. Then, 100µl of the supernatant and 100µl of 1 N NaOH were placed in 96-well flat-bottom plate. Absorbance was read at 410nm using an ELISA reader. A positive control (100%) was prepared by substituting serum with buffer. The percentage inhibition of trypsin activity was determined by comparing it to the positive control.

4.2 Lysozyme activity

Lysozyme activity was assessed according to the method of **Parry *et al.* (1965)**. In brief, 60µL of serum was combined to 2mL of a suspension of *Micrococcus lysodeikticus* (0.2mg/ ml) in 0.05 M PBS. The absorbance was recorded at 530nm using a spectrophotometer at 30 seconds and after 4min and 30 seconds. One unit of lysozyme activity was defined as the amount of sample causing a reduction of 0.001 absorbance units per minute.

4.3. Total protein, albumin, and globulin

Total protein **(Cannon *et al.*, 1974)** and albumin **(Tietz, 1995)** were estimated using commercial biochemical kits (Bio-diagnostics, Egypt). Globulin was obtained by subtracting albumin concentration from total protein concentration. Each biochemical parameter was calorimetrically analyzed according to its manufacturer's instructions using an Agilent Cary UV-Vis spectrophotometer.

4.4. Immunoglobulin M (IgM)

The indirect ELISA of total IgM was determined as the following; About, 20µl of diluted serum (1/100) was placed in 96-well plates in duplicate and coated by 200µl of coating buffer (35mM $NaHCo_3$ and 15 mM Na_2CO_3, pH 9.6), then incubated overnight at 4ºC. The plate was washed 3 times with low salt buffer (LSB) (20 mM Tris–HCl, 380 mMNaCl, and 0.05% Tween 20, pH 7.3), then blocked with blocking buffer (3% BSA in LSB) for 2h at room temperature. Subsequently, the plate was washed 3 times with LSB and 5 times with high salt buffer (HSB) (20mM Tris–HCl, 500mM NaCl and 0.1% Tween 20, pH 7.7). The plates were incubated for 1h with 100µl per well of diluted mouse anti-tilapia (*O. nilotius*) IgM monoclomonoclonal antibody (Institute of Aquaculture, UK) (1/100 in blocking buffer) before washed and incubated again with the secondary antibody anti-mouse IgG-HRP (1/1000 in blocking buffer). Additionally, the plate was washed 5 times with HSB and the reaction was developed using 100µl of a 0.42mM solution of the substrate; 3,3̀,5,5̀-tetramethylbenzidine hydrochloride prepared freshly in a 100mM citric acid/sodium acetate buffer, pH 5.4, containing 0.01% H_2O_2. Finally, the reaction was terminated by 50µl of 2M H_2SO_4 after 10min. The plate was measured at 450nm in ELISA reader. The data were presented as the stimulation index, represented as the mean ± standard error (S.E.). This index was derived by dividing each sample value by its corresponding control value. Values exceeding 1 indicated an elevation, while values below 1 signified a reduction in the levels of total IgM.

4.5. Interleukin 12 (IL-12)

Serum IL-12 ELISA was measured using a commercial Fish IL-12 kit purchased from Bioassay Technology Laboratory, China. The optical density values were measured by ELISA reader.

4.6. Bactericidal activity

Aeromonas hydrophila was isolated and identified using API 20E in Hydrobiology Department laboratory, National Institute of Oceanography and Fisheries. The bacteria were cultivated in nutrient broth at 37°C for 24h, before centrifuged at 3000 x g for 10min. After discarding the supernatants, the resulting pellets were suspended in a 0.9% (w/v) sodium chloride solution. Bacterial counts were performed using a haemocytometer slide on a light microscope.

Bactericidal activity was conducted following the method outlined by **Kajita *et al.* (1990)** using *A. hydrophila*. In summary, a mixture of 100µl of serum and 100µl of bacterial suspension was prepared then incubated at 25°C for 1h. Afterward, the mixture was diluted with sterile 0.05 M PBS (pH 6.2) at a 1:10 ratio. Around 100µl of the mixture was spread onto nutrient agar plates before being incubated at 25°C for 24h. Then, the number of colonies was counted for each plate.

5. Hematological parameters

The number of red blood cells (RBC) and white blood cells (WBC) were determined immediately after sampling using a haemocytometer slide (Improved Neubauertype) at a magnification of x 400 on a light microscope. The RBC was diluted in Hayem's solution (1:20) and the WBC was diluted in Turke's solution (1:200) **(Harikrishnan *et al.*, 2003)**.

6. Statistical analyses

The data underwent analysis using one-way analysis of variance (ANOVA). If variances were detected among treatments, means were compared using Tukey's test with the assistance of Minitab statistical software (Minitab, Coventry, UK). Significance was established at a level of $P< 0.05$.

RESULTS

The results recorded a significant increase in antiprotease activity ($P< 0.05$) in all fish groups fed doses of riboflavin compared to the control (Fig. 1), especially with the dose of 10mg which recorded the highest activity. Although all groups fed with riboflavin showed an increase in the lysozyme activity (Fig. 2) compared with the control, the highest significant activity ($P< 0.05$) was recorded in fish group fed with 20 and 10mg of

riboflavin, respectively. Although The fish group fed with 10mg of riboflavin reported the highest values of total protein (Fig. 3) and globulin (Fig. 4) compared to control, but without significant difference ($P > 0.05$). Fish receiving different doses of riboflavin showed no significant difference in albumin value (Fig. 5) compared to control. Moreover, the dose of 10mg revealed the highest significant difference in IgM (Fig. 6) compared to the control ($P < 0.05$) and other groups. Similarly, the dose of 10mg recorded the highest IL-12 (Fig. 7) compared to other groups but without a significant difference. Indeed, the control showed the lowest values in IgM and IL-12. Regarding the bactericidal activity (Fig. 8), the smallest number of bacterial colonies demonstrates how effective the immune molecules in serum are at killing the bacteria. The lowest colony number of *A. hydrophila* was recorded in the fish group fed with the lowest dose of riboflavin (10mg) compared to other groups. Although all doses of riboflavin have a lower number in the bacterial colony than the control, only 10mg dose showed a significant difference from the control ($P < 0.05$).

