Egyptian Journal of Aquatic Biology and Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(5): 2779 – 2807 (2025)
www.ejabf.journals.ekb.eg

Unveiling the Impact of Hydroxyproline Supplementation Diet on Haemato-biochemical Aspects of Gilthead Seabream (*Sparus aurata)* Exposed to Ammonia Challenge Under Different Stocking Densities

Abdel-Salam A. Abou Ellif[1], Abd El-Rahman A. Khattaby[2], Mohamed Sh. Abu Husein[1], Hassan M.M. Khalaf-Allah[1], and Mahmoud Mahrous M. Abbas[1]

[1]Zoology and Entomology Depart., Faculty of Science, Al-Azhar University, Cairo, 11884, Egypt
[2]Fish Production and Aquaculture Systems Department, Central Laboratory for Aquaculture Research, Agriculture Research Centre, Egypt

***Corresponding Author: Mahmoud_Mahrous42@azhar.edu.eg**

ARTICLE INFO

Article History:
Received: Aug. 20, 2025
Accepted: Oct.7, 2025
Online: Oct. 19, 2025

Keywords:
Seabream,
Ammonia stress,
Rearing density;
Haematological parameters,
Biochemical indices,
Acute ammonia,
Aquaculture

ABSTRACT

This study investigated the effects of dietary hydroxyproline supplementation on the haematological and biochemical responses of *Sparus aurata* fingerlings reared under two stocking densities (low: 15 fish/m3; high: 30 fish/m3) for 90 days, followed by an acute ammonia challenge. Four diets were prepared with increasing hydroxyproline levels (0, 1, 2, and 3%). Prior to ammonia exposure, significant improvements in red blood cell count, haemoglobin concentration, and haematocrit were observed in supplemented groups, with the highest values recorded at the 3% inclusion level. Concurrent reductions in mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH), along with elevated mean corpuscular haemoglobin concentration (MCHC), indicated adaptive adjustments in erythrocyte morphology. Platelet counts showed variable patterns, whereas white blood cell counts decreased with higher supplementation, suggesting an immunomodulatory role. Biochemical analyses revealed that hydroxyproline supplementation increased serum total protein, albumin, and globulin, while reducing glucose and cortisol concentrations, particularly under high-density conditions, reflecting improved metabolic stability and reduced stress. After ammonia exposure, most haematological and biochemical parameters declined across all groups, though reductions were less severe in the hydroxyproline-supplemented treatments, confirming its protective effect. In conclusion, dietary hydroxyproline at 3% inclusion provided the most consistent improvements in blood health, biochemical stability, and resilience to ammonia stress in *S. aurata* fingerlings. Therefore, a 3% hydroxyproline supplementation diet is recommended for practical application in intensive aquaculture systems.

INTRODUCTION

Aquaculture has been practiced in Egypt for centuries, but modern methods have recently been introduced to increase productivity. The aquaculture industry in Egypt represents more than 77% of the total national fish production and employs more than 580 thousand people **(El-Sayed *et al.*, 2015; Shaalan *et al.*, 2018)**. In Egypt, aquaculture represents about 77.89% of the total fish production **(GAFRD, 2022)**. Aquaculture has emerged as a critical source of high-quality animal protein, supplying over 50% of global fish consumption **(FAO, 2022)**.

Among cultured species, the gilthead seabream, *Sparus aurata* is the 33rd most popular fish in aquaculture worldwide. Moreover, it is one of the most important types of farmed fish, especially in the Mediterranean countries. Egypt is the third producer of global production with a share of 13.87% of the total production. Egypt began high growth in recent years to increase production. Furthermore, the total global production of seabream in 2019 was estimated at 258,754 tons. However, Egypt leads global production with a share of 35,880 trillion tons **(FAO, 2020)**. Egypt recently became the ninth country in aquaculture **(Remonte, 2023)**. Under such conditions, the aquaculture industry faces several challenges that negatively impact production, including environmental stress, poor water quality, and the spread of infectious diseases **(Dawood *et al*., 2021; Radwan *et al*., 2023)**. Among these, ammonia accumulation in ponds is considered one of the most critical issues, primarily resulting from fish waste, uneaten feed, organic matter deposition at the pond bottom, and reduced dissolved oxygen (DO) levels **(Sriyasak *et al*., 2015)**. Intensive farming practices often exacerbate this problem, leading to elevated concentrations of un-ionised ammonia (NH_3)—a natural byproduct of protein metabolism and organic matter decomposition—which can severely impair fish health and performance, ultimately reducing overall farm productivity **(Ip & Chew, 2010)**.

Ammonia toxicity in *S. aurata* disrupts haematological homeostasis, manifesting as reduced haemoglobin (Hb) levels, elevated liver enzymes (aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT)), and oxidative stress **(Randall & Tsui 2002; Xu *et al*., 2021)**. These physiological impairments compromise growth, immune function, and survival rates **(Parvathy *et al*., 2023)**. To mitigate the detrimental effects of ammonia accumulation in aquaculture systems, several management strategies have been employed, including reducing feeding rates, increasing water exchange, enhancing dissolved oxygen (DO) levels, and gradually applying lime to ponds **(Boyd, 2017)**. In recent years, dietary interventions—particularly the supplementation of functional amino acids—have gained considerable attention as complementary approaches. Proline, a conditionally essential amino acid, exhibits diverse biological functions, such as antioxidant activity, osmoregulation, and immune modulation in marine fish **(Steinberg, 2022)**. Evidence from recent studies indicates its potential in alleviating ammonia-induced stress, as demonstrated in species like European seabass (*Dicentrarchus labrax*) **(Sinha *et al.*, 2015)**; however, its protective effects in *Sparus aurata* remain largely unexplored.

Conversely, previous studies on ammonia toxicity have shown that elevated ammonia concentrations can suppress immune function, induce oxidative stress, and trigger physiological stress responses. These adverse effects can be alleviated through dietary supplementation with specific amino acids **(Dawood *et al*., 2021; Elbialy *et al*., 2021)**. Therefore, the present study aimed to evaluate the impact of ammonia exposure on haematological and biochemical indices in gilthead seabream, *Sparus aurata* fingerlings, while assessing the potential of dietary proline supplementation under different stocking densities to alleviate these adverse effects.

MATERIALS AND METHODS

1. Fish sampling

A total of 500 specimens of gilthead seabream, *Sparus aurata* fingerlings were obtained from a local hatchery (El-Wafaa), Ismailia, Egypt, during August 2023. All fish specimens are in a good condition. Fish were put in large plastic bags, each containing approximately 20L of water and a lot of oxygen. They were transported to Suez Canal Aquaculture Farms in the Qantara East area, Ismailia. All fish were acclimatized for two weeks in net fish cages (hapa, 100 × 200 × 50 cm).

2. Diets design

The main experimental fish diet, 45% protein, was bought from a fish diet factory in 10th of Ramadan City. This fish diet was sinking pellets. Four treatment diets were peformed; the basic diet is a plan for all the treatment diets (control group). The remaining diets were prepared by adding 1% of hydroxyproline to 99% of the basic diet, adding 2% of hydroxyproline to 98% of the basic diet and adding 3% of hydroxyproline to 97% of the basic diet. The experimental additive is hydroxyproline; the basal diet was formulated according to **Rong *et al.* (2019)**. The proximate composition of the basal diet was 45g/ 100g for protein, 17.5g/ 100g for fat, 6.5g/ 100g for moisture, 9.3g/ 100g for ash, 2.8g/ 100g for fiber, and 5.3g/ 100g for carbohydrates.

3. Fish experimental design

After acclimatization, the fish specimens were selected based on good health and uniformity in size (length and weight) and were divided into two main groups based on stocking density: the first group contained 15 fish/m³ (low density), and the second group contained 30 fish/m³ (high density). Each group was further divided into four subgroups based on the diet used; each subgroup had two replications (16 hapa, 100 × 200 × 50 cm). The first subgroup was fed a basic diet (control, C), the second subgroup was fed the basic diet with 1% hydroxyproline added (T1), the third subgroup was fed the basic diet with 2% hydroxyproline added (T2), and the fourth subgroup was fed the basic diet with 3% hydroxyproline added (T3). Fish were fed twice daily, six days a week at a fixed feeding rate of 2% body fish weight (dry feed/day) at 9:00 and 16:00 for 90 days. The feeding rate (2%) is constant throughout the experiment, where 10 fish were randomly selected from all the fish in each hapa and were weighed. The average weight of the fish was obtained, and the bi-weekly feed consumption (g feed/fish/week) was calculated for each group. 20% of the water volume in the pond containing all the hapa is replaced daily.

