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Effect of Bioenriched blood worm (*Chironomus transvaalensis)* with sea weed (*Kappaphycus alvarezii)* diet on growth performance digestive enzymes, Immunological assays and challenge study against *Aeromonas hydrophila* in Tilapia, *Oreochromis mossambicus (Peters1852)*

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Abstract

The current study demonstrates a novel feeding trial employing O. mossambicus as a model organism by bio-encapsulating C. transvaalensis with K. alvarezii to enhance health status and growth, to prevent and manage the negative effects of A. hydrophila infection. A total of 150 Oreochromis mossambicus were taken randomly and divided into five experimental units (10 fishes per unit) with triplicates.Fish fed with C. transvaalensis which were bio-encapsulated with K.alvarezii was designated as (CTKA) individually. The Experimental Units were T_0-(1gm of CT), T_1-(1gm of CTKA for 24hr), T_2-(1gm of CTKA for 48hr) and T_3-(1gm of CTKA for 72hr) per 1 liter of water individually. After bio-encapsulation the live larvae were freeze dried for the purpose of removing moisture content and for long time storage. Results was a consistent improvement in the total WBC and RBC count, total serum protein, and phagocytic activity during the course of the 75th day of the investigation.The growth parameters were measured on day 30 and day 60.Digestive enzymes activity,liver specific enzymes activity,liver antioxidant activity were investigated after 60 days feeding trial. All these enzymes were positively performed in all experimental diet, while T3 experimental diet significantly enhanced all enzymes activities.*As part of the post-challenge investigation* (*disease resistance test*)*, the rate of survival and percentage of mortality of the fish were tracked and recorded after being exposed to A. hydrophila for 15 days*. *Therefore, fish farmers may be encouraged to do this to enhance the health status, boostup growth, and prevent microbial infections in the freshwater fish O. mossambicus by feeding (CTKA) for 72hrs.*

Keywords: *Oreochromis Mossambicus*, Bio-Encapsulation, *Aeromonas Hydrophila, Kappaphycus Alvarezii*, *C. Transvaalensis*, Digestive Enzymes, Antioxidant Activity.

INTRODUCTION

Aquaculture is the sector of food production that is expanding at the quickest rate in the globe with a total production of 82.1 million tonnes (Rocha, et al. 2022). Tilapia *Oreochromis mossambicus* is the second most widely raised aquatic animal due to its high nutritional value and characteristics that make it resistant to disease(Suresh, et al. 2023). The overall output of tilapia in the world in 2018 was

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6.06 million tonnes, which contributed around 12% to the production of inland aquaculture as a whole 51.3 million tonnes (Suresh, et al. 2023). According to estimates, tilapia production will reach 7.3 million tonnes globally in 2030. A species of tilapia has grown in popularity and is now about 4.53 million tonnes. China, Indonesia, and Egypt are the top three producers of *Oreochromis mossambicus* (Moses, et al. 2021). In many impoverished nations, it serves as the primary source of high-quality, inexpensive protein.