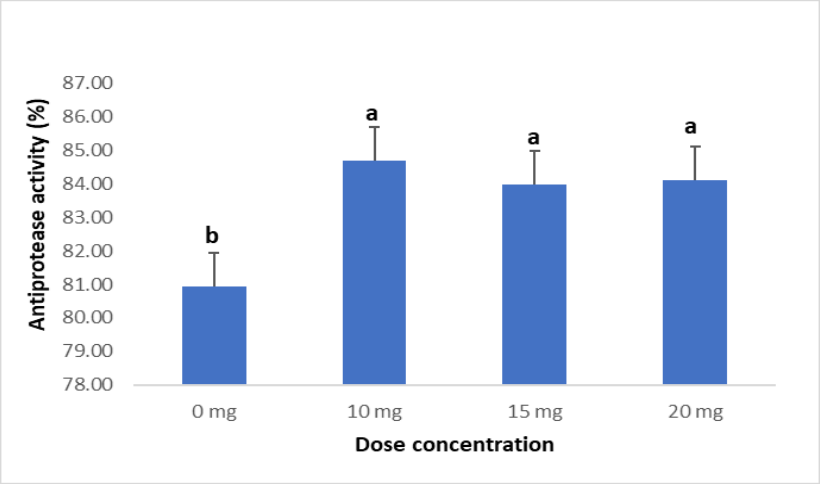

Fig. 1. Antiprotease activity of the Nile tilapia fed with 4 concentrations of riboflavin: 0 (control group), 10, 15, and 20mg. Groups sharing the same letter did not exhibit significant differences. The bars represent the mean values and the standard error (mean ± S.E.)

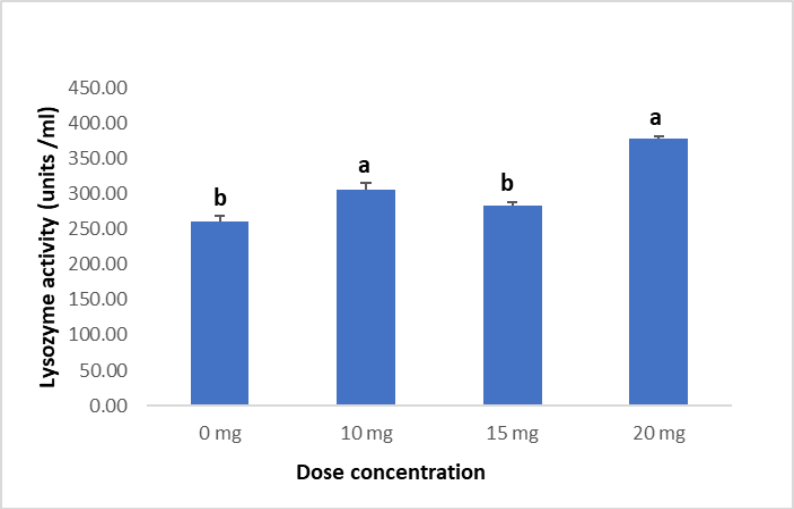

Fig. 2. Lysozyme activity of the Nile tilapia fed with 4 concentrations of riboflavin: 0 (control group), 10, 15, and 20mg. Groups sharing the same letter did not exhibit significant differences. The bars represent the mean values and the standard error (mean ± S.E.)

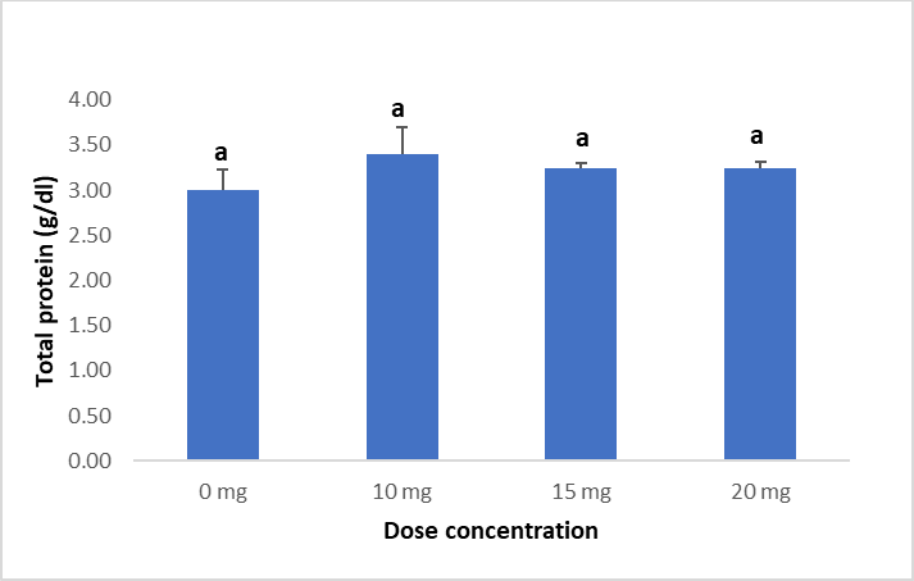

Fig. 3. Total protein of the Nile tilapia fed with 4 concentrations of riboflavin: 0 (control group), 10, 15, and 20mg. Groups sharing the same letter did not exhibit significant differences. The bars represent the mean values and the standard error (mean ± S.E.)

