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Unveiling the Effect of Ammonia on the Nile Tilapia (*Oreochromis niloticus*) Fed on the Prickly Pear (*Opuntia littoralis*) Cladodes as a Dietary Supplement: Haematological and Immunobiochemical Consequences

Mahmoud Mahrous Abbas[1*], Mahmoud Radwan[1], Diaa Farrag Ibrahim Ahmed[1], Hesham Gamal AbdelRasheed[1], Bassem E. Elaraby[1, 2], Ahmed Md. Salem[3]
[1]Zoology and Entomology Depart, Faculty of Science, Al-Azhar University, Cairo, 11884, Egypt
[2]Medical Laboratory Technologies. Kut university college. Kut, Wasit. 52001, Iraq
[3]National Institute of Oceanography & Fisheries, NIOF, Egypt

*Corresponding author: Mahmoud_Mahrous42@azhar.edu.eg,
ORCID: https://orcid.org/0000-0002-2061-4101

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ABSTRACT

Excessive ammonia accumulation poses significant risks to aquaculture, potentially compromising the performance and productivity of aquatic species. The investigation aimed to determine the impacts of dietary supplementation with Opuntia littoralis cladodes (OLCP) at 0% (OLCP-0), 0.5% (OLCP-1), 1% (OLCP-2), and 2% (OLCP-3) on growth performance, haematological, immuno-biochemical indices, digestive enzyme activity, and antioxidant responses. However, a study was conducted on the Nile tilapia (*O. niloticus*) (25.49±0.25g) for sixty days (pre-phase). After that, fish were exposed to ammonia stress (0.5mg/L) for 24 hours (post-phase). The findings indicated that dietary OLCP, particularly at 1% and 2% levels, significantly stimulated growth and enhanced protease, amylase, and lipase activities. Data revealed improved haematological parameters and immunological markers (complement C3, lysozyme activity, and total immunoglobulin) with increased OLCP levels. However, the exposure to ammonia caused a general reduction in these parameters, and the 2% OLCP group maintained the highest improved levels. The higher concentrations of OLCP diets before and after ammonia-induced stress significantly enhanced the antioxidant defenses, especially in the 2% OLCP group. Additionally, OLCP supplementation reduced malondialdehyde (MDA) levels and leukocyte counts. Overall, the results suggested that prickly pear cladodes, can improve the growth, immune function, and antioxidant response of tilapia fish diets, especially in the 2% OLCP group, while effectively reducing the adverse impacts of ammonia exposure on their immuno-biochemical and haematological indices.

INTRODUCTION

One of the food production sectors with the fastest rate of growth is aquaculture, which is essential to supplying people's daily demands for protein **(Dawood & Kari, 2024; FAO, 2024)**. In light of this, fish farming faces many obstacles, such as environmental stress, poor water quality, and the spread of infection that leads to decreased productivity in these farms **(Dawood *et al.*, 2021; Radwan *et al.*, 2023)**. One of these stressors was the accumulation of ammonia in fish farms, mainly caused by fish waste, leftover feed, increased organic matter deposition in pond bottoms, and low dissolved oxygen levels **(Sriyasak *et al.*, 2015)**. According to **Qi *et al.* (2017)**, high levels of ammonia toxicity cause various problems in marine life, such as reduced feed intake and reduced immune and oxidative responses. Additionally, the body's ability to metabolize protein may decline, requiring significant energy to maintain the proper protein level in the fish's body **(Güroy *et al.*, 2014)**. The most effective way to reduce ammonia toxicity is to decrease feeding, change the water, add more dissolved oxygen, or gradually add lime to the ponds **(Boyd, 2017)**.

Medicine plant extract was a potent technique to lower the ammonia level in fish ponds **(Yang *et al.*, 2015; Fayed *et al.*, 2019)**. Also, medicinal herbs or extracts as natural immune stimulants were another promising strategy for enhancing fish growth, immune system performance, and disease resistance in aquaculture **(Dawood *et al.*, 2024)**. Moreover, medicinal plants have been used as therapeutic agents to regulate fish health in aquaculture because of their bioactive components **(Radwan *et al.*, 2024a & b)**.

One of the most popular therapeutic plants in the world, prickly pears are used extensively in the pharmaceutical, food, and healthcare sectors. It belongs to the Cactaceae family and is considered an edible herb used worldwide with medicinal properties **(Mahrose, 2021; Abbas *et al.*, 2024a-c)**. According to **Stintzing and Carle (2005)** and **Galal *et al.* (2017)**, the cladodes contain a variety of active phytochemical components, such as terpenes, carbohydrates, glycosides, coumarins, tannins, and flavonoids. These compounds might significantly defend against nitrogen or reactive oxygen species (ROS) in aquatic life and possess antimicrobial, antioxidant, and immunological properties **(Saheli *et al*., 2021; Ahmadifar *et al.*, 2021)**.

Conversely, previous studies on the toxicity of ammonia have demonstrated that elevated ammonia levels may result in suppression of immunity, oxidative stress, and stress reactions, which can be mitigated using herbal dietary supplements **(Dawood *et al.*, 2021; Elbialy *et al.*, 2021)**. However, no investigation has been conducted into the potential application of nutritional supplements (prickly pears) in tilapia diets and exposure to ammonia stress. Hence, this study aimed to evaluate the benefits of supplementing diets with cladodes of *Opuntia littoralis* to mitigate the negative consequences of ammonia stress. The research entailed feeding period (60 day, pre-phase) of tilapia (*O. niloticus*) on a diet supplemented with OLCP and followed by a twenty-four-hour exposure to ammonia (post-phase).

MATERIALS AND METHODS

Samples collection

Fish were collected from a private farm located in Kafer El-Sheikh (Egyptian province) and transported carefully into the lab for examination. After the fish had arrived at the laboratory, samples were acclimate in glass aquariums. Upon visual inspection, the fish specimens had no apparent injuries or illnesses and appeared healthy **(Radwan *et al.*, 2022a)**. In the same way, fresh cladodes of prickly pears were harvested from the Wady Mageid Marsa-Matrouh province, Egypt (summer-2023).