A heterotrophic, free-living, motile, Gram-negative bacteria called *Aeromonas hydrophila* is typically found in freshwater and sporadically in marine waters (Semwal, et al. 2023). Additionally, this organism may be found in warm climates, saltwater, estuaries, chlorinated and unchlorinated waters, and aerobic and anaerobic conditions(Schwake, et al. 2021). Although *A. hydrophila* is a normal component of the microbial ecology of freshwater fish, it is an opportunist pathogen that can change from a commensal to a pathogenic form when stressed(Sherif and Kassab 2023). *A. hydrophila* has historically been thought to be the culprit behind fish diseases such red sore disease, fin/tail rot, skin ulceration, and *Hemorrhagic septicemia* caused by mobile *Aeromonas* (AlYahya, et al. 2018). Fish producers suffer enormous losses as a result of aquaculture's big problem with bacterial infections in particular (Irshath, et al. 2023).There is an increasing need to do in-depth studies on fish to improve their health state to treat or prevent various types of bacterial illnesses in light of the current growth of intensive aquaculture practices, by providing superior immune stimulation in the management of aquatic animal health, biological matrices-mediated encapsulation of conventional natural extracts improves the feed quality through the sustained release of antioxidant and antibacterial properties over extended periods (Alagawany, et al. 2021). The application of phytonutrients in aquaculture to manage and prevent microbe-associated disorders to increase fish immunity and fitness for avoiding pathogenic infections has been tried to harmonize apparent patterns(Yu, et al. 2023). When *P. indicus* juveniles were fed a seaweed, diet supplemented with artemia, the number of bacteria in several organs was reduced(Jahromi, et al. 2021). The bioactive ingredients of supplements that are accessible in encapsulated form rather than the Free State can also be easily digested by fish, which may have an impact on the growth and immunity of aquatic organisms(Jahromi, et al. 2021). Because of the aforementioned factors, bio-encapsulated live feed is regarded as an environmentally acceptable aquaculture management technique that can be used as a successful substitute to reduce microbial infections in aquatic species(Kauppinen, et al. 2021). A complementary approach to synthetic medications, hormones, probiotics, and antibiotics that are sold commercially is phytotherapy.

Carrageenan, a powerful bioactive substance utilized as an emulsifier in the food industry, is a soluble fiber found in red seaweed called *K. alvarezii*(Rupert, et al. 2022). Along with carrageenan, *K. alvarezii* also has numerous vital minerals like potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), copper (Cu), and zinc (Zn), as well as the antioxidants phycoerythrin and phycocyanin(Wanyonyi, et al. 2017). In addition, it should be noted that the bioactive elements of seaweed effectively interact with the membranes and cells of harmful microbes through a sensing mechanism(Lomartire and Gonçalves 2022). On the other hand, bloodworms, or Chironomus larvae, are a naturally occurring live food source for freshwater fish that contain essential nutrients(Sharifian Fard, et al. 2014). More effectively than that designed feed, their dynamic movements in the water entice the fish to chase and feed. Thus, a unique approach was started in the feeding trail for freshwater fish *Oreochromis mossambicus* fingerlings in the current study. These fish were fed with live a feed Chironomus larvae that was bio-encapsulated with *K. alvarezii* (*Rhodophyta*-red seaweed) at varied concentrations. And then larvae were freeze dried for the purpose of removing moisture content and for long time storageto improve growth and health, to guard against and control the effects of an *A. hydrophila*

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infection**.** Additionally, the fish farmers may be urged to follow these study guidelines to enhance their health condition, stimulate growth, and guard against microbial diseases**.**

MATERIALS AND METHODS

Collection of live food and maintenance

The *Chironomus transvaalensis* were collected from the hobby collection aquariumnear Alagappa University,Tamil Nadu, India.The presence of hemoglobin gives chironomuslarvae the appearance of red color due to iron content. So It is commonly called as blood worm. Glass vessels were used for storing these live specimens. The larvae were collected with a scoopnet and washed thoroughly before feeding. The quality of the water was checked regularly to ensure its survival. Larvae were devoid of feeding before one day to startthe bio-encapsulation procedure. To eliminate *allochthonous* bacteriathey were treated with abroad spectrum of antibiotics Kanamycinat a concentration of 1µg/mL for 5hr to eliminate *allochthonous* bacteria. Water temperature 26.68±3.12 (ºC), 7.46±0.27 pH, electrical conductivity 176.33±10. 83 (EC, µScm-1) and total dissolved solids 83.22±2.77 (TDS, mg L-1) were measured with a pH/EC/TDS meter (Fisher Scientific model HI 98108), and dissolved oxygen 5.93±0.95 (DO, mgL-1) was evaluated in manual Turbidity 13.89±6.33 (FAU), suspended solids 9.22±2.17 (SS, mgL-1) and orthophosphate 0.08±0.05 (PO_4-3, mgL-1) were measured using a spectrophotometer. Chlorophyll-a (µgL-1) was measured using a methanol extraction method (APHA 1998). Three replicates of each parameter were measured. (Sriariyanuwath Em on, et al. 2015).