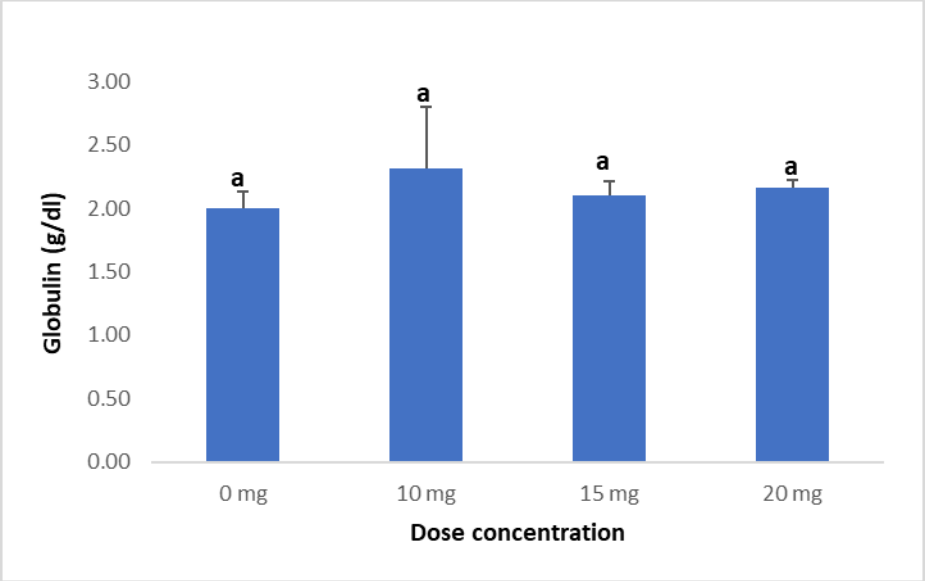

Fig. 4. Globulin of the Nile tilapia fed with 4 concentrations of riboflavin: 0 (control group), 10, 15, and 20mg. Groups sharing the same letter did not exhibit significant differences. The bars represent the mean values and the standard error (mean ± S.E.)

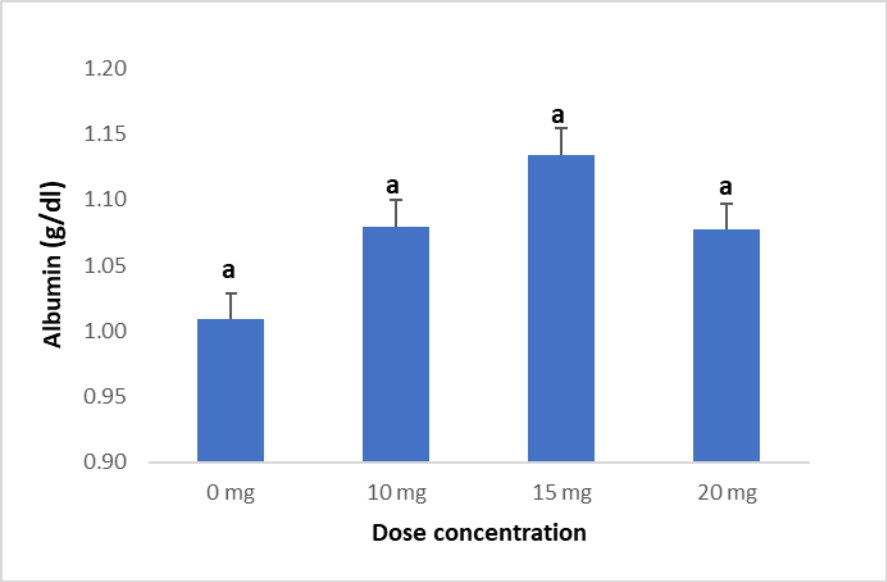

Fig. 5. Albumin value of the Nile tilapia fed with 4 concentrations of riboflavin: 0 (control group), 10, 15, and 20mg. Groups sharing the same letter did not exhibit significant differences. The bars represent the mean values and the standard error (mean ± S.E.)

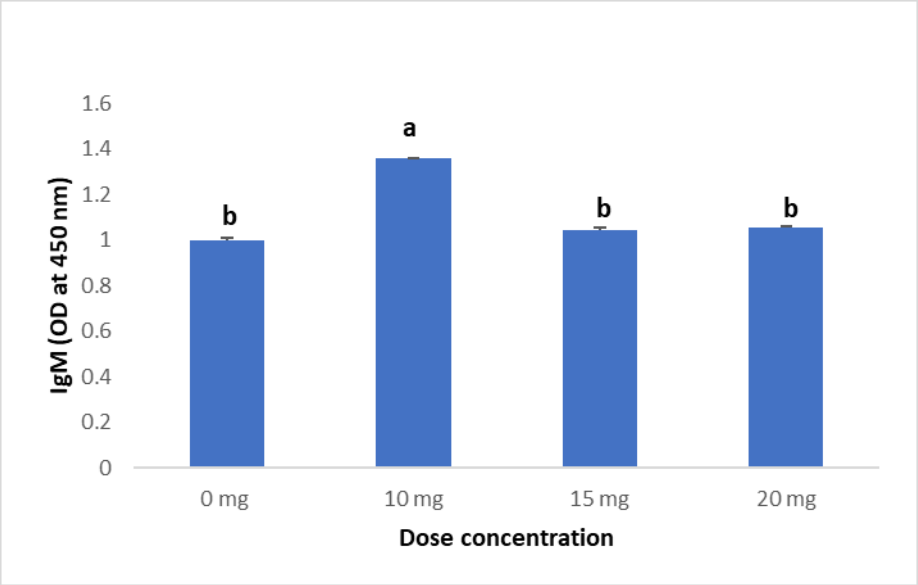

Fig. 6. IgM of the Nile tilapia fed with 4 concentrations of riboflavin: 0 (control group), 10, 15, and 20mg. The data are presented as the stimulation index calculated by dividing each sample value by its corresponding control value, which is set at 1. Values greater than 1 indicate an elevation in the total IgM levels. Groups sharing the same letter did not exhibit significant differences. The bars represent the mean values and the standard error (mean ± S.E.)