Identification, extraction, and dietary procedure of prickly

The cladodes of *O. littoralis* were identified at the Botany Depart., Science Fac., Al-Azhar Uni., Egypt. The cladodes were dried in the shade and extracted using a Soxhlet apparatus with 70% ethanol solvents **(Abd El-Moaty, 2020)**. Then, mix feed ingredients with the extracted cladodes levels of 0% (OLCP-0 category, control), 0.5% (OLCP-1 category), 1% (OLCP-2 category), and 2% (OLCP-3 category) and blinded with water and oil using a miner to create a solid mixture (Table 1). The pellets of OLCP were dried in air and stored in polyethylene bags at 4°C for further use, according to **Hoseinifar *et al.* (2021)**. Gas chromatography and mass spectrometry (GC-MS) analysis were used to detect and identify the composition of bioactive components in the extracted cladode (Table 2, Agricultural Research Centre, Dokki, Giza). The raw protein, total fats, crude fiber, ash, and dry matter contents of diets were calculated according to **Thiex *et al.* (2012)** standard procedures. The formula for calculated nitrogen-free extract (NFE) was NFE (%) = 100 – (lipid (%) + protein (%) + ash (%) + fiber (%)). We estimated 17.2, 23.6, and 39.5 KJ/g f of carbohydrates, protein, and fat to calculate the diet's gross energy content.

Experiment design

Four healthy fish groups, OLCP-0, OLCP-1, OLCP-2, and OLCP-3 (15 fish/aquariums represented as 60 × 50 × 30cm, 50-liter water) with initial weights of 25.49±0.25g were distributed (3 replicates). The fish in each group fed a diet supplemented with cladodes twice daily, at 3% of body weight, at 9:00 AM and 4:00 PM for sixty days. Every day, 70% of dechlorinated water was changed, siphoned out, and continually aerated in each aquarium. Regularly monitored the aquarium water quality, including measurements of temperature (26.60 ± 0.57°C), pH (7.63 ± 0.38), total ammonia (0.17 ± 0.01mg/ L), and dissolved oxygen (6.56 ± 0.74mg/ L). The fish in each aquarium were counted and weighed individually three times (every fifteen days) during the feeding experiment to assess survival rate, growth performance, and feed efficiency for each fish diet category. These measurements were taken using well-established methods as detailed in **Radwan *et al.* (2024b)**.

Table 1. The experimental diets' formulation and composition (%, on a dry matter basis)

Ingredient	OLCP-fortified diets			
	OLCP-0 (Control)	OLCP-1 (0.5%)	OLCP-2 (1%)	OLCP-3 (2%)
Fish meal (72.0% CP)	99.8	99.8	99.8	99.8
Soybean meal (48% CP)	420.2	420.2	420.2	420.2
Yellow corn	200	200	200	200
Wheat flour	90.6	85.6	80.6	70.6
Wheat bran	150.2	150.2	150.2	150.2
Vegetable oil	13.50	13.50	13.50	13.50
Cod liver oil	12.00	12.00	12.00	12.00
Dicalcium phosphate	8.70	8.70	8.70	8.70
*Vitamins and mineral mixture	2.00	2.00	2.00	2.00
Vitamin C	3.00	3.00	3.00	3.00
OLCP	0	5	10	20
	1000	1000	1000	1000
Proximate chemical analysis (%)				
Moisture (%)	9.64	9.68	10.1	10.18
Dry matter (%)	90.36	90.32	89.9	89.82
Crude protein (%)	30.34	30.38	30.36	30.34
Crude lipid (%)	8.19	8.21	8.23	8.24
Fiber (%)	6.14	6.04	6.07	6.08
Ash (%)	10.42	10.44	10.45	10.44
Nitrogen-free extract (%)	44.91	44.93	44.89	44.90
Gross energy (KJ/g)	1917.59	1917.95	1918.09	1918.36

*The mixture of vitamins and minerals was prepared according to **Radwan *et al*. (2024a)**.

Ammonia stress experiment

Fish from each group were fasted for a full day before the ammonia experiment was challenged, representing 0.5mg/ L for 24 hours **(Yousefi *et al*., 2020)**.

Collection of blood samples

After a 24-hour fast, fish were anaesthetised with 50µL/ L of clove oil to determine blood indices in the pre-phase and post-phase ammonia stress experiments. To perform haematological tests, blood collected from the caudal peduncles of three fish in each aquarium using a syringe loaded with an anticoagulant (EDTA). On the other hand, a syringe (without anticoagulants) was used for the immunological biochemical and antioxidant assays. The mixture was centrifuged at 3000g for 15 minutes to extract the serum, which was then stored at -20°C until analysis.

Table 2. GC-MS of *Opuntia littoralis* cladodes extract showed the identified components

No	Compound identified	Molecular weight	Formula	Retention time (min)	Area (%)
1	Palmitic acid	256.40	$C_{16}H_{32}O_2$	7.342	71.24
2	Oleic acid	895.00	$C_{18}H_{34}O_2$	8.111	55.43
3	Oxalic acid	90.03	$C_2H_2O_4$	6.881	52.19
4	Quercetin	117.39	$C_{15}H_{10}O_7$	7.451	49.81
5	β-Sitosterol	414.71	$C_{29}H_{50}O$	8.312	48.45
6	Ellagic acid	302.19	$C_{14}H_6O_8$	11.443	47.37
7	Chlorogenic acid	354.31	$C_{16}H_{18}O_9$	13.109	46.82
8	Isorhamnetin	316.26	$C_{16}H_{12}O_7$	14.95	44.52
9	Luteolin	286.23	$C_{15}H_{10}O_6$	16.117	33.94
10	Kaempferol	286.23	$C_{15}H_{10}O_6$	18.442	25.16
11	Stigmasterol	412.69	$C_{29}H_{48}O$	20.178	20.90
12	Methyl caffeate	194.18	$C_{10}H_{10}O_4$	22.615	18.92
13	Campesterol	400.7	$C_{28}H_{48}O$	24.215	16.69
14	Ursolic acid	456.7	$C_{30}H_{48}O_3$	25.812	15.82
15	Sakuranetin	286.28	$C_{16}H_{14}O_5$	27.701	13.01
16	Myricetin	318.23	$C_{15}H_{10}O_8$	28.172	12.41
17	Squalene	858.00	$C_{30}H5_0$	29.422	10.55
18	Glucocapparin	333.3	$C_8H_{15}NO_9S_2$	34.055	9.54

Digestive enzymes assay

Fish (three intestines) samples were obtained from every aquarium. The samples were dissected, homogenized, then centrifuged at 4°C to get the supernatant for the digestive enzymes (protease, lipase, and amylase) **(Najdegerami *et al.*, 2016)**. However, **Iijima *et al.* (1998)** observed intestinal amylase and protease activity utilizing 0.3% starch and 1% hydrolysis of p-nitrophenyl myristate.

Biochemical-haematological measurement

Haematological markers, including haemoglobin levels, leucocytes, erythrocytes, packed cell volume, and blood cell indices, were calculated using previous methods described by **Dacie and Lewis (1991)** and **Brown (1993)**. Cortisol levels were determiened 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)**. By subtracting albumin from total protein, the globulin level was determined.