Collection of seaweed and maintenance

The seaweed *K. alvarezii* was collected from the seashore Thondi, Ramanathaburam District Tamil Nadu, India. Epiphytes and other debris were removed from algae samples, and necrotic parts were also eliminated from the collected seaweeds after thorough washing with seawater followed by fresh water. They were then placed in sterile plastic bags and brought into the laboratory and then washed with sterile distilled water before being shade dried. They were cut into small pieces and powdered in a lab mixer grinder. The powdered samples were then placed in a freezer for -20˚C further studies (Mei Ling et.al 2019)

Phytochemical analysis of *Kappaphycus alvarezii***.**

10 g of air dried powder of *K.alvarezii* was extracted with 60mL of methanol. The sample was stored and cool place in dark room for 72 h with intermittent shaking. The solvent was evaporated completely by using Rotary vacuum evaporator. The resulted extracts were tested for steroids, alkaloids, phenolic compounds, saponins, flavonoids and quinones etc Phytochemical screening of the extracts was carried out according to the standard method described by (Harborne., 1998) with minor modifications.

Collection and maintenance of experimental fish

The freshwater fish *O. mossambicus* having an average weight of 28±2.0g and average length of 7±2.0cm were obtained from a commercial fish farm and were instantly analyzed for their state of health. Glass aquaria employed to rear fish were treated with 1% $KMnO_4$ solution and sun-dried to prevent microbial contamination. Healthy fishes were chosen after screening and transferred into the aquarium tank (36×18×24cm)containing de-chlorinated tap water (Temperature 28 ± 2^0C; total hardness 525 ± 31 mg/L; salinity 1.12 ± 0.18 ppt and p^H 7.6 ± 0.05) (Assefa, Ayalew, and Fufa Abunna 2018). Fish were acclimatized to the laboratory conditions for 10 to 15 days before experimentation. The water was changed regularly to remove feces and food remnants for preventing ammonia built.

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Bio-encapsulation of (CTKA)

One gm of live *C. transvaalensis* was bio-encapsulated with 1gm of *K. alvarezii*(CTKA)by placing them in one liter of water in four different one-liter glass beakers labeled as T_1, T_2, T_3,and T_4. They represented varying hours of bio-encapsulation such as 24hr, 48hr, 72hr, and 96hr respectively. The Experimental group T_0were fed with *C.transvaalensis* without any bio-encapsulation.And then larvae were freeze dried for the purpose of removing moisture content and for long time storage they were allowed to bio-encapsulate properly at different hours as mentioned above and this procedure was repeated for 75thday. Each bio-encapsulation test was performed in triplicate.

Experimental design

Fish were categorized totally into five groups as control and four treated groups with 10 fish in each group. All groups were maintained in triplicate. The four experimental groups (T_1, T_2, T_3, and T_4) were fed with (CTKA) at different hours (24hr, 48hr, 72hr, and 96hr). And then larvae were freeze dried for the purpose of removing moisture content and for long time storage.

(i) Control Group -T_0-Fish fed with (CT).

(ii) Treated Group-T_1- Fish fed with (CTKA)

(iii) Treated Group-T_2- Fish fed with (CTKA)

(iv) Treated Group -T_3-Fish fed with (CTKA)

(v) Treated Group -T_4-Fish fed with (CTKA)

The fish at 2% of the body weight were fed with the *C. transvaalensis*larvae by the setup design, twice a day at 09:00 and 17.00, until the end of the experiment. Unfed feed was collected with care regularly for the calculation of the feed conversion ratio. During the experimental period, water temperature 28±2ºC, p^H 7.1 ± 1.44, salinity 0.25 ± 0.05 ppt, dissolved oxygen concentration 6.63 ± 7.78 mg/L, and photoperiod 14L:10Dwere maintained. Blood was collected randomly from five fish from each group for every 15th day to determine the non-specific and specific immunological parameters.