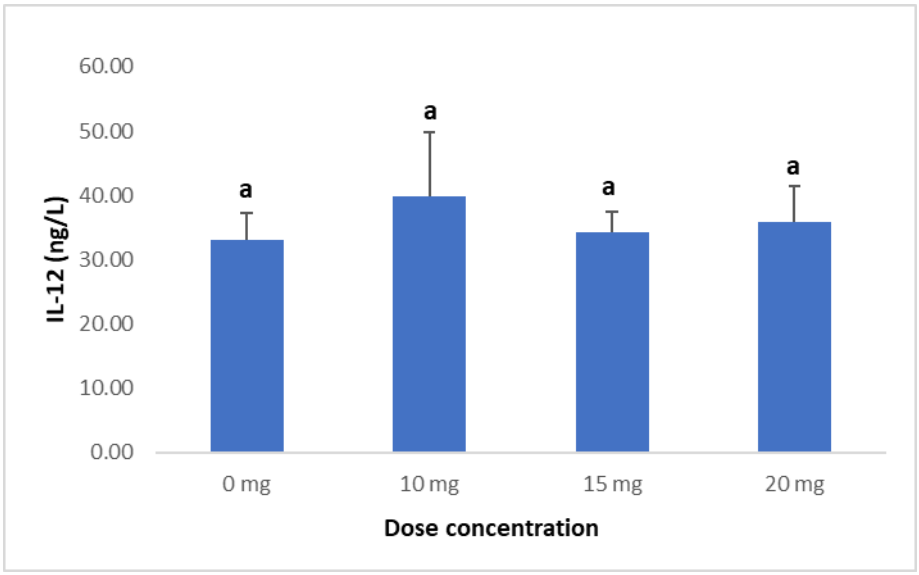

Fig. 7. IL-12 of the Nile tilapia fed with 4 concentrations of riboflavin: 0 (control group), 10, 15, and 20mg. Groups sharing the same letter did not exhibit significant differences. The bars represent the mean values and the standard error (mean ± S.E.)

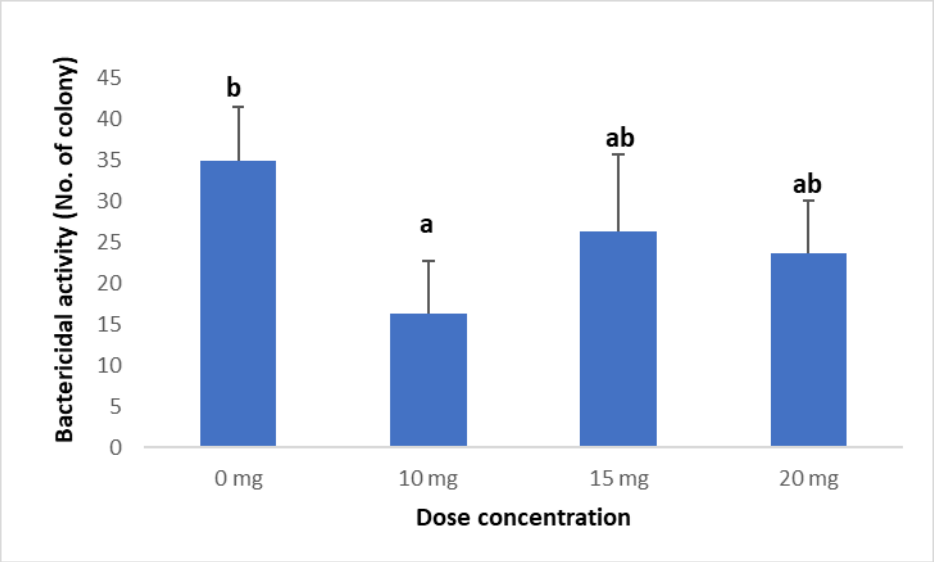

Fig. 8. Bactericidal activity of the Nile tilapia fed with 4 concentrations of riboflavin: 0 (control group), 10, 15, and 20mg. Groups sharing the same letter did not exhibit significant differences. The bars represent the mean values and the standard error (mean ± S.E.)

In RBC, the highest significant number ($P< 0.05$) was recorded in the groups fed with 10mg of riboflavin compared to the control and other groups (Fig. 9). Additionally, the fish group fed 20mg of riboflavin showed a high significant number of RBC compared to the control ($P< 0.05$). All fish groups fed riboflavin reported a high significant number of WBC (Fig. 10) compared to the control group ($P< 0.05$). The highest number of WBC was counted in 10 and 20mg of riboflavin treated groups.