Immune- and antioxidants assay

Tilapia blood serum samples were tested for lysozyme, total immunoglobulin, and complement C3 activity using techniques outlined by **Siwicki** and **Anderson (1993)** and **Tang *et al.* (2008)**. Diagnostic kits were used to assess the antioxidant activities in the tilapia serum, including glutathione peroxidase, superoxide dismutase, catalase, and malonaldehyde activities.

Analytical statistics

SPSS software was used to perform a one-way ANOVA on the final data **(Dytham, 2011)**. When a significant difference was found between the fish groups ($P < 0.05$), Tukey's test was applied. To identify differences between the pre- and post-phase samples, a T-test was used.

RESULTS AND DISCUSSION

Fish growth and the activity of digestive enzymes

The final body weight, weight gain, and specific growth rate (FBW, WG and SGR, respectevily) of fish fed OLCP for 60 days were significantly ($P < 0.05$) higher than those fed an OLCP-free diet (Table 3). The highest levels of FBW, WG, and SGR (72.92±2.27, 47.18±3.10, and 1.74±0.11g, respectively) were recorded in the fish-fed OLCP diet at 2%. Conversely, the value feed conversion ratio (1.49±0.15 %) was significantly reduced in fish-fed OLCP at 2% compared to the control value (1.88±0.21%) without significant differences from the other groups. Between the groups, there were no significant variations in the survival rate. Correspondingly, the highest value (3.91±0.18, 9.21± 0.41, 15.72±1.19) of digestive enzyme activities (lipase, amylase, and protease, respectively) of the OLCP-3 group and the lowest values (1.88±0.32, 4.19±0.54, 9.35±1.78) observed in the control OLCP-0 (Fig. 2). Incorporating cladodes extracts to the feed, growth of fish was encouraged, feed intake rose, and palatability was enhanced. The increased growth performance is most likely due to the function of Cladodes in stimulating the digestive enzymes, which improves the palatability of diets **(Abbas *et al,*. 2024)**. More precisely, by enhancing intestinal wall permeability, steroidal saponins contribute to increased nutrient absorption **(Francis *et al.*, 2002)**. Furthermore, **Salem *et al.* (2024)** reported that *O. littoralis's* improved growth performance is due to the inclusion of vitamins, fatty acids, and amino acids. Phenolic and flavonoids compounds, two phytochemical chemicals found in extracts of *O. littoralis*, may be responsible for the recorded results **(Ahmed *et al.*, 2020)**. In this sense, medicinal herbs increase feed intake and weight gain in supplemented fish by mediating the formation of beneficial bacteria colonies in the digestive tract **(Safari *et al.*, 2017; Radwan *et al*., 2023)**. Additionally, herbal extracts are known for suppressing the activity of pathogenic microorganisms in the gastrointestinal tract, which is behind their influence on the activity of digestive enzymes **(Dhama *et al.*, 2018)**.

Table 3. Growth performance of tilapia fed with OLCP-fortified diets (OLCP) for sixty days

	OLCP levels (%)				p-value
	OLCP-0	OLCP-1	OLCP-2	OLCP-3	
Initial body weight(g)	25.50±1.35	25.74±1.85	25.55±1.11	25.51±1.66	0.11
Final weight (g)	55.00±2.97 ^{c}	64.06±3.37 ^{b}	65.95±1.85 ^{b}	72.92±2.27 ^{a}	0.01
Weight gain (g)	29.26±3.13 ^{d}	36.32±3.74 ^{c}	40.20±1.94 ^{b}	47.18±3.10 ^{a}	0.01
Specific growth rate (%day^{-1})	1.26±0.11 ^{d}	1.47±0.13 ^{c}	1.57±0.07 ^{b}	1.74±0.11 ^{a}	0.03
Feed intake (g)	51.76±3.32 ^{c}	62.72±3.09 ^{b}	64.11±3.23 ^{b}	70.20±4.23 ^{a}	0.03
Feed conversion ratio	1.88±0.21 ^{a}	1.65±0.25 ^{b}	1.60±0.10 ^{b}	1.49±0.15 ^{c}	0.02
Survival rate (%)	100	100	100	100	

* A one-way ANOVA comparing the OLCP groups with $P < 0.05$ shows a significant difference (mean±S.D.) when different letters were displayed.

Haematological assay

Haematological indices are crucial to understanding fish farming because they provide information on the physiological states, nutritional status, and overall health of aquatic species **(Fazio, 2019)**. Table (4) displayed the haematological parameters of tilapia groups between pre- and post-phases. The haematological indices of tilapia fed with different levels of OLCP showed notable variations. Before the ammonia stress test, the OLCP-3 group exhibited the highest erythrocyte count (2.36±0.08 × 10^6 cells/mm³) and PCV (24.98±0.10 %), while the OLCP-0 group had the lowest values for both (2.18±0.13 × 10^6 cells/mm³ and 22.73±0.39 %, respectively). After the ammonia stress, erythrocyte counts and PCV decreased in all groups, with OLCP-3 still maintaining the highest values (2.29±0.06 × 10^6 cells/mm³ and 23.04±0.08%) and OLCP-0 the lowest (1.97±0.07 × 10^6 cells/mm³ and 19.91±0.49 %, respectively). For leucocytes, OLCP-0 had the highest count pre-ammonia (22.86±0.28 × 10^3 cells/mm³, respectively), which increased post-ammonia to 24.18±0.53 × 10^3 cells/mm³, while OLCP-3 had the lowest pre-ammonia (19.65±0.31 × 10^3 cells/mm³) and post-ammonia (21.14±0.56 × 10^3 cells/mm³). Haemoglobin levels were highest in OLCP-2 pre-ammonia (7.93±0.08 g/dl) and lowest in OLCP-0 (6.51±0.14 g/dl), with OLCP-3 showing the highest post-ammonia value (6.96±0.09 g/dl) and OLCP-0 the lowest (5.35±0.44 g/dl). MCV and MCH followed similar patterns, with OLCP-3 having the highest values before (105.85±2.42 fL and 32.88±0.74 pg) and after (100.61±1.55 fL and 30.39±0.40 pg) the ammonia stress. In contrast, OLCP-2 had the lowest pre-ammonia MCV (101.07±1.72 fL) and MCH

(33.89±0.31 pg), and post-ammonia MCV (99.55±0.48 fL) and MCH (29.77±0.36 pg). MCHC values were highest for OLCP-2 before (33.26±0.33 %) and OLCP-3 after (30.21±0.27 %), and lowest for OLCP-0 before (28.64±0.46 %) and after (26.87±0.60 %). Overall, while there were significant differences in these haematological indices across different OLCP levels, the relative rankings of the groups remained consistent before and after the ammonia stress experiment.