4. Ammonia experimental design

In the second experiment, the same fish groups used in the first experiment (feeding experiment) were transferred to 16 plastic aquaria (100 × 60 × 40 cm) and exposed to ammonia stress for one day (24 hours). The fish from each subgroup (both low-density and high-density groups) were fasted for a full day before the ammonia experiment and exposed to a concentration of 0.5mg/ L of ammonia for 24 hours **(Yousefi *et al.*, 2020; Abbas *et al.*, 2024a)**. The experimental setup remained the same as the first experiment, with each subgroup receiving the corresponding diet (control, 1% hydroxyproline, 2% hydroxyproline, and 3% hydroxyproline) while being exposed to ammonia for a 24-hour period.

5. Measurements

5.1. Water quality

In water quality studies, evaluating various environmental parameters is essential to understand the impact of environmental factors on the health of aquatic ecosystems and organisms. Water quality is a crucial factor in the sustainability of marine and freshwater life, as factors such as salinity, water temperature, DO, pH, and concentrations of toxic and total ammonia influence the growth and reproduction of aquatic species.

Water quality of the pond was checked every 7 days during the study period to determine salinity, water temperature (°C), hydrogen ion concentration (pH), DO, and concentrations of ammonia using multi-parameter device (HANNA, USA, HI 9829)**.** During the study period, salinity ranged between 20.90 to 27.52 ‰. Water temperature fluctuated between 22.10 to 28.64°C. Hydrogen ion concentration (pH) values lie in alkaline side; it ranged between 8.28 to 8.69. The DO level ranged between 5.64 to 6.77mg/ L. Toxic ammonia concentration varied from 0.020 ± 0.001 mg/L to 0.050 ± 0.002 mg/L. Total ammonia concentration ranged between 0.023 ± 0.003 mg/L to 0.067 ± 0.018 mg/L.

5.2. Collection of blood samples

Fish were fasted for 24 hours and then anesthetized with 50µL/ L of clove oil to measure the blood indices in treated fish (pre-ammonia) and ammonia stress experiment (post- ammonia). The caudal peduncles of three fish in each group were used in a syringe filled with EDTA as an anticoagulant for haematological examinations. Conversely, the immunological biochemical and antioxidant analyses were performed using a syringe (without anticoagulants). Serum was obtained by centrifuging the mixture for 15 minutes at 3000 ×*g* and frozen at –20 °C until analysis.

5.3. Haemato-biochemical assays

Haematological indices (leucocytes, erythrocytes, packed cell volume, blood cell indices, and Hb levels) were determined according to previous methods described by **Dacie and Lewis (1991)** and **Brown (1993)**. Cortisol levels were determined using France kits from Bio-Merieux **(Vecsei 1979)**. Blood glucose **(Trinder, 1969)** and biochemical parameters (total protein, albumin, alanine, and aspartate aminotransferase) were measured in blood-serum samples **(Reitman & Frankel 1957; Henry 1964)**. Globulins level was calculated by deducting the serum albumin from the serum total protein.

6. Statistical analysis

The obtained results were subjected to a two-way ANOVA using the SPSS program ($P< 0.05$). However, the T-test was used to determine the differences between the pre- and post-ammonia samples.

RESULTS

1. Haematological parameters before and after-ammonia challenge

The results in Table (1) show the variations in the haematological parameters of *S. aurata* fingerlings fed over a 90-day experimental period under two distinct stocking densities: low-density (15 fish/hapa) and high-density (30 fish/hapa) with a standardized feeding rate of 2% using dietary hydroxyproline supplementation at four inclusion levels: 0% (control), 1% (T_1), 2% (T_2), and 3% (T_3). This was followed by an acute ammonia exposure (24 hours), with haematological parameters examined both pre- and post-ammonia challenge.

1.1. Red blood cell (RBC) counts (×10^{6}cell/mm^3, mean ± standard deviation)

In the pre-ammonia challenge phase, fish reared at low stocking density exhibited RBC counts in the blood of *S. aurata* fingerlings increased with the increasing of hydroxyproline supplementation in treatments. The RBC count in T_1 recorded $2.61\pm0.32\times10^6$ cell/mm^3, which increased in T_2 ($2.77\pm0.34\times10^6$ cell/mm^3), and reached the highest mean value in T_3 ($2.91 \pm 0.36 \times10^6$ cell/mm^3) compared with the control group ($2.10 \pm 0.26 \times10^6$ cell/mm^3). The same trend was observed at high stocking density, RBC values recorded $2.68\pm0.33\times10^6$ cell/mm^3 in T_1, $2.90\pm0.36\times10^6$ cell/mm^3 in T_2, and reached the highest mean value in T_3 ($2.97 \pm 0.37 \times10^6$ cell/mm^3) compared with the control group ($2.19 \pm 0.27 \times10^6$ cell/mm^3). In the low-density group, post-challenge RBC values increased by increasing the hydroxyproline supplementation. The RBC count in the blood of *S. aurata* fingerlings in T_1 recorded $2.20\pm0.27\times10^6$ cell/mm^3, $2.29\pm0.28 \times10^6$ cell/mm^3 in T_2, and reached the highest mean value in T_3 ($2.45 \pm 0.30 \times10^6$ cell/mm^3) compared with the control group ($1.84 \pm 0.23 \times10^6$ cell/mm^3). Similarly, RBC counts in the high-density group recorded $2.36\pm0.29\times10^6$ cell/mm^3 in T_1, $2.65\pm0.33\times10^6$ cell/mm^3 in T_2, and reached the highest mean value in T_3 ($2.89 \pm 0.36\times10^6$ cell/mm^3) compared with the control group ($1.98 \pm 0.25\times10^6$ cell/mm^3). Following the ammonia challenge, a reduction in RBC counts in *S. aurata* fingerlings was observed across all treatments in low and high stock densities as compared with the case before the ammonia challenge (Table 1).

1.2. Hb concentrations (g/dL, mean ± standard deviation)

In the pre-ammonia challenge, fish reared at low stocking density exhibited Hb concentrations in the blood of *S. aurata* fingerlings that increased with the increasing of hydroxyproline supplementation in treatments. The Hb concentrations in T_1 recorded values of 6.69±0.22 g/dL which increased in T_2 (6.96±0.23 g/dL) and reached the highest mean value in T_3 (7.66 ± 0.25 g/dL) compared with the control group (6.15 ± 0.20 g/dL). Similarly, fish in the high-density group showed Hb concentrations with recorded values of 7.00±0.23 in T_1; it increased gradually in T_2 (7.71±0.26 g/dL) and reached the highest mean value in T_3 (8.16 ± 0.27 g/dL) compared with the control group (6.28 ± 0.21 g/dL). In the low-density group, post-challenge values of Hb concentrations in the blood of *S. aurata* fingerlings increased with the increasing of hydroxyproline supplementation in treatments. The Hb concentrations in T_1 were recorded at 5.13±0.36 g/dL; values increased gradually in T_2 (5.61±0.39 g/dL) and reached the highest mean value in T_3 (6.33±0.44 g/dL) compared with the control group (4.82±0.34 g/dL). Similarly, fish in the high-density group showed Hb concentrations with recorded values of 6.22±0.43 in T_1, and increased gradually in T_2 (6.78±0.47 g/dL) reaching the highest mean value in T_3 (7.64±0.53 g/dL) compared with the control group (5.33±0.37 g/dL). Following the after-ammonia challenge, a reduction in Hb levels in the blood of *S. aurata* fingerlings were observed across all treatments and stock density compared with the case before ammonia challenge (Table 1).

1.3. Haematocrit (HCT) (%, mean ± standard deviation)

In the pre-ammonia challenge phase, fish reared at low stocking density exhibited HCT values in the blood of *S. aurata* fingerlings which increased with the increasing hydroxyproline supplementation in treatments. The lowest value of HCT was recorded in T_1 (21.30±0.71%), which increased in T_2 (22.31±0.74%) and reached the highest mean value in T_3 (23.54±0.78%) compared with the control group (19.88 ± 0.66%). Similarly, fish in the high-density group showed HCT values with recorded values of (23.12±0.77%) in T_1, (23.55±0.78%) in T_2 and (24.67±0.82%) in T_3 compared with the control group (20.59±0.68%). In the low-density group, post-challenge HCT values in the blood of *S. aurata* fingerlings increased with the increase of hydroxyproline supplementation in treatments. The HCT values in T_1 were recorded at 20.84±1.46%, which increased gradually in T_2 (21.67±1.52) and reached the highest mean value in T_3 (22.11 ± 1.55%) compared with the control group (19.80 ± 1.38%). Similarly, in the high-density group, the HCT values recorded were 22.29±1.56 in T_1, 23.64±1.65% in T_2 and 23.89 ± 1.67% in T_3 compared with the control group (20.46 ± 1.43%). Following the post-ammonia challenge, HCT values in the blood of *S. aurata* fingerlings showed a slight decline across treatments, although the reduction was not as marked as seen in other haematological parameters compared with the pre-ammonia challenge (Table 1).