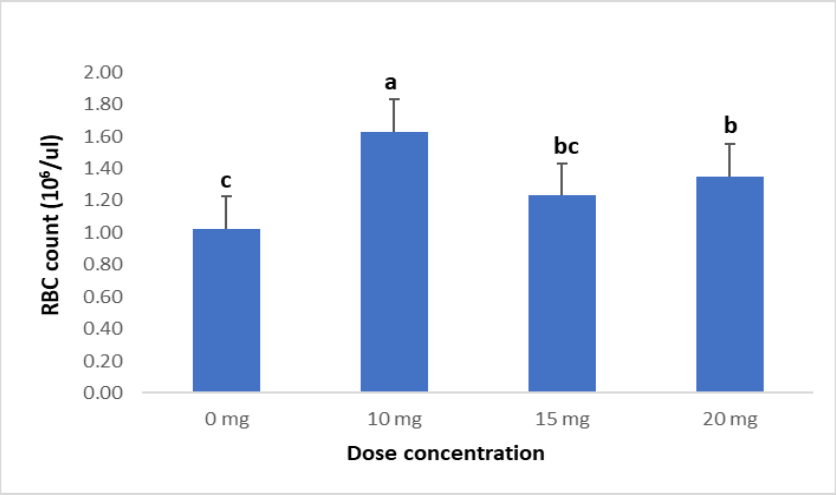

Fig. 9. RBC count of the Nile tilapia fed with 4 concentrations of riboflavin: 0 (control group), 10, 15, and 20mg. Groups sharing the same letter did not exhibit significant differences. The bars represent the mean values and the standard error (mean ± S.E.)

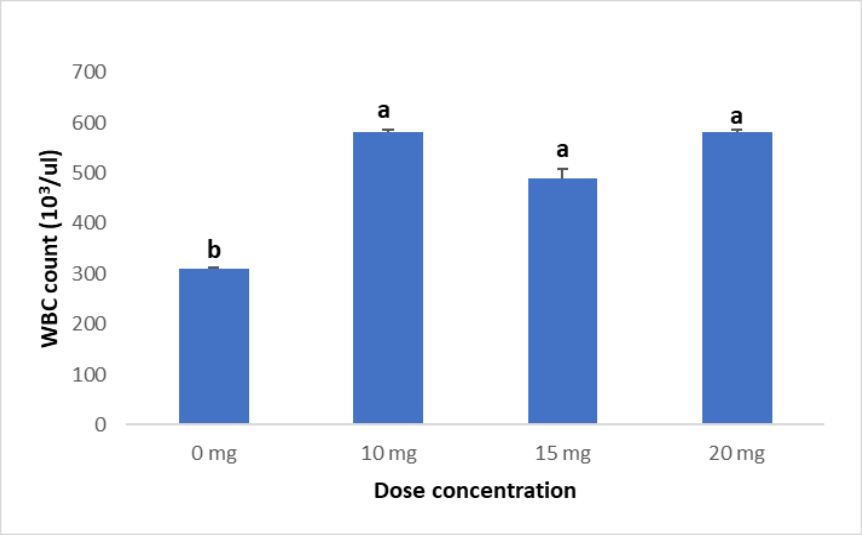

Fig. 10. WBC count of the Nile tilapia fed with 4 concentrations of riboflavin: 0 (control group), 10, 15, and 20mg. Groups sharing the same letter did not exhibit significant differences. The bars represent the mean values and the standard error (mean ± S.E.)

DISCUSSION

Using immunostimulants in aquaculture is a strategy that aims mainly to booster the defense mechanisms of fish against environmental stressors and to explore alternatives to chemotherapeutic treatments for fish diseases **(Awad & Awaad, 2017)**. Vitamins are used in fish diets not only as immunostimulants but also as growth promotors and stress reduction **(Dioguardi *et al.*, 2017; Lameeihassankiadeh *et al.*, 2019; Kumar, 2021; Rahimnejad *et al.*, 2021)**. Our study was concerned with the influence of vitamin B2 (riboflavin) on the Nile tilapia's immune response by investigating some immunological and hematological parameters. Since serum contains several substances with enzymes and anti-enzymes that play a vital role in the body's defense mechanism **(Dioguardi *et al.*, 2017)**, we chose to investigate the immune parameters in serum. Antiproteases, which are known as protease inhibitors in serum, play essential functions in protecting various organisms by controlling and suppressing the actions of potentially harmful proteases. Antiproteases prevent protease activity either by binding to their active sites or by entrapping the protease to halt protein hydrolysis **(Greene & McElvaney 2009)**, thus it limits bacteria's capacity to invade and proliferate in fish **(Ellis, 2001)**. Our research indicates that feeding fish doses of riboflavin resulted in a significant increase ($P< 0.05$) in antiprotease activity in all groups compared to the control. This increase was particularly pronounced with a dose of 10mg, which exhibited the highest significant activity level. Indeed, vitamin additives in fish diets revealed variable immune responses depending on the type of vitamin used, its dose, the time administered, and fish species. For example, using dietary of vitamin D3 for two weeks in the European sea bass diet showed an increase in protease, antiprotease, complement activities, and total IgM level

(Dioguardi *et al.*, 2017) although feeding the rainbow trout with doses of vitamins C and E for ten weeks caused a significant decrease in serum lysozyme and antiprotease activities **(Rahimnejad *et al.*, 2021)**.

In fish, lysozyme acts as an opsonin, stimulating the complement system and enhancing the activity of phagocytes **(Saurabh & Sahoo, 2008)**. Thus, it plays a significant role in the host's defense against infectious diseases **(Smith *et al.*, 2019)**. It is primarily generated by monocytes and macrophages, with macrophages typically displaying elevated levels of lysozyme secretion **(Ogundele, 1998)**. The present study showed that fish groups fed riboflavin had enhanced serum lysozyme activity compared to the control, especially in the group fed with 10 and 20mg which showed the highest significant activity. A similar observation was recorded in previous studies, where the highest percentage of lysozyme activities was reported in the grass carp fed with 10 mg of riboflavin **(Chen *et al.*, 2015)**, and in the rainbow trout fingerlings fed with 20mg of riboflavin **(Lameeihassankiadeh *et al.*, 2019)**. From previous studies, we can conclude that the optimal dose of riboflavin that induces high activity is variable among fish species.