The present investigation found that exposure to ambient ammonia dramatically reduced erythrocytes, Hb, MCHC, MCV, MCH, and PCV levels in tilapia groups. The decrease in erythrocyte count in aquatic species could be attributed to anemia, which inhibited erythropoietin following ammonia exposure. According to **Tilak *et al.* (2007)**, increased oxygen intake and methemoglobin resulted in a significantly lower percentage of haemoglobin in common carp following ammonia stress.

Nevertheless, the leucocyte levels showed a considerable increase following exposure to ammonia stress in all experimental groups. The OLCP-0 group exhibited the maximum rise in leucocyte levels, while the group with the highest OLCP concentration (2%) showed the lowest growth. Increased lymphocytes and lymphopoies from lymphoid tissues may cause leukocyte elevation following ammonia stress. Also, **Zeitoun *et al.* (2016)** and **Gehad *et al.* (2023)** reported similar results. Compared to the OLCP-0 groups, the current study's diet groups with OLCP supplementation showed significantly higher levels of PCV, erythrocytes, Hb, and MCH (P < 0.05). Haematological measures, which primarily display the stress levels and overall health of fish, may be improved by adding cladodes to diets. Comparable reports **(Osman *et al.*, 2018; Fazio, 2019)** showed that feeding aquatic organisms a diet enriched with herbal supplements enhanced their haematological measures.

All tilapia on OLCP diets, erythrocytes, PCV, and Hb were increased significantly compared to the control group not on OLCP diets, indicating that OLCP has an immune-stimulating effect and can be protected against toxins. According to **Bhatt and Nagar (2013)** and **Osuna-Martinez *et al.* (2014)**, the bioactive compounds (flavonoids, glycosides, phenols, terpenoids, saponins, and tannins) that are immunostimulants could be the reason for the improvement in tilapia haematological markers. Improved haematological indicators promote haemoglobin in the Nile tilapia-fed OLPC in the diet. The current study revealed that the bioactive compounds also explained this effect, in agreement with **Goda (2008)**. Numerous medicinal herbs contain secondary metabolites, which have been linked to immunee-modulating effects under adverse conditions **(Hoseinifar *et al.*, 2021; Radwan *et al.*, 2022a; Gehad *et al.*, 2023; Abbas *et al.*, 2024a-c; Radwan *et al.*, 2024c-d)**.

Table 4. Hematological indices of tilapia fed with OLCP groups

		OLCP levels			
		OLCP-0	OLCP-1	OLCP-2	OLCP-3
RBCs ($\times 10^6$ cell / mm^3)	Pre-Phase	2.18 ± 0.13^{bA}	2.31 ± 0.09^{aA}	2.34 ± 0.02^{aA}	2.36 ± 0.08^{aA}
	Post-Phase	1.97 ± 0.07^{dB}	2.11 ± 0.09^{cB}	2.21 ± 0.07^{bB}	2.29 ± 0.06^{aB}
WBCs ($\times 10^3$ cell / mm^3)	Pre-Phase	22.86 ± 0.28^{cB}	20.39 ± 0.71^{bB}	19.82 ± 0.24^{aB}	19.65 ± 0.31^{bB}
	Post-Phase	24.18 ± 0.53^{cA}	22.05 ± 0.62^{bA}	21.62 ± 0.68^{aA}	21.14 ± 0.56^{cA}
Hb (g/dl)	Pre-Phase	6.51 ± 0.14^{cA}	7.36 ± 0.14^{bA}	7.93 ± 0.08^{bA}	7.76 ± 0.20^{aA}
	Post-Phase	5.35 ± 0.44^{cB}	6.42 ± 0.04^{bB}	6.58 ± 0.18^{bB}	6.96 ± 0.09^{aB}
PCV (%)	Pre-Phase	22.73 ± 0.39^{cA}	24.13 ± 0.12^{bA}	23.84 ± 0.13^{bA}	24.98 ± 0.10^{aA}
	Post-Phase	19.91 ± 0.49^{cB}	21.65 ± 0.39^{bB}	22.00 ± 0.30^{bB}	23.04 ± 0.08^{aB}
MCV (fL)	Pre-Phase	104.27 ± 1.04^{dB}	104.46 ± 1.27^{cB}	101.88 ± 1.61^{bB}	105.85 ± 2.42^{aB}
	Post-Phase	101.07 ± 1.72^{dA}	102.61 ± 1.26^{cA}	99.55 ± 0.48^{bA}	100.61 ± 1.55^{aA}
MCH (pg)	Pre-Phase	29.86 ± 0.78^{cA}	31.86 ± 0.18^{cA}	33.89 ± 0.31^{bA}	32.88 ± 0.74^{aA}
	Post-Phase	27.16 ± 0.57^{dB}	30.43 ± 0.44^{cB}	29.77 ± 0.36^{bB}	30.39 ± 0.40^{aB}
MCHC (%)	Pre-Phase	28.64 ± 0.46^{Aa}	30.50 ± 0.27^{bA}	33.26 ± 0.33^{cA}	31.06 ± 0.06^{dA}
	Post-Phase	26.87 ± 0.60^{aB}	29.65 ± 0.37^{bB}	29.91 ± 0.39^{cB}	30.21 ± 0.27^{dB}

* Tables' data revealed significant variations in bars with different large letters in the same diets (T-test between Pre and Post-Phase, $P < 0.05$) and different small letters in the same phase (means±S.E., n = 5; ANOVA, $P < 0.05$).

Biochemical assay

After the toxin stress, changes in the biochemical investigations in tilapia blood and blood tests are a reliable and accurate method of assessing the condition of the species **(Shin *et al.*, 2016; Abdel-Aziz *et al.*, 2022; El-Gaar *et al.*, 2022; Abbas *et al.*, 2023, 2024; Elaraby *et al.*, 2024)**.