1.4. Mean corpuscular volume (MCV) (fL, mean ± standard deviation)

During the pre-ammonia challenge phase, fish reared at low stocking density exhibited MCV values in the blood of *S. aurata* that decreased in treatments (87.73±10.89 fL in T_1, 86.71 ± 10.76 fL in T_2 and 86.89±10.79 fL in T_3) compared with the control group (101.85 ± 12.64 fL). Similarly, fish under high stocking density, MCV values recorded values of 92.66±11.50 fL in T_1, 87.24 ± 10.83 fL in T_2 and 89.48±11.11 fL in T_3 compared with the control group (100.96 ± 12.53 fL). In the low-density group, post-challenge MCV values in the blood of *S. aurata* fingerlings were decreased in treatments (99.49±12.35 fL in T_1, 99.68±12.37 fL in T_2 and 94.80 ± 11.77 fL in T_3) compared to the control group (113.35 ± 14.07 fL). In the high-density group, MCV values were decreased with the increasing of hydroxyproline supplementation in treatments. MCV value in T_1 recorded 99.36±12.33 fL, decreasing in T_2 (93.75±11.64 fL) and reached the lowest value in T_3 (86.87 ± 10.78 fL) compared with the control group (108.46 ± 13.46 fL). Following the post-ammonia challenge, MCV values in the blood of *S. aurata* fingerlings increased noticeably in most groups, particularly in the control, T_1 and T_2 treatments, compared with the pre-ammonia challenge (Table 1).

1.5. Mean corpuscular haemoglobin (MCH) (pg, mean ± standard deviation)

In the pre-ammonia challenge phase, fish were maintained at low stocking density. MCH values in the blood of *S. aurata* fingerlings decreased in treatments (27.57±3.42 pg in T_1, 27.04 ± 3.36 pg in T_2 and 28.26±3.51pg in T_3) compared with the control group (31.49 ± 3.91 pg). Similarly, in the high stocking density group, MCH values were slightly decreased in treatments (28.04 ± 3.48 pg in T_1, 28.55±3.54 pg in T_2 and 29.60±3.68pg in T_3) compared with the control group (30.79±3.82 pg). For the fish at low stocking density, post-challenge MCH value in the blood of *S. aurata* fingerlings were decreased in treatments compared with the control, but in T_3 (27.16±3.37 pg) the value was slightly less than the control group (27.59 ± 3.43 pg). In the high stocking density group, MCH values were decreased in treatments (27.73±3.44 pg in T_1, 26.89 ± 3.34 pg in T_2 and 27.79±3.45 pg in T_3) compared with the control group (28.25 ± 3.51 pg). After the ammonia challenge, MCH levels in the blood of *S. aurata* fingerlings generally declined across all groups in low and high stock densities compared with before the ammonia challenge (Table 1).

1.6. Mean corpuscular haemoglobin concentration (MCHC) (%, mean ± standard deviation)

In the pre-ammonia challenge phase, in fish reared at low stocking density, MCHC values in the blood of *S. aurata* fingerlings increased in treatments (31.87±1.06%

in T_1, 31.62±1.05% in T_2 and 32.99 ± 1.09 % in T_3) compared with the control group (31.36 ± 1.04 %). While in the high-density group, MCHC values increased in T_2 (33.19±1.10%) and T_3 (33.56±1.11%) compared with the control group (30.92±1.02%). In the post-ammonia challenge phase, fish reared in the low-density group, MCHC values in the blood of *S. aurata* fingerlings in T_1 recorded 24.98±0.83%, which increased gradually in T_2 (26.25±0.87%) and reached the highest mean value in T_3 (29.05 ± 0.96 %) compared with the control group (24.69 ± 0.82%). Similarly, in the high stocking density group, MCHC values were gradually increased in T_1 (28.30±0.94%), T_2 (29.09±0.96%) and T_3 (32.44 ± 1.07 %) compared with the control group (26.42 ± 0.87 %)**.** Following the after-ammonia challenge, a marked decline in MCHC values in the blood of *S. aurata* fingerlings was observed in all groups, though the extent of reduction varied by treatment in low and high stock densities compared with before the ammonia challenge (Table 1).

1.7. Platelet count (×10^3cell/mm^3, mean ± standard deviation)

In the pre-ammonia challenge phase, fish reared at low stocking density exhibited platelet counts in the blood of *S. aurata* fingerlings increased in T_1 (36.18±0.65×10^3 cell/mm^3), T_2 (41.21±0.74×10^3 cell/mm^3) and T_3 (10.05±0.18×10^3 cell/mm^3) compared with the control group (9.05 ± 0.16 ×10^3cell/mm^3). Under high stocking density, blood platelet values ranged more widely, it increased in T_1 (77.39 ± 1.39×10^3 cell/mm^3) and decreased in T_2 (16.08 ± 0.29×10^3 cell/mm^3) and T_3 (27.14±0.49×10^3 cell/mm^3) compared with the control group (34.17±0.61×10^3 cell/mm^3). In the low-density group, post-challenge blood platelet values in the blood of *S. aurata* fingerlings increased in T_1 (58.29 ± 1.05 ×10^3cell/mm^3) and T_2 (46.23±0.83×10^3 cell/mm^3) and decreased in T_3 (18.09 ± 0.32×10^3 cell/mm^3) compared with the control group (22.11±0.40×10^3 cell/mm^3). Similarly, fish reared at high stocking density showed platelet counts that increased in T_1 (55.28±0.99×10^3 cell/mm^3), T_2 (45.23±0.81×10^3 cell/mm^3) and T_3 (23.12±0.41×10^3cell/mm^3) compared with the control group (22.11 ± 0.40 9.05 ± 0.16×10^3 cell/mm^3). Following the post-ammonia challenge, blood platelet counts in the blood of *S. aurata* fingerlings increased in most groups, especially in the low-density group and in T_2 and T_3 in the high-density group in compared with the pre-ammonia challenge, possibly reflecting a physiological stress response (Table 1).

1.8. White blood cell count (WBC, ×10^3cell/mm^3, mean ± standard deviation)

During the pre-ammonia challenge phase, fish reared under low stocking density exhibited WBC counts in the blood of *S. aurata* fingerlings decreased with the increasing of hydroxyproline supplementation in treatments. The WBC counts in T_1 recorded values of 21.88±0.39×10^3 cell/mm^3, decreasing in T_2 (20.26±0.36×10^3 cell/mm^3) and reaching the lowest value in T_3 (19.10 ± 0.34×10^3 cell/mm^3) compared to the highest value, which occurred in the control group (23.37 ± 1.23×10^3 cell/mm^3). Similarly, at high stocking density, WBC values were decreased in T_1 (20.09±0.36×10^3 cell/mm^3), T_2

($18.47\pm0.33\times10^3$ cell/mm^3) and T_3 ($17.31 \pm 0.31\times10^3$ cell/mm^3) compared with the highest value recorded in the control group ($21.02\pm0.38\times10^3$ cell/mm^3). Following the ammonia challenge, in the low-density group, post-challenge WBC counts in the blood of *S. aurata* fingerlings were decreased with the increasing of hydroxyproline supplementation in treatments. The WBC counts in T_1 recorded values of $23.22\pm0.42\times10^3$ cell/mm^3, decreasing in T_2 ($22.65\pm0.41\times10^3$ cell/mm^3) and reaching the lowest value in T_3 ($21.81\pm0.39\times10^3$ cell/mm^3) compared with the highest value, which occurred in the control group ($24.90\pm0.45\times10^3$ cell/mm^3). Similarly, at high stocking density, WBC values were decreased in T_1 ($21.43\pm0.38\times10^3$ cell/mm^3), T_2 ($20.86\pm0.37\times10^3$cell/mm^3) and T_3 ($20.02\pm0.36\ \times10^3$cell/mm^3) compared with the highest value recorded in the control group ($23.12\pm0.41\ \times10^3$cell/mm^3). Following the ammonia challenge, WBC counts in the blood of *S. aurata* fingerlings increased significantly in all groups compared with the pre-ammonia challenge, possibly reflecting a physiological stress response with the lowest values that was observed in T_3 treated groups under both densities (Table 1).

The table presents the results of the two-way ANOVA analysis showing the effects of stocking density (density), dietary treatment (treatment), and their interaction (density × treatment) on haematological parameters (RBCs, Hb, HCT, MCV, MCH, MCHC, platelet count, WBCs) at both the pre-ammonia and post-ammonia exposure stages. Stocking density showed a significant impact on Hb, HCT, platelet count, and WBCs before ammonia exposure ($P< 0.05$), while no effect was detected on RBCs, MCV, MCH, or MCHC. After ammonia exposure, density continued to influence haemoglobin ($P< 0.001$), MCHC ($P< 0.001$), platelet count ($P= 0.003$), and WBCs ($P< 0.0001$), while no effect was detected on the remaining parameters. This indicates that density primarily affects oxygen-carrying capacity and immune-related parameters, particularly under stress. However, treatment affected significantly before ammonia exposure; RBCs ($P= 0.004$), Hb ($P< 0.0001$), HCT ($P< 0.001$), MCHC ($P= 0.009$), platelet count ($P< 0.0001$), and WBCs ($P< 0.0001$)), while no effect was detected on the remaining parameters. Following exposure, treatment continued to have strong effects on Hb, MCHC, platelet count, and WBCs (all $P< 0.001$), highlighting its role in modulating haematological performance both under normal and stressed conditions. Moreover, the interaction between density and treatment showed limited influence before ammonia exposure, with significance observed only for platelet count and WBCs ($P< 0.0001$). After exposure, interactive effects became more pronounced, particularly for MCHC ($P< 0.01$), platelet count ($P< 0.0001$), and WBCs ($P< 0.0001$). These findings suggest that the combined effect of density and treatment is more evident when fish are subjected to ammonia stress.