Blood Collection and serum separation

Blood samples were drawn from the common cardinal vein from randomly chosen five fishes from each group by using a 2.5 mL smaller 24-gauge needle after anesthetizing with MS222 (Sigma Aldrich, St. Louis, MO, U.S.A) at the end of every 15thday up to 75th day before 8.00AM during the experimental period. The blood was stored in EDTA coated tube at -4^{o}C. The blood was collected and subjected to centrifugation and stored in an Eppendorf tube at -4^{o}C for 24 hrs following the protocol (Dagur and McCoy 2015) for serum preparation. Succeeding the overnight storage, the blood was allowed to clot for 2hr at room temperature.

Non-specificImmune Responses

Total WBC &RBC Count

The blood was drawn from the common cardinal vein of fish *O. mossambicus*. Collected 0.2ml of blood was used for the estimation of total WBC & RBC count**.** The cells were counted in the Neubauer Counting chamber of the Haemocytometer under 400 x magnifications following the protocol(Zhang, et al. 2020) .

Number of WBC cells (cu.mm^{-1}) =

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Total Serum Protein (g/dl)

Estimation of total serum protein followed the protocol with some modifications by employing the Folin-Ciocalteau reagent method(Lovrien and Matulis 1995).

Phagocytic Activity (%)

The phagocytic activity of the leukocytes was analyzed following the procedure (Gupta-Wright, et al. 2017).

Specific immune response

Challenge study with *Aeromonas hydrophila*

Preparation of *A. hydrophilia* for challenge study

A. hydrophila (ATCC 7966) was cultured in nutrient broth (Himedia) for 24 hr at 37 ºC. The culture broth was centrifuged at 3000 g for 10 min at 4ºC. The supernatant was discarded and the pellet was suspended in phosphate-buffered saline (PBS, pH 7.4) for the challenge test. The optical density (OD: 456 nm) of the bacterial suspension was adjusted to 0.5 and kept in a water bath at 60ºC for 2 hr. This bacterial suspension (10^7 CFU) was used for the challenge test. A stock culture in nutrient broth was stored at -86 ºC in 0.85% (w/v) NaCl with 20% glycerol (v/v) to provide stable inoculation throughout the study period(Russell and Gildenhuys 2020).

Challenge Study

A disease resistance test was performed in the four experimental groups including control after the 75thday of treatment. Ten fish alone taken from each treated group and challenge study was conducted. Each experimental group was injected with the bacterial inoculum at a rate of 10^7CFU/mL intramuscularly in their abdominal region. During the challenge study, survival and mortalities were observed and recorded in all groups for 15 days. The relative percentage of survival (RPS) of the fish was calculated using the following formula (Torres-Corral, et al. 2021).

$$\text{RPS} - 1 \quad \frac{\text{Percentage of mortality in the treated group}}{\text{Percentage of mortality in the control group}} \times 100$$

Growth Parameters.

Assessment of growth-related parameters

The growth performance of fish and feed utilization parameters were recorded and calculated as described below following the protocol (Syed, et al. 2022).A digital scale (Sartorius Digital Gram Scale TE1502S) with 0.1 g precision was used to measure fish weight. Growth parameters, weight gain, specific growth rate, feed conversion ratio, and survival rate were then calculated through the following equations; and mortalities (if any) were recorded throughout the trial to compare the treatment survival rates.

Weight gain (g) = final weight (g) – initial weight (g)

Specific growth rate (SGR) = [ln (final weight) - ln (initial weight)/days] × 100

Feed conversion rate (FCR) = Feed intake (g)/Weight gain (g)

Survival rate = [Number of survived fish/initial number of fish] × 100

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Digestive Enzymes.

Digestive tract samples were homogenized in 25mM Tris-HCl buffer at pH7.2 and centrifuged at 25,000 g for 20 minutes. The resulting supernatants were then collected, and lipase, protease, and amylase activities were determined (Yousef and Ghafarifarsani et al., 2021).

Liver-Specific Enzymes.

Liver-specific enzymes activity, consisting of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST), was assessed based on the method designed by (Peyghan and Takamy, 2002)

Liver Antioxidant Parameters.