Table (5) reports the ALT, cortisol, AST, glucose, total protein, globulin, and albumin of tilapia studied groups. The biochemical indices of tilapia fed with various levels of OLCP revealed several significant trends. AST levels increased in all groups post-phase, with the OLCP-0 group showing the highest increase (164.95±0.53 U/L) compared to OLCP-3, which had the lowest (133.27±0.67 U/L). ALT also rose post-phase, with the OLCP-0 group again having the highest value (36.87±0.49 U/L) and the OLCP-3 group the lowest (24.84±0.34 U/L). Cortisol levels increased after the stress test across all groups, with OLCP-0 showing the highest levels (6.51±0.17ng/ ml) and OLCP-3 the lowest (3.11±0.11ng/ ml). Glucose levels significantly increased in all groups (post-phase), with OLCP-0 having the highest glucose levels (122.75±1.49mg/ dl) and OLCP-3 the lowest (71.64±0.36mg/ dl). For total protein, the OLCP-0 group had the highest pre-phase value (3.79±0.06g/ dl) and the highest post-phase value (4.51±0.24g/ dl), while the OLCP-3 group showed the lowest values in both pre-phase (2.41±0.09g/ dl) and post-phase (3.21±0.09g/ dl). Albumin levels increased post-phase in all groups, with OLCP-0 showing the highest levels both pre-phase (2.04±0.09g/ dl) and post-phase (2.69±0.09g/ dl), while OLCP-3 had the lowest pre-phase (1.34±0.09g/ dl) and post-phase (1.89±0.09g/ dl). Globulin levels increased post-phase in all groups, with OLCP-0 showing the highest pre-phase value (1.75g/ dl) and post-phase value (1.82±0.09g/ dl), and OLCP-3 the lowest pre-phase (1.07±0.09g/ dl) and post-phase (1.32±0.09g/ dl). These results suggested that OLCP levels affected biochemical markers and protein status in tilapia and the higher OLCP concentrations correlating with lower stress responses and better protein profiles.

All OLCP groups showed significantly greater levels of albumin, ALT, cortisol, AST, glucose, total protein, and globulin after exposure to ammonia. Compared to control samples (tilapia-fed non-OLCP-fortified diets), the levels of AST and ALT in the former were much lower. Following ammonia exposure, AST and ALT levels significantly rise in all fish diets, with the control diets displaying the highest values. Conversely, the activities of ALT and AST in tilapia-fed OLCP diets showed a decreased in escalation rate after exposure to ammonia compared to OLCP-0 diets (Table 5). One possible explanation for the degree of liver necrosis in tilapia subjected to ammonia stress is the ALAT activity. 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 inside the bloodstream.

On the other hand, lower blood levels of liver enzyme synthesis were due to the existence of phenolic and flavonoid groups, which have hepato-protective properties. According to earlier studies, exposure to ammonia causes harm to aquatic creatures' organs and increases their enzyme activity. **Lin *et al.* (2011)** made similar discoveries. Moreover, **Agrahari *et al.* (2007)** suggested the measurment of AST and ALT activities in fish to evaluate kidney and liver injury.

Additionally, Table (5) demonstrates that the tilapia-fed OLCP diets had considerably lower levels of cortisol and hyperglycemia than the control samples. The fish showed a significant increase in glucose and cortisol levels in all treatments after being exposed to ambient ammonia. Furthermore, tilapia fed OLCP diets exhibited a lower rate of glucose and cortisol escalation after ammonia exposure compared to the OLCP-0 group (control). Elevated blood cortisol levels in fish exposed to ammonia stress has been linked to physiological stress **(Sinha *et al.*, 2012; Shin *et al.*, 2016)**. Elevated glucose levels (hyperglycemia), can be caused by the activation of cortisol in response to stressful events, as stated by **Elbialy *et al.* (2021)**.

Table 5. Biochemical indices of tilapia fed with OLCP groups

		OLCP levels			
		OLCP-0	OLCP-1	OLCP-2	OLCP-3
AST (U/L)	Pre-Phase	145.45±0.57^{aB}	132.32±0.82^{aB}	131.90±0.24^{bB}	130.32±0.62^{bB}
	Post-Phase	164.95±0.53^{aA}	137.04±0.98^{bA}	136.75±0.66^{cA}	133.27±0.67^{dA}
ALT (U/L)	Pre-Phase	27.39±0.71^{aB}	22.79±0.49^{bB}	21.31±1.27^{bB}	20.40±0.23^{cB}
	Post-Phase	36.87±0.49^{aA}	26.46±0.65^{bA}	25.49±0.45^{cA}	24.84±0.34^{dA}
Cortisol	Pre-Phase	2.98±0.13^{bA}	2.36±0.08^{aA}	2.34±0.02^{aA}	2.31±0.09^{aA}
(ng/ml)	Post-Phase	6.51±0.17^{cA}	3.55±0. 25^{bA}	3.32±0.23^{bA}	3.11±0.11^{aA}
Glucose	Pre-Phase	76.50±0.48^{aB}	67.72±0.31^{bB}	62.62±0.35^{cB}	58.08±0.55^{cB}
(mg/dl)	Post-Phase	122.75±1.49^{aA}	93.98±1.00^{bA}	84.66±0.79^{cA}	71.64±0.36^{cA}
Total protein	Pre-Phase	3.79±0.06^{cB}	3.18±0.18^{bB}	2.87±0.02^{bB}	2.41±0.09^{aB}
(g/dl)	Post-Phase	4.51±0.24^{cA}	3.88±0. 12^{bA}	3.45±0.13^{bA}	3.21±0.09^{aA}
Albumin	Pre-Phase	2.04±0.09^{aB}	1.81±0.09^{aB}	1.69±0.09^{aB}	1.34±0.09^{aB}
(g/dl)	Post-Phase	2.69±0.09^{aA}	2.26±0.09^{aA}	2.05±0.09^{aA}	1.89±0.09^{aA}
Globulin	Pre-Phase	1.75±0.09^{aB}	1.37±0.09^{aB}	1.18±0.09^{aB}	1.07±0.09^{aB}
(g/dl)	Post-Phase	1.82±0.09^{aA}	1.63±0.09^{aA}	1.40±0.09^{aA}	1.32±0.09^{aA}

* Tables' data revealed significant variations in bars with different large letters in the same diets (T-test between Pre and Post-Phase, $P < 0.05$) and different small letters in the same phase (means±S.E., n = 5; ANOVA, $P < 0.05$).