Table 1. Haematological parameters of *S. aurata* fingerlings, pre-and post-ammonia challenge

		Low density				High density				Two-way ANOVA		
		C	T_1	T_2	T_3	C	T_1	T_2	T_3	Density	Treatment	Den * Treat
Red cell count ($\times 10^6$cell/mm^3)	**Pre-Ammonia**	2.10±0.26^{A}	2.61±0.32^{A}	2.77±0.34^{A}	2.91±0.36^{A}	2.19±0.27^{A}	2.68±0.33^{A}	2.90±0.36^{A}	2.97±0.37^{A}	0.520	0.004	0.997
	Post-Ammonia	1.84±0.23^{B}	2.20±0.27^{B}	2.29±0.28^{B}	2.45±0.30^{B}	1.98±0.25^{B}	2.36±0.29^{B}	2.65±0.33^{B}	2.79±0.36^{B}	0.170	0.052	0.125
Haemoglobin (g/dL)	**Pre-Ammonia**	6.15±0.20^{A}	6.69±0.22^{A}	6.96±0.23^{A}	7.66±0.25^{A}	6.28±0.21^{A}	7.00±0.23^{A}	7.71±0.26^{A}	8.16±0.27^{A}	< 0.001	< 0.0001	0.166
	Post-Ammonia	4.82±0.34^{B}	5.13±0.36^{B}	5.61±0.39^{B}	6.33±0.44^{B}	5.33±0.37^{B}	6.22±0.43^{B}	6.78±0.47^{B}	7.64±0.53^{B}	< 0.001	< 0.001	0.065
Haematocrit (HCT, %)	**Pre-Ammonia**	19.88±0.66	21.30±0.71	22.31±0.74	23.54±0.78	20.59±0.68	23.12±0.77	23.55±0.78	24.67±0.82	< 0.001	< 0.001	0.639
	Post-Ammonia	19.80±1.38	20.84±1.46	21.67±1.52	22.11±1.55	20.46±1.43	22.29±1.56	23.64±1.65	23.89±1.67	0.119	0.080	0.470
MCV (FL)	**Pre-Ammonia**	101.85±12.64^{B}	87.73±10.89^{B}	86.71±10.76^{B}	86.89±10.79^{B}	100.96±12.53^{B}	92.66±11.50^{B}	87.24±10.83^{B}	89.48±11.11^{B}	0.706	0.152	0.973
	Post-Ammonia	113.35±14.07^{A}	99.49±12.35^{A}	99.68±12.37^{A}	94.80±11.77^{A}	108.46±13.46^{A}	99.36±12.33^{A}	93.75±11.64^{A}	86.87±10.78^{A}	0.599	0.498	0.319
MCH (pg)	**Pre-Ammonia**	31.49±3.91^{A}	27.57±3.42^{A}	27.04±3.36^{A}	28.26±3.51^{A}	30.79±3.82^{A}	28.04±3.48^{A}	28.55±3.54^{A}	29.60±3.68^{A}	0.661	0.360	0.947
	Post-Ammonia	27.59±3.43^{B}	24.50±3.04^{B}	25.80±3.20^{B}	27.16±3.37^{B}	28.25±3.51^{B}	27.73±3.44^{B}	26.89±3.34^{B}	27.79±3.45^{B}	0.375	0.962	0.667
MCHC (%)	**Pre-Ammonia**	31.36±1.04^{A}	31.87±1.06^{A}	31.62±1.05^{A}	32.99±1.09^{A}	30.92±1.02^{A}	30.69±1.02^{A}	33.19±1.10^{A}	33.56±1.11^{A}	0.767	0.009	0.168
	Post-Ammonia	24.69±0.82^{B}	24.98±0.83^{B}	26.25±0.87^{B}	29.05±0.96^{B}	26.42±0.87^{B}	28.30±0.94^{B}	29.09±0.96^{B}	32.44±1.07^{B}	< 0.001	< 0.001	< 0.01
Platelet count ($\times 10^6$cell/mm^3)	**Pre-Ammonia**	9.05±0.16^{B}	36.18±0.65^{B}	41.21±0.74^{B}	10.05±0.18^{B}	22.11±0.40^{B}	55.28±0.99^{B}	16.08±0.29^{B}	23.12±0.41^{B}	<0.0001	< 0.0001	<0.0001
	Post-Ammonia	22.11±0.40^{A}	58.29±1.05^{A}	46.23±0.83^{A}	18.09±0.32^{A}	34.17±0.61^{A}	77.39±1.39^{A}	45.23±0.81^{A}	27.14±0.49^{A}	0.003	< 0.0001	<0.0001
White blood cell count ($\times 10^3$cell/mm^3)	**Pre-Ammonia**	23.37±1.23^{B}	21.88±0.39^{B}	20.26±0.36^{B}	19.10±0.34^{B}	21.02±0.38^{B}	20.09±0.36^{B}	18.47±0.33^{B}	17.31±0.31^{B}	<0.0001	< 0.0001	<0.0001
	Post-Ammonia	24.90±0.45^{A}	23.22±0.42^{A}	22.65±0.41^{A}	21.81±0.39^{A}	23.12±0.41^{A}	21.43±0.38^{A}	20.86±0.37^{A}	20.02±0.36^{A}	<0.0001	< 0.0001	<0.0001

* Tables' data showed significant variations in bars with different letters in the same parameters (T-test between Pre- and Post-ammonia, $P < 0.05$).

2. Biochemical parameters

The results in Table (2) show the variations in the biochemical parameters in the serum of *S. aurata* fingerlings fed over a 90-day experimental period under two distinct stocking densities (low-density (15 fish/hapa) and high-density (30 fish/hapa)) with a standardized feeding rate of 2% using dietary hydroxyproline supplementation at four inclusion levels: 0% (control), 1% (T_1), 2% (T_2), and 3% (T_3). This was followed by an acute ammonia exposure (24 hours), with biochemical parameters examined both pre- and post-challenge.

2.1. Alanine aminotransferase (ALT) levels

In the pre-ammonia challenge phase, the ALT activity in the serum of *S. aurata* fingerlings were decreased progressively with the increasing of hydroxyproline supplementation in treatments. The ALT activity in T_1 was recorded with 16.89±0.56 U/L, decreasing in T_2 (15.16±0.50 U/L) and the lowest value was observed in T_3 (14.13 ± 0.47 U/L) compared with the highest value, which occurred in the control group (18.48 ± 0.61 U/L). A similar trend was observed in fish under high stocking density, where the ALT levels decreased gradually in treatments (15.29±0.51 U/L in T_1, 14.00±0.46 U/L in T_2 and 13.18 ± 0.44 U/L in T_3) compared with the control group (18.61 ± 0.62 U/L). In the low-density group, post-challenge, the ALT values in the serum of *S. aurata* fingerlings were decreased progressively with the increasing of hydroxyproline supplementation in treatments. The ALT activity in T_1 was recorded with 20.03±1.40 U/L, decreasing in T_2 (18.27±1.28 U/L) and the lowest value was observed in T_3 (17.21 ± 1.20 U/L U/L) compared with the highest value, which occurred in the control group (21.66 ± 1.51 U/L). A similar trend was observed in fish under high stocking density, where ALT levels decrease gradually in treatments (19.75±1.38 U/L in T_1, 16.64±1.16 U/L in T_2 and 15.70 ± 1.10 U/L in T_3) compared with the control group (21.48 ± 1.50 U/L). Following ammonia exposure, ALT activity in the serum of *S. aurata* fingerlings increased significantly in all groups in low and high stock densities compared with before the ammonia challenge, indicative of hepatic stress or damage (Table 2).