The function of glutathione reductase (GR), superoxide dismutase (SOD), and catalase

(CAT) activity, as well as the function of glutathione peroxidase (GPx) (where glutathione is converted to glutathione disulfide), was measured through the use of commercial equipment (Zellbio®, Berlin, Germany). Malondialdehyde (MDA) concentrations were also measured using thiobarbituric acid, based on the calorimetric method defined by Buege and Aust, 1978.

Statistical analysis

The effects of bio-encapsulated *C. transvaalensis* with *K. alvarezii* at four different treated groups were plotted using one-way analysis of variance (ANOVA) using SPSS (version 16 for Windows) software. Mean values were considered significantly different when $P < 0.05$.

RESULTS

Table 1: Primary phytochemical screening of *K. alvarezii* Methanol extract

S. No	Compound	Methanol extract
1	Carbohydrate	++
2	Saponins	+
3	Oils and Fats	+
4	Terpenoids	+
5	Alkaloids	+
6	Steroids and Sterols	-
7	Flavonoids	+
8	Tannins and phenolics compounds	+
9	Amino acids	++
10	Quinones	-
11	Protein	++
12	Sugar	-
13	Reducing sugar	-

+ = Presence - = Absence

Important phytochemicals, such as alkaloids, terpenoids, steroids, tannin, saponin, quinone, phytosterols, phenolic compound and flavonoids were screened for their presence and presented in Table 1

Total WBC count

Estimation of the total WBC count in fish-fed with (CTKA) at four different times (24hr, 48hr, 72hr, and 96hr) showed a **Fig 1 a-e**. The count significantly increased in the experimental groups compared to

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the control group, which had 9.5 ±0.05 (10^3x mm^3). When compared to the other experimental period, the WBC count significantly increased on day 60 (**Fig.1d**). Additionally, in comparison to the other experimental groups, the treated (T_3) group fed (CTKA) for 72hr has recorded highest count of 22.2±1.0 (10^3x mm^3).