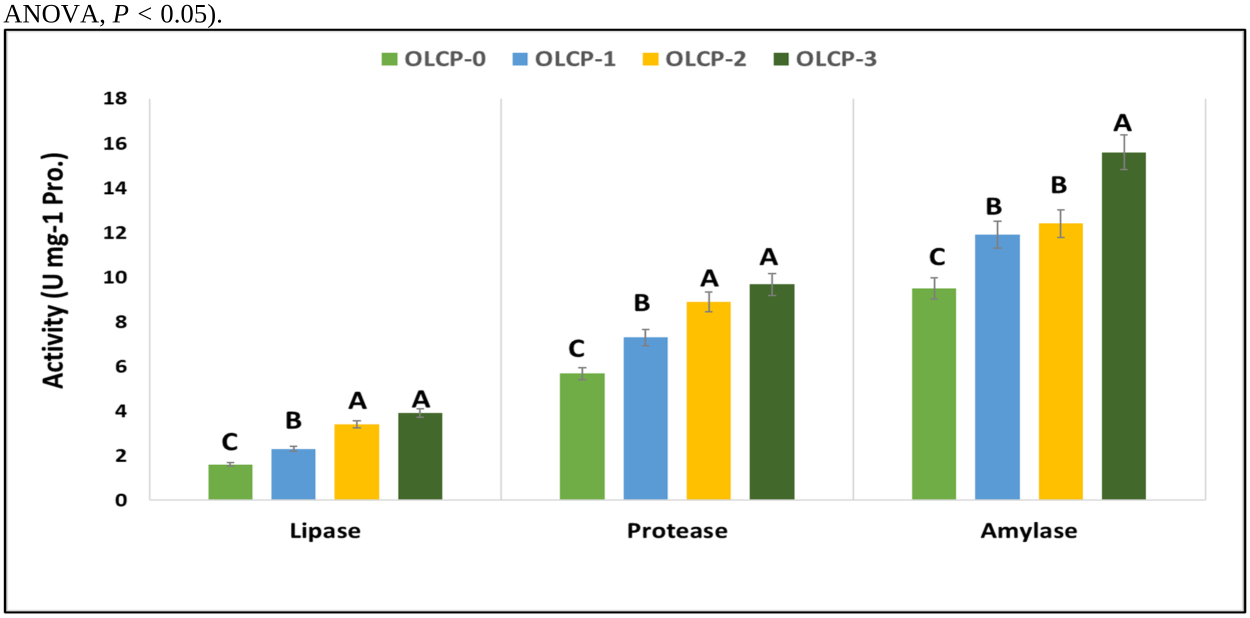

Fig. 1. Activities of digestive enzymes in tilapia fed with OLCP groups. Figures' data revealed significant variations in bars with different letters in the same treatments (means±S.E., n = 5; ANOVA, $P < 0.05$)

In contrast, the decline in levels of glucose observed in the studied groups under both phases in comparison to the control group may be explained by the bioactive components (phenols, flavonoids, and saponins) of *O. littoralis* mediating its hypoglycemic action. Flavonoid and phenolic substances are widely recognized for their antidiabetic properties, enabling them to effectively reduce blood glucose levels **(Parwata *et al.*, 2018)**. Moreover, a group of phytochemicals known as saponins has various biological effects, such as lowering blood sugar and preventing the enzymes from converting disaccharides into simple sugars **(Oishi *et al.*, 2007)**.

The level of protein in the blood of aquatic animals may be a sign of their overall well-being **(Ngugi *et al.*, 2017)**. Compared to the OLCP-0 group (control samples, $P < 0.05$), all OLCP-fortified treatments significantly reduced albumin, total protein, and globulin levels. Nonetheless, albumin, total protein, and globulin contents were significantley increased in tilapia fed on OLCP-fortified diets, while exposure to ambient ammonia caused the most significant amounts in the OLCP-0 group (Table 5). Likewise, fish diets that include immune stimulants increase total protein levels in their blood, which links to an innate immune response **(Rudneva & Koverchina, 2011)**. OLCP diets increase tilapia fish's innate immunity, as evidenced by the significantly higher total protein levels in fish fed OLCP diets during the investigation's pre- and post-phases. These findings align with **Goda (2008)** and **Sonmez *et al.* (2015)**, whom found that the fish had greater globulin and total protein levels when herbal plants were added to fish diets. Following the ammonia challenge, all OLCP-fortified meals significantly decreased in all biochemical indicators (albumin, globulin, glucose, total protein, cortisol, and ALT). However, the OLCP-3 groups (which accounted for 2% of OLCP) had the highest effectiveness. Also, **Harikrishnan *et al.* (2011)**, **Gehad *et al.* (2023)**, and **Radwan *et al.* (2024c-d)** stated that incorporating medicine plants into the diet reduced the rise in ammonia-induced levels of various biochemical variables, including AST, cortisol, ALT, total protein, glucose, globulin, and albumin.

Antioxidant assay

The antioxidant indices of tilapia fed with different levels of OLCP showed notable variations (Fig. 2). Before the ammonia stress test, the OLCP-3 group exhibited the highest GPX (38.84±1.02 IU/L), CAT (36.44±1.13 IU/L), and SOD (13.92±0.79 IU/L) levels. In contrast, the OLCP-0 group had the lowest values for GPX, CAT, and SOD (23.14±1.31, 28.19±1.15, and 11.24±1.08, respectively). Conversely, the highest MDA value (19.10±0.42 IU/L) was recorded in the OLCP-0 group, and the lowest values (13.54±0.92 IU/L) were recorded in the OLCP-3 group. After the ammonia stress, the OLCP-3 group exhibited the highest GPX (58.93±1.87 IU/L), CAT (46.52±1.85 IU/L), and SOD (16.44±1.23 IU/L) levels, while the OLCP-0 group had the lowest values for GPX, CAT, and SOD (25.54±2.07, 26.72±1.44, and 12.21±0.77, respectively). Conversely, the highest MDA value (22.17±0.19 IU/L) was recorded in the OLCP-0 group, and the lowest values (15.12±1.03 IU/L) were recorded in the OLCP-3 group. Overall, while there were significant differences in these antioxidant indices across different OLCP levels, the relative rankings of the groups remained consistent before and after the ammonia stress experiment. The findings were associated with prickly pear extract's flavonoids and phenolics, which improved immunological reactions, decreased lipidic superoxide injury, promoted the antioxidant state, and inhibited the generation of free radicals **(Daniloski *et al.*, 2022)**. Similarly, **Ahmed *et al.* (2020)** showed that the strong antioxidant content of prickly extract can improve the body's antioxidant status and stop lowering the peroxidation of lipids Moreover, **Abbas *et al.* (2024)** noted that Prickly pear plants contain flavonoids, which work as antioxidants and stop free radicals volatile, reactive chemicals—from harming healthy cells. Furthermore, **Ben Saad *et al.* (2017)** found that prickly pear extract administration significantly reduced oxidative lithium-mediated rat model damage. This finding results from the antioxidant activity of the extract's active ingredients, which include polysaccharides, flavonoids, and phenolics. Owing to the presence of polyphenolic chemicals, vitamins C and E, β-carotene, and total carotenoids, prickly pears showed to have antioxidant activities both *in vivo* and *in vitro*, protecting the body from oxidative stress, as reported by **Avila-Nava *et al.* (2014)**. Similarly, these findings are in agreement with **Rodriguez-Mateos *et al.* (2014)**, **Mata *et al.* (2016)**, **Andreu *et al.* (2018)** and **Berrabah *et al.* (2019)**, who revealed that the bioactive compounds in OLCP are tannins, flavonoids, terpenoids, and phenols, which are essential for their capacity to function as antioxidants and as lipid peroxidation. Moreover, according to **Ramadan and Mörsel (2003)** and **Yahia and Mondragon-Jacobo (2011)**, the prickly showed to be capable of providing protection and antioxidant properties through a range of substances, including phenolic compounds, vitamins, and other non-nutritional substances. Prickly pears' phenolic components have antioxidant agents due to the separation of the primary flavonoids **(Saih *et al.*, 2017; Mahrose, 2021)**.