Serum proteins have a variety of physiological functions which regulate and protect the fish. Indeed, acute phase proteins' protective effects limit infectious agents through tissue repair and microbial eradication **(Larsen *et al.*, 2001; Gerwick *et al.*, 2002)**. In our study, total protein and globulin did not show significant differences in the fish fed with riboflavin although they were slightly higher in fish group fed 10mg of riboflavin compared to other groups. Furthermore, previous study carried out on *Pangasianodon hypophthalmus* given diet supplemented with riboflavin reported an increase in the total protein, albumin, and globulin values, especially with 10mg dose that showed the highest value **(Kumar, 2021)**.

Several humoral factors linked to both non-specific and specific immunity are increased in the serum to defend the host against infections. Bactericidal activity in serum refers to identifying and eliminating pathogenic organisms in fish, thereby contributing to their defense against infections **(Ellis, 2001)**. The bactericidal activity was evaluated by recording the number of bacterial colonies grown after incubation serum with a liquid suspension of *A. hydrophila*., the smallest number of bacterial colonies demonstrates how effectively serum immune molecules can kill the bacteria. In the present experiment, the lowest significant number of bacterial colonies ($P< 0.05$) was observed in the fish group administered the lowest dose of riboflavin (10mg) compared to the control. Such findings could show the effectiveness of riboflavin in enhancing the immune molecules in fish serum to defend against disease. Similar results were observed in the juvenile Japanese flounder where dietary supplements with vitamin A led to a significant increase in bactericidal activity **(Hernandez *et al.*, 2007)**. In addition, dietary vitamin C supplements

recorded a significant enhancement in the immune parameters of *Labeo rohita* (including serum bactericidal activity, phagocytic activity, and respiratory burst) compared with controls and increased the protection against *A. hydrophila* **(Tewary & Patra, 2008)**.

IgM serves a vital role as an immune effector molecule in the bloodstream **(Liu *et al.*, 2019)**. The concentration of IgM in teleost serum may vary depending on water temperature and quality, as well as fish species, size, stress, stimulation, and immunization **(Solem & Stenvik 2006)**. Our results demonstrated a higher significant increase in IgM in the fish group fed with 10mg of riboflavin compared to the control. Such findings could suggest using 10mg of riboflavin as the optimal dose for enhancing the immune response in the Nile tilapia, especially since the same dose of riboflavin showed an increase in total immunoglobulin in *Pangasianodon hypophthalmus* **(Kumar, 2021)**. However, the optimal dose is still variable with fish species, size, and with the type of vitamin or immunostimulant used. For example, 20mg of riboflavin was considered as the optimal dose in the rainbow trout fingerlings that elevated the immune parameters including IgM, lysozyme, and complements activities **(Lameeihassankiadeh *et al.*, 2019)**. Moreover, a significant enhancement in IgM and lysozyme activity was observed in the Caspian brown trout fed vitamin C at an optimal dose of 300mg/ kg **(Khara *et al.*, 2016)**.

Interleukin 12 **(**IL-12) family plays crucial roles in cancer, infections, and inflammatory conditions by modulating both non-specific and specific immune responses **(Wojno *et al.*, 2019)**. Moreover, IL-12 family owns special subunit compositions and effectiveness in modulating cell differentiation and combating bacteria **(Hamza *et al.*, 2010)**, fungi **(Thompson & Orr, 2018)**, and viruses **(Guo *et al.*, 2019)**. IL-12 is predominantly produced by immune cells including monocytes, macrophages, and dendritic cells as well as other antigen-presenting cells (APCs) in response to stimulation **(Trinchieri, 2003; Kang *et al.*, 2005)**. Our investigation revealed no significant enhancement among the fish groups regarding IL-12 value although 10mg of riboflavin showed a slight increase in IL-12 values compared to other groups.

Haematological parameters, including erythrocyte count and leucocyte count, present valuable knowledge in evaluating fish health and in monitoring stress responses **(Witeska *et al.*, 2022)**. Hematological responses might be associated with either a deficiency or an excess of vitamins in fish diet **(Garcia *et al.*, 2007)**. The enhancement in hematological parameters particularly the red and white blood cells was reported in the Nile tilapia after fed diets supplemented with vitamins A **(Guimarães *et al.*, 2014)** and in the brown trout after fed diets supplemented with vitamin E and Vitamin C (separately) **(Khara *et al.*, 2016)**. A previous study carried on the rainbow trout fed diets with 20 and 30mg of riboflavin showed an increase in WBC and RBC numbers compared to the control **(Lameeihassankiadeh *et al.*, 2019)**. Interestingly, all groups fed with riboflavin revealed a significantly higher count of red and white blood cell (except in 15mg) compared to the

control ($P< 0.05$). The highest number of RBC was counted in 10mg group while in WBC was in 10 and 20mg groups. Such observation could be attributed to the power of riboflavin to improve the production of cell numbers in lymphoid tissues. **Grimble (1997)** suggested that a deficiency of riboflavin might lead to a decrease in cell numbers within lymphoid tissues of experimental animals, potentially causing functional abnormalities in the cell-mediated immune response.