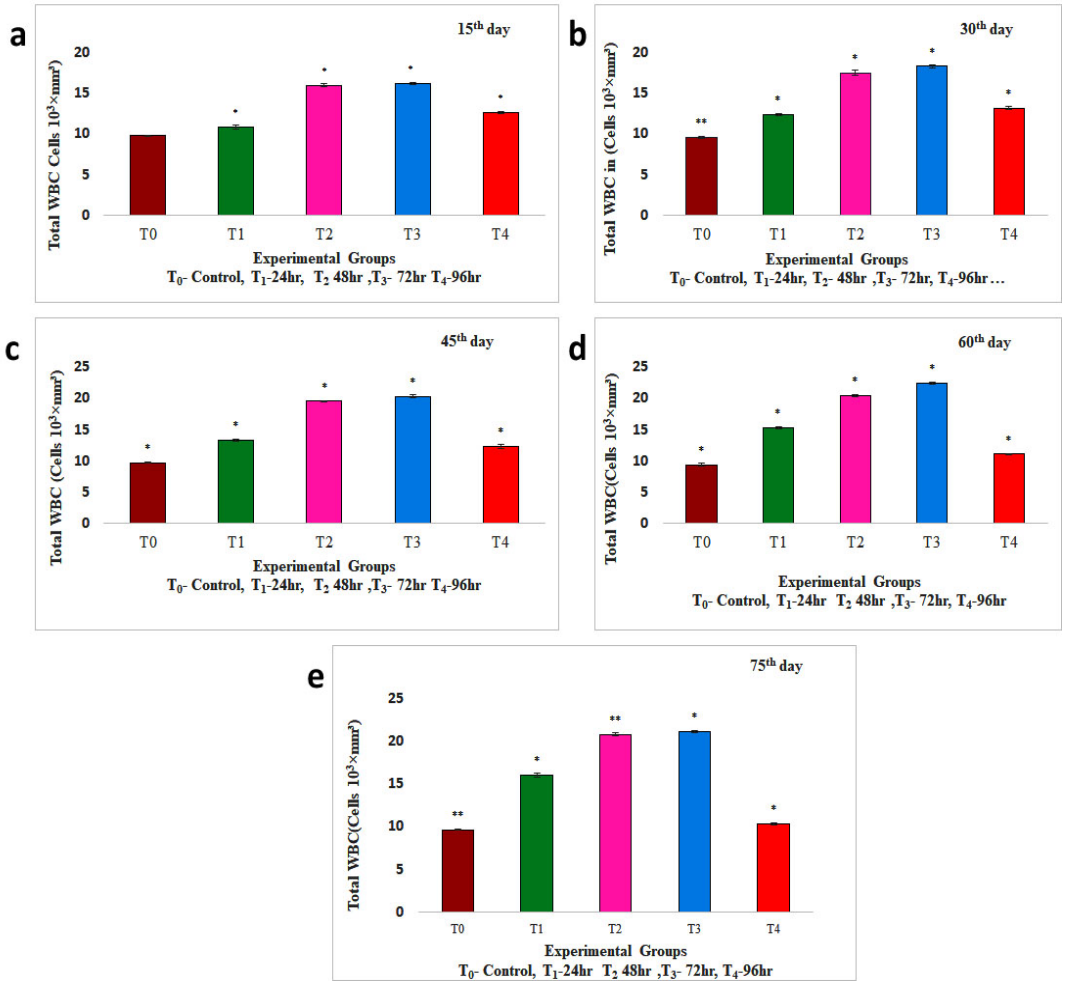


Figure 1: Effect of Total WBC Count (Cells 10^3×mm^3) in *O. mossambicus* fed with (CTKA) after (a) 15th day, (b) 30th day, (c) 45th day, (d) 60th day and (e) 75th day. Data are presented as mean ± stranded error (SE) of three replicates (n = 10). Within treatment groups, values with different letters are significantly different (*P* <0.05) as per one-way ANOVA followed by Tukey's HSD test.

Total RBC count

Estimates of the total RBC count in fish-fed (CTKA) at four distinct time points (T_1-24hr, T_2-48hr, T_3-72hr, and T_4-96hr) showed in **Fig 2 a-e**. All experimental groups greatly outnumbered the control group in terms of RBC count. Comparing the 60th day of the experimental phase to other experimental days, a stunning increase in the count was seen in (**Fig. 2d**). Further(CTKA) for T_3-72hr. significantly increased total RBC count from 2.4±0.1 (10^6x mm^3) in control (T_0) to 8.4±0.05 (10^6x mm^3) in experimental (T_3-72hr) group at the 60th day in comparison to fish fed with (CTKA) for T_1-24hr, T_2-48hr, and T_4-96hr.