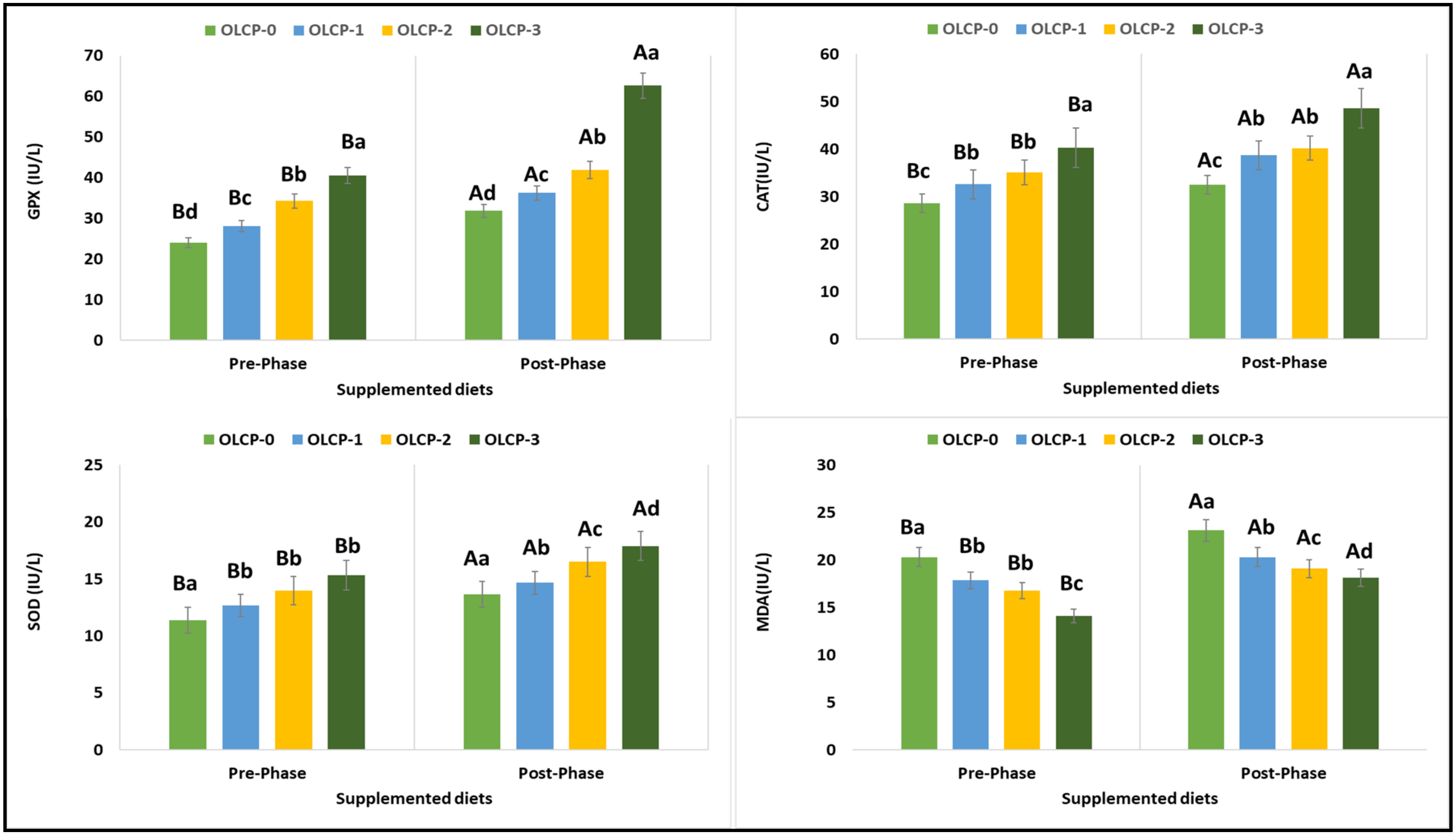

Fig. 2. Variations in SOD (IU/L), CAT (IU/L), GPX (IU/L), and MDA (IU/L) activities of tilapia fed with OLCP groups. The figures' data revealed significant variations in bars with different large letters in the same diets (T-test between Pre- and Post-Phase, $P < 0.05$) and different small letters in the same phase (means±S.E., n = 5; ANOVA, $P < 0.05$).

Immunological assay

Lysozyme and complement C3 activity are innate immune responses that regulate the body's general immunity against infection or stress **(Radwan *et al.*, 2023b)**. The immunological status of aquatic organisms can be assessed by measuring their blood Ig level, even though Ig is a part of the adaptive immune system **(Yousefi *et al.*, 2023)**. Moreover, the supplementing fish diets with herbal plants may increase the lysozyme total Ig and complement C3 activities, boosting fish's resistance to subsequent stressors **(Taheri Mirghaed *et al.*, 2019; Dawood *et al.*, 2021)**.

The immunological characteristics of tilapia under ammonia stress in both the pre- and post-phase are represented in Fig. (3). Following ammonia exposure, the tilapia groups showed a decrease in the activities of total Ig, complement C3, and lysozyme. In tilapia fish during the pre-phase, the lysozyme, total Ig, and complement C3, activities were increased through OLCP groups, suggesting that OLCP has immunostimulant qualities. Following exposure to ammonia, the activites of Ig, complement C3, and lysozyme were both dramatically decreased; the control group observed the least degree of this loss. Compared to the control one, they were much higher in all OLCP groups ($P < 0.05$). Therefore, the current investigation stated that the OLCP diets promoted the immune system of *O. niloticus*, as evidenced by the activities of immune responses being elevated. Consistent with the present findings, tilapia exposed to ammonia exhibits immunosuppressive biomarkers **(Xu *et al.*, 2021)**. Furthermore, the fish benefited from the OLCP diets because they reduced the immunosuppression caused by exposure to ammonia stress. On the other hand, dietary phytochemicals may have reduced the negative effects of ammonia stress on fish species' complement C3 lysozyme and total Ig activity **(Adineh *et al.*, 2021; Abdel-Tawwab *et al.*, 2022; Yousefi *et al.*, 2023)**.