2.2. Aspartate aminotransferase (AST) levels

In the pre-ammonia challenge phase, AST activity in the serum of *S. aurata* fingerlings decreased progressively with the increasing of hydroxyproline supplementation in dietary treatments across both stocking densities. Under low stocking density, the AST activity in T_1 recorded values of 198.44±24.63U/L, decreasing in T_2 (176.51±21.91 U/L) and reaching the lowest value was observed in T_3 (161.89 ± 20.10 U/L) compared with the highest value, which occurred in the control group (214.11 ± 26.58 U/L). Similarly, in fish reared under high stocking density, the AST values declined gradually in treatments (174.42±21.65 U/L in T_1, 152.49±18.93 U/L in T_2

and 137.87 ± 17.11 U/L in T_3) compared with the control group (197.40 ± 24.50 U/L). In the low-density group, post-challenge, the AST activity in the serum of *S. aurata* fingerlings in T_1 recorded values of 223.51±27.75 U/L, decreasing in T_2 (202.62±25.15 U/L) and the lowest value was observed in T_3 (182.78 ± 22.69 U/L) compared with the highest value, which occurred in the control group (275.73 ± 34.23 U/L). Similarly, in fish reared under high stocking density, the AST values declined gradually in treatments (199.49±24.76 U/L in T_1, 178.60±22.17 U/L in T_2 and 169.20 ± 21.00 U/L in T_3) compared with the control group (220.38 ± 27.36 U/L). Following the post-ammonia challenge, a marked increase in AST activity in the serum of *S. aurata* fingerlings was recorded in all groups in low and high stock densities compared with the pre-ammonia challenge, indicative of hepatic or tissue stress due to toxic insult (Table 2).

2.3. Glucose concentrations

During the pre-ammonia challenge phase, a gradual decrease in blood glucose levels was observed with increasing treatment intensity. Under low stocking density, glucose values in the serum of *S. aurata* fingerlings were decreased progressively with the increasing of hydroxyproline supplementation in treatments. The glucose value in T_1 was recorded at 56.13±1.86 mg/dL, decreasing in T_2 (46.28±1.53 mg/dL) and the lowest value was observed in T_3 (37.33 ± 1.24 mg/dL) compared with the highest value, which occurred in the control group (66.70 ± 2.21 mg/dL). Similarly, fish reared under high stocking density exhibited glucose levels that declined gradually in treatments (61.58±2.04 mg/dL in T_1, 48.89±1.62 mg/dL in T_2 and 37.28 ± 1.23 mg/dL in T_3) compared with the control group (73.55 ± 2.43 mg/dL). In the low-density groups, post-ammonia challenge, the glucose concentrations in the serum of *S. aurata* fingerlings in T_1 recorded 94.78±6.63 mg/dL, decreasing in T_2 (72.80±5.09 mg/dL) and reaching the lowest value was observed in T_3 (52.05 ± 3.64 mg/dL) compared with the highest value, which occurred in the control group (153.17 ± 10.71 mg/dL). In the high-density groups, Similarly, fish reared under high stocking density, the glucose values declined gradually in treatments (85.76±6.00 mg/dL in T_1, 65.53±4.58 mg/dL in T_2 and 48.48 ± 3.39 mg/dL in T_3) compared with the control group (127.02 ± 8.88 mg/dL)**.** Following the post-ammonia challenge, a high increase in serum glucose levels in *S. aurata* fingerlings was recorded across all groups in low and high stock densities compared with the pre-ammonia challenge, indicative of a classical stress response involving enhanced gluconeogenesis and cortisol-mediated mobilization of energy reserves (Table 2).

2.4. Blood cholesterol concentrations

In the pre-ammonia challenge phase, cholesterol levels demonstrated a treatment-dependent increase across both stocking densities. Under low density, cholesterol concentrations in the serum of *S. aurata* fingerlings increased with the increasing of hydroxyproline supplementation in treatments. The cholesterol concentrations in T_1 were recorded with values of 133.87±4.43 mg/dL, which increased gradually in T_2 (145.03±4.80 mg/dL) and the highest value was observed in T_3 (159.22 ± 5.27 mg/dL) compared with the lowest value, which occurred in the control group (112.57 ± 3.73 mg/dL). A similar trend was observed under high density; the cholesterol concentrations increased gradually in treatments (135.90±4.50 mg/dL in T_1, 145.03±4.80 mg/dL in T_2 and 186.61 ± 6.18 mg/dL in T_3) compared with the control group (128.80 ± 4.26 mg/dL). In the low-density groups, post-challenge, the cholesterol level in the serum of *S. aurata* fingerlings in T_1 was recorded at 106.79±7.47 mg/dL; it increased gradually in T_2 (118.19±8.26m g/dL) and the highest value was observed in T_3 (145.15±10.15 mg/dL) compared with the lowest value, which occurred in the control group (102.64±7.18 mg/dL). A similar trend was observed under high density; the cholesterol concentrations increase in treatments (122.34±8.55 mg/dL in T_1, 129.59±9.06 mg/dL in T_2 and 126.48±8.84 mg/dL in T_3) compared with the control group (109.90±7.68 mg/dL)**.** Following the ammonia challenge, a general significant decrease in cholesterol levels in the serum of *S. aurata* fingerlings was observed across all groups in low and high stock densities compared to their pre-challenge counterparts (Table 2).

2.5. Blood triglycerides concentrations

During the pre-ammonia challenge phase, a clear dose-dependent elevation in triglyceride levels was observed across all treatments. Under low stocking density, the triglyceride values in the serum of *S. aurata* fingerlings increased with the increasing of hydroxyproline supplementation in treatments. The triglyceride levels in T_1 were recorded at 28.97±3.6 mg/dL, which increased gradually in T_2 (34.82±4.32 mg/dL) and the highest value was observed in T_3 (54.21±6.73 mg/dL) compared with the lowest value, which occurred in the control group (21.98±2.73 mg/dL). Similarly, fish reared under high-density showed gradually increased in treatments (39.17±4.86 mg/dL in T_1, 42.61±5.29 mg/dL in T_2 and 57.24 ± 7.11 mg/dL in T_3) compared with the control group (26.74 ± 3.32 mg/dL). In the low-density group, post-challenge, triglyceride values in the serum of *S. aurata* fingerlings increased with the increasing of hydroxyproline supplementation in treatments. The triglyceride in T_1 recorded levels of 17.48±02.17 mg/dL, which increased gradually in T_2 (20.83±2.59 mg/dL) and the highest value was observed in T_3 (22.39 ± 2.78 mg/dL) compared with the lowest value, which occurred in the control group (12.95 ± 1.61 mg/dL). Similarly, fish reared under high-density showed gradually increased in treatments (21.62±2.68 mg/dL in T_1, 28.62±3.55 mg/dL in T_2 and 39.17 ± 4.86 mg/dL in T_3) compared with the control group (14.73 ± 1.83 mg/dL). Following the post-ammonia challenge, triglyceride concentrations in the serum of *S. aurata* fingerlings were decreased significantly in all groups regardless of treatment or stocking density compared with the pre-ammonia challenge. Notably, the levels were reduced post-challenge, the T_3 treatment consistently preserved higher triglyceride levels compared to other treatments, especially under high stocking density (Table 2).

2.6. Blood albumin concentrations

In the pre-ammonia challenge phase, albumin levels in the serum of *S. aurata* fingerlings decreased progressively across treatments. Under low stocking density, albumin values were decreased with the increasing of hydroxyproline supplementation in treatments. The albumin values in T_1 were recorded at 1.60±0.20 g/dL, with a decrease in T_2 (1.33±0.17 g/dL) while the lowest value was observed in T_3 (0.79±0.10 g/dL) compared with the highest value, which occurred in the control group (2.23±0.28 g/dL). Similarly, fish reared under high-density, albumin concentrations declined gradually in treatments (1.51±0.19 g/dL in T_1, 1.18±0.15 g/dL in T_2 and 1.04±0.13 g/dL in T_3) compared with the highest value in the control group (1.99±0.25 g/dL). In the low-density group, post-challenge, albumin values in the serum of *S. aurata* fingerlings in T_1 recorded 2.10±0.26 g/dL, decreasing in T_2 (1.66±0.21 g/dL) and the lowest value was observed in T_3 (1.13±0.14 g/dL) compared with the highest value, which occurred in the control group (2.90±0.36 g/dL). Similarly, fish reared under high-density albumin concentrations declined gradually in treatments (1.88±0.23 g/dL in T_1, 1.44±0.18 g/dL in T_2 and 1.15±0.14 g/dL in T_3) compared with the highest value in the control group (2.55±0.32 g/dL)**.** Following the ammonia challenge, albumin levels in the serum of *S. aurata* fingerlings increased significantly in all groups but remained lower in the more intensively treated groups. Although the values increased relative to their pre-challenge levels, particularly in the control groups, the pattern of lower albumin in T_3 persisted (Table 2).