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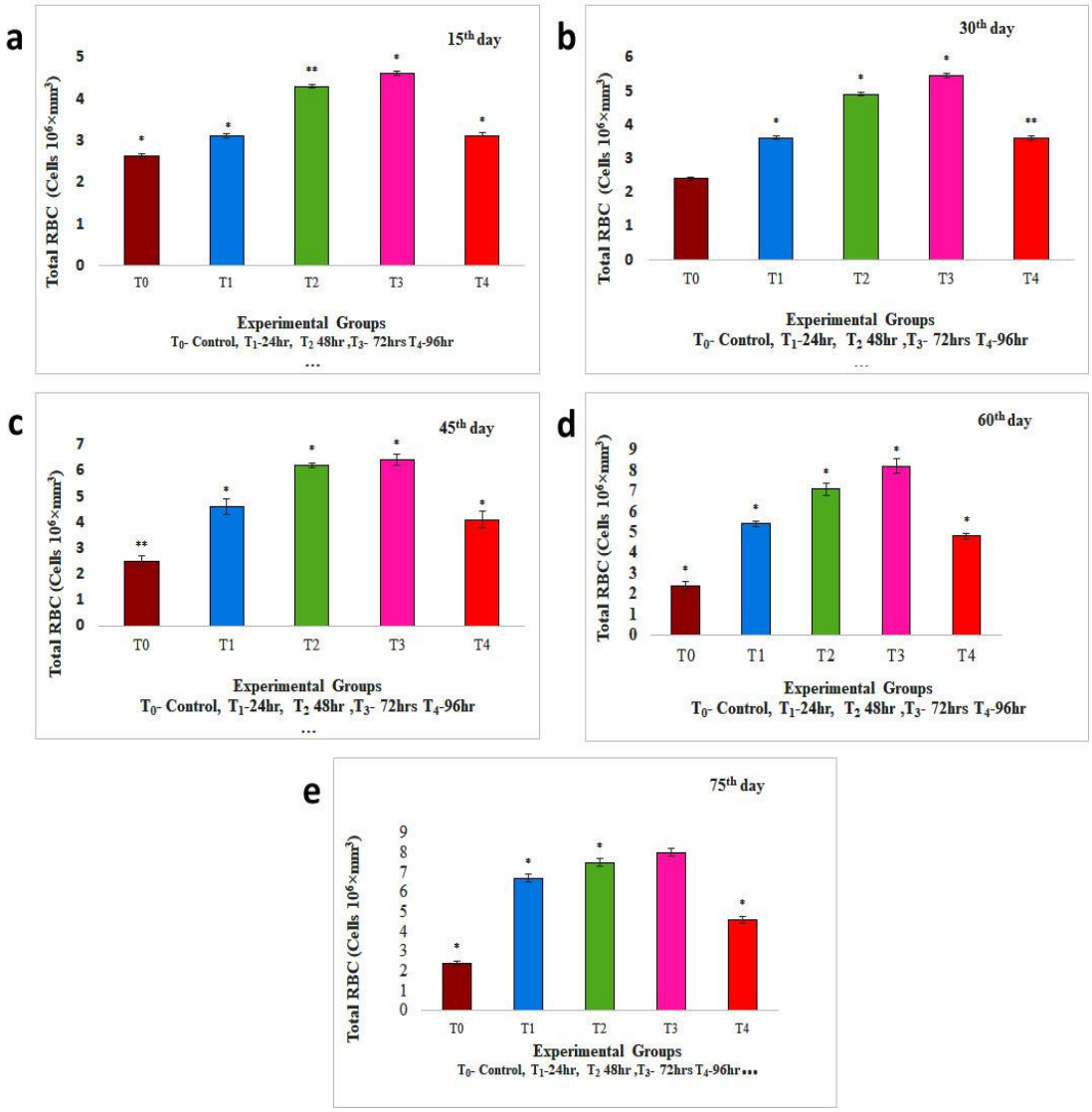


Figure 2: Effect of total RBC Count (Cells 10^6×mm^3) in *O. mossambicus* fed with (CLKA) for (a) 15th day, (b) 30th day, (c) 45th day, (d) 60th day, and (e) 75th day. Data are presented as mean ± stranded error (SE) of three replicates (n = 10). Within treatment groups, values with different letters are significantly different (*P* < 0.05) as per one-way ANOVA followed by Tukey's test.

Total Serum Protein

An estimate of the total protein content of the serum in fish fed (CTKA) at four different time points (T_1-24hr, T_2-48hr, T_3-72hr, and T_4-96hr) showed in **Fig 3 a-e**. All experimental groups significantly increased their protein level as compared to the control (0.74±0.01 (g/dl). In comparison to earlier experimental days, the total protein content increased noticeably on the 60th day (**Fig. 3d**). In addition, the experimental (T_3-72hr) group fed with bio-encapsulated (CTKA) during T_3-72hr at the 60th day showed the highest protein content of 2.9±0.05 (g/dl) in contrast to the other experimental groups.