CONCLUSION

In conclusion, our study revealed that the dietary riboflavin enhanced the humoral immune response and the production of WBC and RBC of the Nile tilapia. The highest enhancement was recorded in 10mg of riboflavin; thus, the current study considers this dose as the optimal dose to improve the Nile tilapia immune response and elevate its resistance to disease. Subsequent research would concentrate on examining the influence of riboflavin on fish growth and overall health status.

REFERENCES

Amezaga, M. and Knox, D. (1990). Riboflavin requirements in on-growing rainbow trout, *Oncorhynchus mykiss*. Aquaculture, 88(1): 87-98.

Awad, E. and Awaad, A. (2017). Role of medicinal plants on growth performance and immune status in fish. Fish shellfish immunol., 67: 40-54.

Cannon, D.C.; Olitzky, I.G. and Inkepen, J.A. (1974). Proteins: Clinical Chemistry, Principles and Techniques. Henry, RJ, Cannon, DC & Winkelma, JW (Editors), 2nd ed. New York, Harper & Row Publishers 405-503.

Chen, L.; Feng, L.; Jiang, W.D.; Jiang, J.; Wu, P.; Zhao, J.; Kuang, S.Y.; Tang, L.; Tang, W.N. and Zhang, Y.A. (2015). Dietary riboflavin deficiency decreases immunity and antioxidant capacity and changes tight junction proteins and related signaling molecules mRNA expression in the gills of young grass carp (*Ctenopharyngodon idella*). Fish shellfish immunol., 45(2): 307-320.

Cruz-Garcia, L.; Sánchez-Gurmaches, J., Bouraoui, L.; Saera-Vila, A.; Pérez-Sánchez, J.; Gutiérrez, J. and Navarro, I. (2011). Changes in adipocyte cell size, gene expression of lipid metabolism markers, and lipolytic responses induced by dietary fish oil replacement in gilthead sea bream (*Sparus aurata* L.). Comp. Biochem. Physiol. A: Mol. Integ. Physiol., 158(4): 391-399.

Deng, D.F. and Wilson, R. (2003). Dietary riboflavin requirement of juvenile sunshine bass (*Morone chrysops*♀× *Morone saxatilis*♂). Aquaculture, 218(1-4): 695-701.

Dioguardi, M.; Guardiola, F.; Vazzana, M.; Cuesta, A.; Esteban, M. and Cammarata, M. (2017). Vitamin D 3 affects innate immune status of European sea bass (*Dicentrarchus labrax* L.). Fish Physiol. Biochem., 43: 1161-1174.

Ellis, A. (2001). Innate host defense mechanisms of fish against viruses and bacteria. Develop. Comp. Immunol., 25(8-9): 827-839.

FAO (2020). The State of World Fisheries and Aquaculture, sustainability in action, 23pp.

Farah, N.; Chin, V.K.; Chong, P.P.; Lim, W.F.; Lim, C.W.; Basir, R.; Chang, S.K. and Lee, T.Y. (2022). Riboflavin as a promising antimicrobial agent? A multi-perspective review. Current Res. Micro. Sci., 3: 100111.

Garcia, F.; Pilarski, F.; Onaka, E.M.; de Moraes, F.R. and Martins, M.L. (2007). Hematology of *Piaractus mesopotamicus* fed diets supplemented with vitamins C and E, challenged by *Aeromonas hydrophila*. Aquaculture, 271(1-4): 39-46.

Gerwick, L.; Steinhauer, R.; Lapatra, S.; Sandell, T.; Ortuno, J.; Hajiseyedjavadi, N. and Bayne, C. (2002). The acute phase response of rainbow trout (*Oncorhynchus mykiss*) plasma proteins to viral, bacterial and fungal inflammatory agents. Fish Shellfish Immunol., 12(3): 229-242.

Greene, C.M. and McElvaney, N.G. (2009). Proteases and antiproteases in chronic neutrophilic lung disease–relevance to drug discovery. Br. J. Pharmacol., 158(4): 1048-1058.

Grimble, R. (1997). Effect of antioxidative vitamins on immune function with clinical applications. Int. J. Vitam. Nutr. Res., 67(5): 312-320.

Guimarães, I.; Lim, C.; Yildirim-Aksoy, M.; Li, M. and Klesius, P. (2014). Effects of dietary levels of vitamin A on growth, hematology, immune response and resistance of Nile tilapia (*Oreochromis niloticus*) to *Streptococcus iniae.* Anim. Feed Sci. Technol., 188: 126-136.

Guo, Y.; Cao, W. and Zhu, Y.J.V. (2019). Immunoregulatory functions of the IL-12 family of cytokines in antiviral systems. Viruses, 11(9): 772.

Hamza, T.; Barnett, J.B. and Li, B. (2010). Interleukin 12 a key immunoregulatory cytokine in infection applications. Int. J. Mol. Sci., 11(3): 789-806.

Harikrishnan, R.; Rani, M.N. and Balasundaram, C. (2003). Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection. Aquaculture, 221(1-4): 41-50.

Hernandez, L.H.H.; Teshima, S.I.; Koshio, S.; Ishikawa, M.; Tanaka, Y. and Alam, M.S. (2007). Effects of vitamin A on growth, serum anti-bacterial activity and transaminase activities in the juvenile Japanese flounder, *Paralichthys olivaceus*. Aquaculture, 262(2-4): 444-450.

Kajita, Y.; Sakai, M.; Atsuta, S. and Kobayashi, M. (1990). The immunomodulatory effects of levamisole on rainbow trout, *Oncorhynchus mykiss*. Fish Pathol., 25(2): 93-98.

Kang, B.Y.; Kim, E. and Kim, T.S. (2005). Regulatory mechanisms and their therapeutic implications of interleukin-12 production in immune cells. Cell. Signal., 17(6): 665-673.