2.7. Total protein levels

In the pre-ammonia phase, total protein concentrations in the serum of *S. aurata* fingerlings were highest in the control groups across both densities. Under low stocking density, protein levels decreased with the increasing of hydroxyproline supplementation in treatments. The total protein values in T_1 recorded 2.78±0.34 g/dL, decreasing in T_2 (2.65±0.33 g/dL) and the lowest value was observed in T_3 (2.05±0.25 g/dL) compared with the highest value, which occurred in the control group (3.69±0.46 g/dL). Similarly, fish reared under high-density, total protein concentrations declined gradually in treatments (2.66±0.33 g/dL in T_1, 2.48±0.31 g/dL in T_2 and 2.02±0.25 g/dL in T_3) compared with the highest value in the control group (3.63±0.45 g/dL). Under low density, post-challenge, total protein values in the serum of *S. aurata* fingerlings in T_1 recorded 3.67±0.46 g/dL, decreasing in T_2 (3.19±0.40 g/dL) and the lowest value was observed in T_3 (2.22±0.28 g/dL) compared with the highest value, which occurred in the control group (4.77±0.59 g/dL). Similarly, fish reared under high-density, total protein values declined gradually in treatments (3.32±0.41 g/dL in T_1, 2.74±0.34g/dL in T_2 and 2.10±0.26 g/dL in T_3) compared with the highest value in the control group (4.17±0.52 g/dL)**.** After ammonia exposure, total protein levels in the serum of *S. aurata* fingerlings were increased significantly in all groups in low and high stock densities compared to their pre-challenge, particularly in the control treatments. Despite the post-challenge increase, protein levels in T_3 groups remained comparatively lower (Table 2).

2.8. Globulin levels

In the pre-ammonia phase, globulin concentrations in the serum of *S. aurata* fingerlings were relatively higher in the control groups. Under low stocking density, globulin levels declined in treatments (1.14±0.04 g/dL in T_1, 1.28±0.04 g/dL in T_2 and 1.22 ± 0.04 g/dL in T_3) compared with the highest value in the control group (1.42 ± 0.05 g/dL). Similarity in the high-density groups; globulin values were slightly variable and decreased in treatments (1.12±0.04 g/dL in T_1, 1.25±0.04 g/dL in T_2 and 0.95 ± 0.03 g/dL in T_3) compared with the highest value in the control group (1.59 ± 0.05 g/dL), indicating reduced globulin in treated groups**.** In the low-density groups, post-challenge, the globulin values in the serum of *S. aurata* fingerlings were decreased with the increasing of hydroxyproline supplementation in treatments. The total protein values in T_1 recorded 1.52±0.05 g/dL, decreasing in T_2 (1.48±0.05 g/dL) and the lowest value was observed in T_3 (1.06±0.04 g/dL) compared with the highest value, which occurred in the control group (1.82±0.06 g/dL). Similarly, fish reared under high density had globulin values that declined gradually in treatments (1.40±0.05 g/dL in T_1, 1.27±0.04 g/dL in T_2 and 0.93±0.03 g/dL in T_3) compared with the highest value in the control group (1.58±0.05 g/dL)**.** Following ammonia exposure, globulin levels in the serum of *S. aurata* fingerlings were increased significantly in all groups in low and high stock densities compared to their pre-challenge, although the T_3 group remained markedly low (Table 2).

2.9. Cortisol levels

Prior to ammonia exposure, cortisol concentrations in the serum of *S. aurata* fingerlings were highest in the control groups across both densities. In the low-density group, cortisol levels decreased with the increasing of hydroxyproline supplementation in treatments. The cortisol values in T_1 recorded 2.31±0.04 µg/dL, decreasing in T_2 (1.63±0.03 µg/dL) and the lowest value was observed in T_3 (1.27 ± 0.02 µg/dL) compared with the highest value, which occurred in the control group (2.71 ± 0.05 µg/dL). Similarly, fish reared under high-density, cortisol values declined gradually in treatments (2.13±0.04 µg/dL in T_1, 1.98±0.04 µg/dL in T_2 and 1.46 ± 0.03 µg/dL in T_3) compared with the highest value in the control group (2.45 ± 0.04 µg/dL). In low-density fish, post-challenge, the cortisol values in the serum of *S. aurata* decreased with the increasing of hydroxyproline supplementation in treatments. The cortisol values in T_1 recorded 3.65±0.07 µg/dL, decreasing in T_2 (2.85±0.05 µg/dL) and the lowest value was observed in T_3 (2.18 ± 0.04 µg/dL) compared with the highest value, which occurred in the control group (4.63 ± 0.08 µg/dL). Similarly, fish reared under high-density, the cortisol values declined gradually in treatments (3.43±0.06 µg/dL in T_1, 2.95±0.05 µg/dL in T_2 and 2.34 ± 0.04 µg/dL in T_3) compared with the highest value in the control group (4.29 ± 0.08 µg/dL). After the ammonia challenge, cortisol levels in the serum of *S.*

aurata fingerlings increased significantly in all groups in low and high stock densities compared to their pre-challenge, indicative of a stress response. Notably, although cortisol levels increased post-challenge, fish in T_3 treatment groups exhibited relatively lower stress responses compared to controls (Table 2).

The biochemical parameters were analyzed using a two-way ANOVA to evaluate the effects of stocking density, treatment, and their interaction (density × treatment) before and after ammonia exposure. The results revealed clear variations in hepatic enzyme activities, carbohydrate and lipid metabolism, protein profile, and stress markers depending on the experimental conditions. A detailed description of these effects is presented below. However, the effect of stocking density on biochemical parameters showed selective significance across different indicators. Before ammonia exposure, density significantly influenced AST, ALT, blood glucose, cholesterol, triglycerides, and globulin, while non-significant effects were recorded for albumin, total protein, and cortisol. After ammonia exposure, density continued to exhibit a significant effect on AST, ALT, blood glucose, cholesterol, triglycerides, globulin, and cortisol, whereas albumin, and total protein remained statistically unaffected. These findings suggest that density plays a more pronounced role in modulating energy- and stress-related markers than protein metabolism under ammonia stress. Moreover, treatment exhibited a highly consistent and significant effect on almost all measured biochemical parameters both before and after ammonia exposure. This consistent trend highlights the dominant impact of dietary or experimental treatments in regulating hepatic enzyme activity, carbohydrate and lipid metabolism, and stress indicators regardless of ammonia challenge. Furthermore, the interaction between density and treatment showed variable significance among the biochemical parameters. Before ammonia exposure, significant interactive effects were noted for blood glucose, cholesterol, globulin, and cortisol, while the remaining parameters remained unaffected. Post-ammonia exposure, the interaction effect became more evident, with all measured biochemical parameters all showing significant differences. This indicates that the combined effect of stocking density and treatment exerts a synergistic influence on metabolic and stress-related parameters, especially under ammonia challenge conditions.

Table 2. Biochemical parameters of *S. aurata* fingerlings, pre-and post-ammonia challenge