The results corroborate the theory that plant extracts can improve the immune systems of fish farms. Numerous bioactive substances (phenolics, alkaloids, terpenoids, pigments, and steroids) activated the different biological reactions (phagocytic activation, complement system, anti-stress responses, and immunostimulant) in cultivated organisms **(Chakraborty *et al.*, 2014; Radwan *et al.*, 2022b; Abbas *et al.*, 2024)**. According to **Liu *et al.* (2021)**, **Esam *et al.* (2022)**, and **Guo *et al.* (2023)**, various environmental contaminants impact the levels of complement C3, IgM, and lysozyme activities. These changes can potentially influence the immunological properties of fish species. In the present investigation, lysozyme, complement C3, and total Ig in activities the studied groups were reduced, followed by ammonia stress, suggesting a chronic decline in immunological function. Furthermore, **Gao *et al.* (2022)** showed a significant decrease in lysozyme activities, complement C3, and IgM of *Takifugu rubripes* fish after ammonia stress

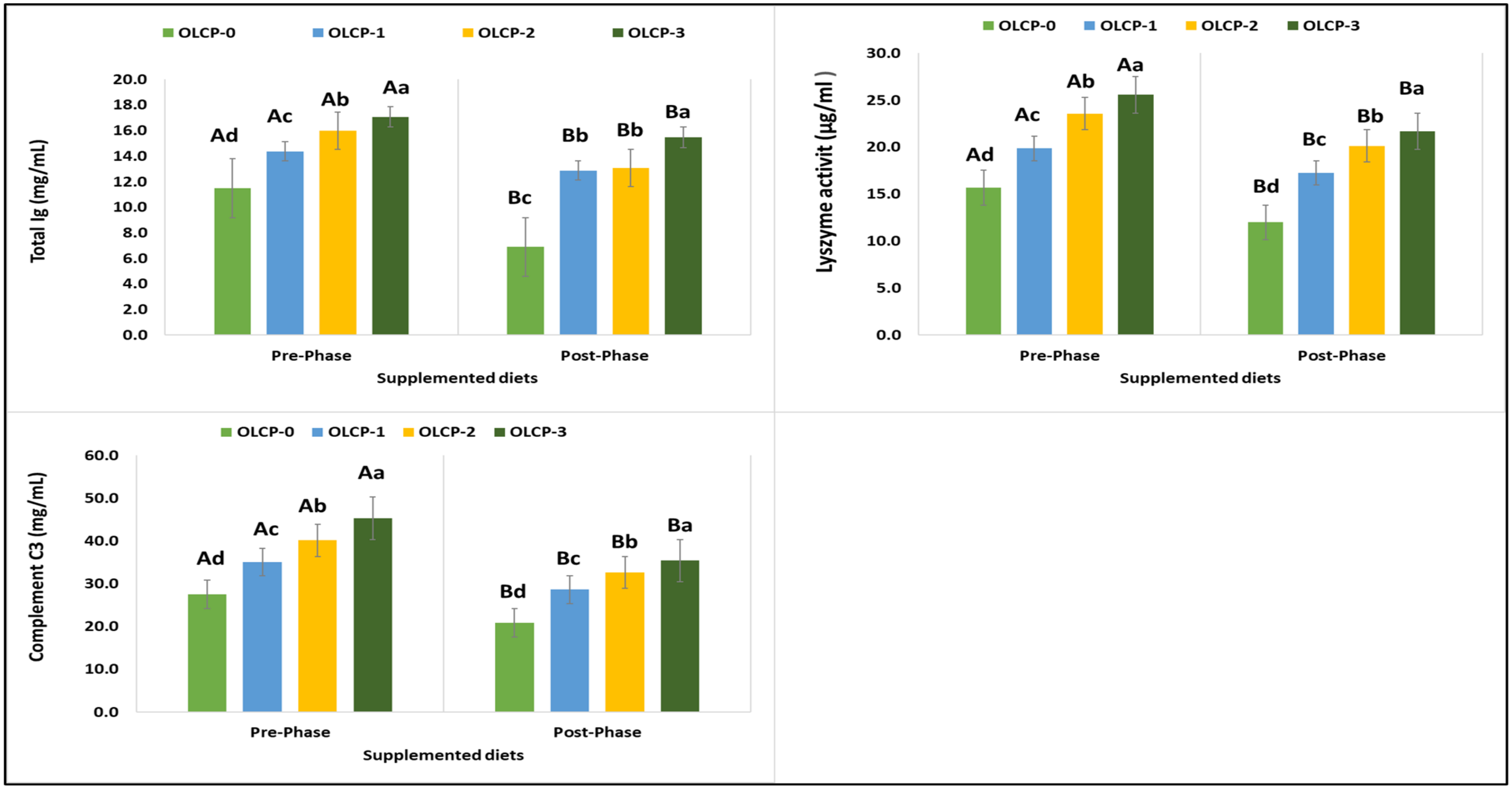

Fig. 3. Variations in lysozyme, complement C3, and total Ig activities of tilapia fed with OLCP groups. The data in the Figs. revealed significant variations in bars with different large letters in the same diets (T-test between Pre- and Post-Phase, $P < 0.05$) and different small letters in the same phase (means±S.E., n = 5; ANOVA, $P < 0.05$)

CONCLUSION

The findings comprehensively show how dietary *Opuntia littoralis* cladodes protect tilapia fish against ammonia-induced damage. The exposure of tilapia fish to ammonia led to a significant decrease in hemato-biochemical indices and immune and antioxidant markers depending on the level of *O. littoralis* cladodes in tilapia diets. The study's results suggest that tilapia diets supplemented with varying concentrations of OLCP may improve growth, stress resistance, and feed utilization. Increased levels of OLCP are associated with activating the immune system and antioxidants. In the current study, tilapia (*Oreochromis niloticus*) fed up to 2% OLCP showed the best outcomes across all metrics. The research suggests that adding *O. littoralis* cladodes to fish diets promotes sustainable aquaculture by mitigating the negative impacts of ammonia exposure in an environmentally responsible manner.

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