Khara, H.; Sayyadborani, M. and SayyadBorani, M. (2016). Effects of α-tocopherol (vitamin E) and ascorbic acid (vitamin C) and their combination on growth, survival and

some haematological and immunological parameters of Caspian brown trout, *Salmo Trutta Caspius* juveniles. Turk. J. Fisher. Aq. Sci., 16(2): 385-393.

Kumar, N. (2021). Dietary riboflavin enhances immunity and anti-oxidative status against arsenic and high temperature in *Pangasianodon hypophthalmus*. Aquaculture, 533: 736209.

Kumar, V.; Akinleye, A.; Makkar, H.; Angulo-Escalante, M. and Becker, K. (2012). Growth performance and metabolic efficiency in Nile tilapia (*Oreochromis niloticus* L.) fed on a diet containing Jatropha platyphylla kernel meal as a protein source. J. Anim. Physiol. Anim. Nut., 96(1): 37-46.

Lameeihassankiadeh, S., Mohammadalikhani, M., Najjar Lashgari, S. and Abbasian, F. (2019). Effects of riboflavin on growth, hematological and immunological parameters of rainbow trout (*Oncorhynchus mykiss*) fingerlings. Iran. J. Fisher. Sci., 18(4): 727-734.

Lange, S.; Guđmundsdottir, B.K. and Magnadottir, B. (2001). Humoral immune parameters of cultured Atlantic halibut (*Hippoglossus hippoglossus* L.). Fish Shellfish Immunol., 11(6): 523-535.

Larsen, M.H.; Larsen, J.L. and Olsen, J.E. (2001). Chemotaxis of *Vibrio anguillarum* to fish mucus: role of the origin of the fish mucus, the fish species and the serogroup of the pathogen. FEMS Microbiol. Ecol., 38(1): 77-80.

Li, W.; Zhou, X.Q.; Feng, L.; Liu, Y. and Jiang, J. (2010). Effect of dietary riboflavin on growth, feed utilization, body composition and intestinal enzyme activities of juvenile Jian carp (*Cyprinus carpio* var. Jian). Aquac. Nutr., 16(2): 137-143.

Liu, J.; Wang, Y.; Xiong, E.; Hong, R.; Lu, Q.; Ohno, H. and Wang, J.Y. (2019). Role of the IgM Fc receptor in immunity and tolerance. Front. Immunol., 10: 428007.

Misra, C.; Das, B.; Mukherjee, S. and Pradhan, J. (2007). Effects of dietary vitamin C on immunity, growth and survival of Indian major carp *Labeo rohita*, fingerlings. Aquac. Nutr., 13(1): 35-44.

Muñoz de la Peña, A. and Espinosa-Mansilla, A. (2009). Analysis of antibiotics in fish samples. Anal. Bioanal. Chem., 395(4): 987-1008.

Ogundele, M. (1998). A novel anti-inflammatory activity of lysozyme: modulation of serum complement activation. Mediat. Inflamm., 7: 363-365.

Parry, R.M.; Chandan, R.C. and Shahani, K.M. (1965). A rapid and sensitive assay of muramidase, Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, NY), Royal Society of Medicine. pp. 384-386.

Rahimnejad, S.; Dabrowski, K.; Izquierdo, M.; Hematyar, N.; Imentai, A.; Steinbach, C. and Policar, T. (2021). Effects of vitamin C and E supplementation on growth, fatty acid composition, innate immunity, and antioxidant capacity of rainbow trout (*Oncorhynchus mykiss*) fed oxidized fish oil. Front. Mar. Sci., 8: 760587.

Santos, L. and Ramos, F. (2016). Analytical strategies for the detection and quantification of antibiotic residues in aquaculture fishes: A review, Trends Food Sci. Technol., 52: 16-30.

Saurabh, S. and Sahoo, P. (2008). Lysozyme: an important defence molecule of fish innate immune system. Aquac. Res., 39(3): 223-239.

Smith, N.C.; Rise, M.L. and Christian, S.L. (2019). A comparison of the innate and adaptive immune systems in cartilaginous fish, ray-finned fish, and lobe-finned fish. Front. Immunol., 10: 475871.

Solem, S.T. and Stenvik, J. (2006). Antibody repertoire development in teleosts—a review with emphasis on salmonids and *Gadus morhua* L. Develop. Comp. Immunol., 30(1-2): 57-76.

Tewary, A. and Patra, B.C. (2008). Use of vitamin C as an immunostimulant. Effect on growth, nutritional quality, and immune response of *Labeo rohita* (Ham.). Fish Physiol. Biochem., 34: 251-259.

Thompson, A. and Orr, S.J.J.C. (2018). Emerging IL-12 family cytokines in the fight against fungal infections. Cytokines, 111: 398-407.

Tietz, N.W. (1995): Clinical guide to laboratory tests, Clinical guide to laboratory tests, pp. 1096-1096.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nature Rev. Immunol., 3(2): 133-146.

Witeska, M.; Kondera, E.; Ługowska, K. and Bojarski, B. (2022). Hematological methods in fish–Not only for beginners. Aquaculture, 547: 737498.

Wojno, E.D.T.; Hunter, C.A. and Stumhofer, J.S.J.I. (2019). The immunobiology of the interleukin-12 family: room for discovery. Immunity, 50(4): 851-870.

Zehra, S. and Khan, M. (2018). Dietary riboflavin requirement of fingerling *Channa punctatus* (Bloch) based on growth, conversion efficiencies, protein retention, liver riboflavin storage, RNA/DNA ratio and carcass composition. Aquac. Nutr., 24(1): 269-276.