		Low density				High density				Two- way ANOVA *P* -value		
		C	**T_1**	**T_2**	**T_3**	**C**	**T_1**	**T_2**	**T_3**	**Density**	**Treatment**	**Den * Treat**
GPT (ALAT) (U/L)	**Pre-ammonia**	18.48±0.61^{B}	16.89±0.56^{B}	15.16±0.50^{B}	14.13±0.47^{B}	18.61±0.62^{B}	15.29±0.51^{B}	14.00±0.46^{B}	13.18±0.44^{B}	< 0.001	< 0.0001	0.065
	Post-ammonia	21.66±1.51^{A}	20.03±1.40^{A}	18.27±1.28^{A}	17.21±1.20^{A}	21.48±1.50^{A}	19.75±1.38^{A}	16.64±1.16^{A}	15.70±1.10^{A}	0.208	< 0.01	< 0.01
GOT (ASAT) (U/L)	**Pre-ammonia**	214.11±26.58^{B}	198.44±24.63^{B}	176.51±21.91^{B}	161.89±20.1^{B}	197.40±24.50^{B}	174.42±21.65^{B}	152.49±18.93^{B}	137.87±17.11^{B}	0.026	< 0.01	0.989
	Post-ammonia	275.73±34.23^{A}	223.51±27.75^{A}	202.62±25.15^{A}	182.78±22.69^{A}	220.38±27.36^{A}	199.49±24.76^{A}	178.60±22.17^{A}	169.20±21.00^{A}	0.028	0.022	0.019
Blood Glucose (mg/dL)	**Pre-ammonia**	66.70±2.21^{B}	56.13±1.86^{B}	46.28±1.53^{B}	37.33±1.24^{B}	73.55±2.43^{B}	61.58±2.04^{B}	48.89±1.62^{B}	37.28±1.23^{B}	< 0.001	< 0.0001	0.022
	Post-ammonia	153.17±10.71^{A}	94.78±6.63^{A}	72.80±5.09^{A}	52.05±3.64^{A}	127.02±8.88^{A}	85.76±6.00^{A}	65.53±4.58^{A}	48.48±3.39^{A}	< 0.001	< 0.0001	<0.0001
Cholesterol (mg/dL)	**Pre-ammonia**	112.57±3.73^{A}	133.87±4.43^{A}	145.03±4.80^{A}	159.22±5.27^{A}	128.80±4.26^{A}	135.90±4.50^{A}	145.03±4.80^{A}	186.61±6.18^{A}	<0.0001	< 0.0001	< 0.001
	Post-ammonia	102.64±7.18^{B}	106.79±7.47^{B}	118.19±8.26^{B}	145.15±10.15^{B}	109.90±7.68^{B}	122.34±8.55^{B}	129.59±9.06^{B}	126.48±8.84^{B}	0.027	< 0.001	<0.001
Triglycerides (mg/dL)	**Pre-ammonia**	21.98±2.73^{A}	28.97±3.6^{A}	34.82±4.32^{A}	54.21±6.73^{A}	26.74±3.32^{A}	39.17±4.86^{A}	42.61±5.29^{A}	57.24±7.11^{A}	< 0.01	< 0.0001	0.615
	Post-ammonia	12.95±1.61^{B}	17.48±02.17^{B}	20.83±2.59^{B}	22.39±2.78^{B}	14.73±1.83^{B}	21.62±2.68^{B}	28.62±3.55^{B}	39.17±4.86^{B}	< 0.01	< 0.0001	< 0.01
Albumin (g/dL)	**Pre-ammonia**	2.23±0.28^{B}	1.60±0.20^{B}	1.33±0.17^{B}	0.79±0.10^{B}	1.99±0.25^{B}	1.51±0.19^{B}	1.18±0.15^{B}	1.04±0.13^{B}	0.496	< 0.0001	0.163
	Post-ammonia	2.90±0.36^{A}	2.10±0.26^{A}	1.66±0.21^{A}	1.13±0.14^{A}	2.55±0.32^{A}	1.88±0.23^{A}	1.44±0.18^{A}	1.15±0.14^{A}	0.060	< 0.0001	< 0.0001
Total Protein (g/dL)	**Pre-ammonia**	3.69±0.46^{B}	2.78±0.34^{B}	2.65±0.33^{B}	2.05±0.25^{B}	3.63±0.45^{B}	2.66±0.33^{B}	2.48±0.31^{B}	2.02±0.25^{B}	0.516	< 0.0001	0.983
	Post-ammonia	4.77±0.59^{A}	3.67±0.46^{A}	3.19±0.40^{A}	2.22±0.28^{A}	4.17±0.52^{A}	3.32±0.41^{A}	2.74±0.34^{A}	2.10±0.26^{A}	0.060	< 0.001	<0.001
Globulin (g/dL)	**Pre-ammonia**	1.42±0.05^{B}	1.14±0.04^{B}	1.28±0.04^{B}	1.22±0.04^{A}	1.59±0.05	1.12±0.04^{B}	1.25±0.04	0.95±0.03	0.035	< 0.0001	<0.0001
	Post-ammonia	1.82±0.06^{A}	1.52±0.05^{A}	1.48±0.05^{A}	1.06±0.04	1.58±0.05	1.40±0.05^{A}	1.27±0.04	0.93±0.03	<0.0001	< 0.0001	<0.0001
Cortisol (µg/dL)	**Pre-ammonia**	2.71±0.05^{B}	2.31±0.04^{B}	1.63±0.03^{B}	1.27±0.02^{B}	2.45±0.04^{B}	2.13±0.04^{B}	1.98±0.04^{B}	1.46±0.03^{B}	0.113	< 0.0001	<0.0001
	Post-ammonia	4.63±0.08^{A}	3.65±0.07^{A}	2.85±0.05^{A}	2.18±0.04^{A}	4.29±0.08^{A}	3.43±0.06^{A}	2.95±0.05^{A}	2.34±0.04^{A}	< 0.001	< 0.0001	<0.0001

* Tables' data showed significant variations in bars with different letters in the same parameters (T-test between Pre- and Post-ammonia, $P < 0.05$).

DISCUSSION

Improving the efficiency of aquaculture systems through nutritional interventions has emerged as a prominent research trend in aquatic animal farming, particularly under the environmental challenges associated with intensive culture systems. Ammonia is among the most critical environmental pollutants in aquaculture, accumulating mainly due to microbial decomposition of organic waste and uneaten feed **(Abbas *et al.*, 2024a)**. Elevated ammonia levels can impair growth, feed conversion efficiency, and immune responses, and in severe cases, lead to mass mortalities **(Ip *et al*., 2001; Naiel *et al*., 2024)**. High ammonia concentrations are known to cause significant haematological and biochemical disturbances, including reduced Hb levels, altered blood cell ratios, elevated hepatic enzyme activities, and increased oxidative stress markers **(Gao *et al.*, 2021)**. Nutritional interventions using functional amino acids, such as arginine, glutamine and proline, have been recognized as promising strategies for enhancing fish physiological health by supporting immune function, stimulating antioxidant defences, and improving protein metabolism, thereby alleviating the detrimental effects of environmental stress **(Li *et al.*, 2009; Wu, 2013; Hoseini *et al.*, 2019)**. In the present study, the physiological effects of feeding diets supplemented with different levels of an added amino acid (0%, 1%, 2%, and 3%) were evaluated in the serum of *S. aurata* fingerlings reared under two stocking densities: low (15 fish/hapa) and high (30 fish/hapa). After a 90-day feeding period, fish were subjected to an acute ammonia challenge for 24 hours. Haematological and biochemical parameters were analysed before and after exposure to determine the role of dietary supplementation in improving physiological responses and mitigating the negative effects of ammonia-induced stress.

Haematological parameters are widely recognized as reliable biomarkers for assessing the physiological status, stress response, and overall health of fish under different rearing conditions. Variations in RBC and WBC indices, Hb concentration, and HCT values provide critical insights into oxygen transport efficiency, immune competence, and the ability of fish to cope with environmental challenges such as high stocking density and ammonia exposure. Recent studies have emphasised the importance of monitoring haematological responses when evaluating the efficacy of dietary interventions aimed at enhancing fish resilience **(Hoseini *et al*., 2022)**. Also, haematological indices are crucial to understanding fish farming because they provide information on physiological states, nutritional status, and overall health of aquatic species **(Fazio, 2019)**. The present study demonstrated that dietary hydroxyproline supplementation exerted significant haemato-protective effects in *Sparus aurata* fingerlings reared under both low and high stocking densities, particularly before and after acute ammonia challenge.

In the present study, before the ammonia stress test, the pre-ammonia challenge findings revealed that dietary hydroxyproline supplementation significantly improved haematological parameters in *Sparus aurata* fingerlings. The results demonstrated that hydroxyproline-supplemented treatments caused a significant increase with higher hydroxyproline levels under both low and high stocking densities in RBCs, Hb, HCT, and MCHC compared to the control, indicating enhanced oxygen transport and physiological resilience. This improvement agrees with the outcomes of **Al-Dohail *et al.* (2009)** and **Bai *et al.* (2025)**; they reported that amino acid supplementation or protein-rich additives enhanced erythropoiesis and improved oxygen-transport parameters in fish.

In the present study, platelet counts varied depending on stocking density, whereas WBC counts consistently declined with higher supplementation levels, potentially reflecting reduced inflammatory responses and a more efficient immune regulation. These outcomes align with previous studies reporting that amino acid supplementation, including proline and hydroxyproline, can enhance haematopoiesis and blood performance in fish **(Hoseini *et al.*, 2018; Chen *et al.*, 2020; Ahmedifar *et al.*, 2021; Radwan *et al*., 2024)**. However, other research has shown limited or no significant effects of hydroxyproline or proline supplementation on haematological indices in some fish species **(Peres & Oliva-Teles, 2008; Li *et al.*, 2019)**, suggesting that such effects may depend on species-specific physiology and rearing conditions such as density and environmental stressors.

Following the ammonia challenge, haematological indices (RBC, Hb, HCT) declined across all groups, yet hydroxyproline-supplemented fish showed higher values compared to controls, indicating partial mitigation of ammonia-induced stress. This decline in erythrocyte count in aquatic species may be associated with anaemia, potentially caused by suppressed erythropoietin production following ammonia exposure. **Tilak *et al.* (2007)** reported that in common carp, ammonia stress increased oxygen uptake and induced methaemoglobin formation, which consequently resulted in a significant reduction in Hb percentage. This decline in Hb and RBCs suggests a hepatotoxic effect of ammonia, which is consistent with previous reports attributing such changes to increased erythrocyte destruction (haemolysis), inhibition of erythropoiesis in haematopoietic tissues, and disruption of ionic balance **(Ip *et al.*, 2001; Randall & Tsui, 2002)**. The reduction in RBCs and Hb concentration is consistent with earlier reports by **Shalaby (2001)** and **Randall and Tsui (2002)**, who indicated that ammonia induces haemolysis and suppresses erythropoiesis due to oxidative stress and ionic imbalance. The platelet count ($\times10^6$ cell/mm^3) showed a significant increase in all proline-supplemented treatments after ammonia exposure under both stocking densities. This elevation suggests a stimulatory response of thrombopoiesis under stress conditions, potentially representing a compensatory mechanism against ammonia-induced haematological disturbances **(Abbas *et al.*, 2024a, b)**. The WBC count showed a significant increase in all treatments after ammonia exposure, indicating an activation of the fish immune system in response to stress. Elevated WBC levels are commonly interpreted as a defence mechanism against tissue damage and oxidative stress caused by ammonia toxicity. The elevation in platelet count may suggest a stress-induced stimulation of thrombopoiesis, enhancing clotting ability and maintaining circulatory stability, similar to the observations of **Zou *et al.* (2023)**. Furthermore, the rise in WBCs points to immune system activation against ammonia-induced toxicity, in agreement with **Rehulka (2000)** and **Gabriel *et al.* (2004)**. Similar findings were reported by **Gabriel *et al.* (2004)** and **Fazio (2019)**, who emphasized that increased leukocyte counts are reliable indicators of stress and immune activation in fish exposed to environmental pollutants. However, the increase in platelet and WBC counts post-challenge in several treatments reflects typical stress-induced thrombopoietic and immune responses, though the suppressed WBC values in high-supplemented groups suggest hydroxyproline may attenuate excessive inflammatory activation. These findings agree with earlier reports highlighting the positive role of hydroxyproline in enhancing haematopoiesis and stress resilience in fish **(Hoseini *et al.*, 2018; Abdel-Tawwab *et al.*, 2019; Chen *et al.*, 2020; Ahmedifar *et al*., 2021; Hoseinifar *et al.*, 2021)**. Additionally, this leucocytosis may be attributed to elevated lymphocytes and lymphopoiesis originating from lymphoid tissues in response to ammonia stress. Similar findings were documented by **Zeitoun *et al.* (2016)** and **Gehad *et al.* (2023)**. Conversely, some studies reported limited or no haematological benefits of hydroxyproline or proline supplementation in other species, such as European seabass or tilapia under varying conditions **(Peres & Oliva-Teles, 2008; Li *et al.*, 2019; Zhang *et al.*, 2022; Abbas *et al.*, 2023)**, suggesting that the efficacy of supplementation is both dose- and species-dependent. Collectively, the present findings underscore that dietary hydroxyproline enhances haematological resilience in *S. aurata*, particularly at 2–3% inclusion levels, though its protective effect remains partial under acute ammonia stress.

Biochemical indices are key indicators of the metabolic status and physiological well-being of fish and are widely employed to evaluate the effects of dietary interventions and environmental stressors in aquaculture. They reflect the functionality of vital organs, particularly the liver and kidney, and provide insight into nutrient utilisation, energy balance, and stress-mediated metabolic alterations. Recent studies have emphasised that dietary supplementation with functional amino acids, such as hydroxyproline and proline, can modulate serum biochemical indices, improve protein and lipid metabolism, and enhance tolerance to stressors like high stocking density and ammonia exposure **(Abdel-Tawwab *et al.*, 2024)**. These findings highlight the importance of biochemical responses as a sensitive tool to assess the protective role of dietary additives in cultured fish.

In the present study, before ammonia exposure, dietary hydroxyproline supplementation induced significant dose-dependent effects on serum biochemical parameters of *S. aurata* fingerlings. Both ALT and AST activities decreased progressively with higher supplementation levels under both stocking densities, suggesting reduced hepatic turnover and improved liver health. Similarly, glucose and cortisol concentrations declined with hydroxyproline inclusion, reflecting enhanced metabolic stability and lower stress sensitivity. Conversely, cholesterol and triglyceride levels increased significantly in a dose-dependent manner, possibly indicating enhanced lipid metabolism and energy storage. However, albumin, total protein, and globulin concentrations decreased in treated groups compared to controls, suggesting a trade-off between protein allocation and stress mitigation. These findings agree with studies highlighting the hepato-protective and stress-reducing roles of amino acids, including proline and hydroxyproline, in aquaculture species **(Hoseini *et al.*, 2018; Abdel-Tawwab *et al.*, 2019; Chen *et al.*, 2020)**. Nevertheless, some research reported negligible or even adverse impacts of amino acid supplementation on protein metabolism and serum biochemistry **(Peres & Oliva-Teles, 2008; Li *et al.*, 2019)**, emphasizing the species- and dose-dependent nature of these responses.

After the toxin stress, changes in the biochemical investigations in seabream blood and blood tests are a reliable and accurate method of determining the health of the species **(Shin *et al.*, 2016; Abbas *et al.*, 2023; Elaraby *et al.*, 2024)**. Following acute ammonia exposure, serum ALT and AST activities increased markedly across all groups, confirming hepatic stress and tissue damage. However, hydroxyproline-supplemented groups consistently exhibited lower enzyme activities than controls, suggesting partial hepato-protection. According to **Ye *et al.* (2011)**, when hepatocytes are injured, substantial liver enzymes, which change the amino groups of alpha-amino into alpha-keto acids, often leak into the bloodstream. Previous studies have reported that exposure to ammonia damages the organs of aquatic organisms and elevates their enzyme activities, a finding consistent with **Lin *et al.* (2011)**. Furthermore, **Agrahari *et al.* (2007)** recommended the measurement of ASAT and ALAT activities in fish as indicators for assessing kidney and liver injury.

Conversely, cholesterol and triglyceride concentrations generally declined post-challenge, likely due to their mobilisation for energy demands under stress, yet hydroxyproline-treated groups maintained relatively higher values, indicating better metabolic compensation. This decline may be linked to increased lipid mobilisation as an alternative energy source during stress, especially under conditions of elevated glucose utilisation and higher metabolic demands **(Wendelaar Bonga, 1997; El-Nobi *et al.*, 2021)**. Additionally, the reduction might reflect impaired lipid metabolism associated with ammonia-induced hepatic dysfunction.

Similarly, glucose and cortisol levels rose sharply in response to ammonia stress, indicating activation of gluconeogenesis and stress hormones, though increases were less pronounced in supplemented groups, particularly at 3%. Additionally, seabream fed hydroxyproline-supplemented diets exhibited significantly lower levels of cortisol and glucose (hyperglycaemia) compared to the control group. Although all treatments showed significant increases in both parameters following exposure to ambient ammonia, the escalation rates of glucose and cortisol were notably lower in hydroxyproline-fed fish than in the hydroxyproline-0 group. Elevated blood cortisol in fish exposed to ammonia stress has been associated with physiological stress responses **(Sinha *et al.*, 2012; Shin *et***

***al.*, 2016)**, while hyperglycaemia can result from cortisol activation during stressful events **(Elbialy *et al.*, 2021)**.

In the present study, albumin, total protein, and globulin levels increased after ammonia exposure across all groups, reflecting an acute-phase protein response, but values remained lower in hydroxyproline treatments, consistent with altered protein turnover. Likewise, fish diets that include immune stimulants increase total protein levels in their blood, which links to an innate immune response **(Rudneva & Koverchina, 2011)**. Fish-fed proline diets had significantly higher total protein in both the pre- and post-phases of the study, demonstrating that proline-based diets boost seabream fish innate immunity.

These results corroborate previous studies showing that amino acid supplementation, including proline derivatives, enhances resilience to ammonia toxicity by supporting hepatic and metabolic functions **(Abdel-Tawwab *et al.*, 2019; Hoseinifar *et al.*, 2021; Radwan *et al.*, 2021; Abdel-Aziz *et al.*, 2025)**. Conversely, other reports found limited or no protective effects against ammonia-induced biochemical disturbances **(Gao *et al.*, 2021; Abbas *et al.*, 2023)**, highlighting variability among species and stress intensities. These outcomes align with previous findings that ammonia exposure elevates both glucose and cortisol levels and destabilises serum proteins, ultimately impairing fish health and performance **(e.g., Zeitoun *et al.*, 2016)**.

CONCLUSION

The present study demonstrated that dietary supplementation with hydroxyproline significantly modulated haematological and biochemical parameters of *Sparus aurata* fingerlings reared under different stocking densities and subjected to ammonia-induced stress. Hydroxyproline supplementation, particularly at the 3% inclusion level, improved erythropoiesis and oxygen transport capacity, as reflected by increased RBCs, Hb, and HCT. Concurrent reductions in MCV and MCH, alongside elevated MCHC, indicated adaptive modifications in erythrocyte morphology and haemoglobinisation. Moreover, biochemical indices such as glucose, cholesterol, triglycerides, and liver enzymes showed significant amelioration in supplemented groups, suggesting enhanced metabolic stability and hepatic protection against ammonia stress. Despite a general decline in haematological status post-challenge, hydroxyproline-supplemented fish exhibited reduced severity of alterations compared to the control, highlighting its haemato-protective and stress-mitigating role. Collectively, these findings emphasize the potential of dietary hydroxyproline, particularly at 3% inclusion, as an effective nutritional strategy to enhance blood health, metabolic resilience, and tolerance to environmental stressors in aquaculture practices.

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