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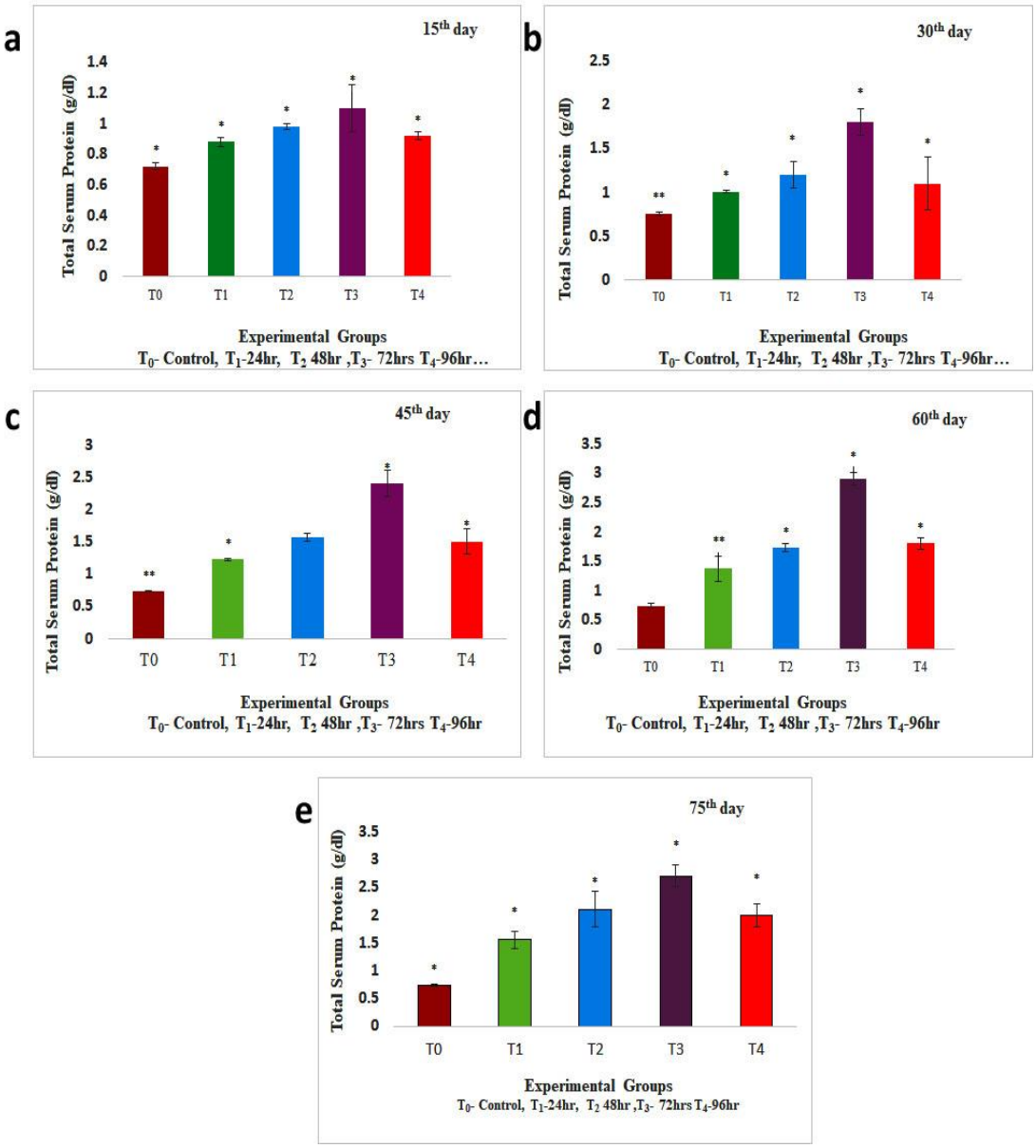


Figure 3: **Effect of total Serum Protein (g/dl) in *O. mossambicus* fed with (CTKA) Data are presented as mean ± stranded error (SE) of three replicates** (**n = 10**). **Within treatment groups, values with different letters are significantly different (*P* < 0.05**) **as per one-way ANOVA followed by Tukey's test**.

Phagocytic Activity

Leukocytes from fish in the control and experimental groups' phagocytic activity were examined, and the results are shown in **Fig 4 a-e**. In comparison to the control, phagocytic activity considerably increased in all experimental groups. When compared to earlier days of the experiment, a startling increase in activity was observed on the trial's 60th day (**Fig. 4d**). Further enriching for T_3-(CTKA) 72hr significantly increased the phagocytic activity in the experiment's (T_3-72hr) group at the 60th day compared to the control group [30±1.15 (%)] and in fish fed (CTKA) at T_1-24hr, T_2-48hr, and T_4 -96hr.

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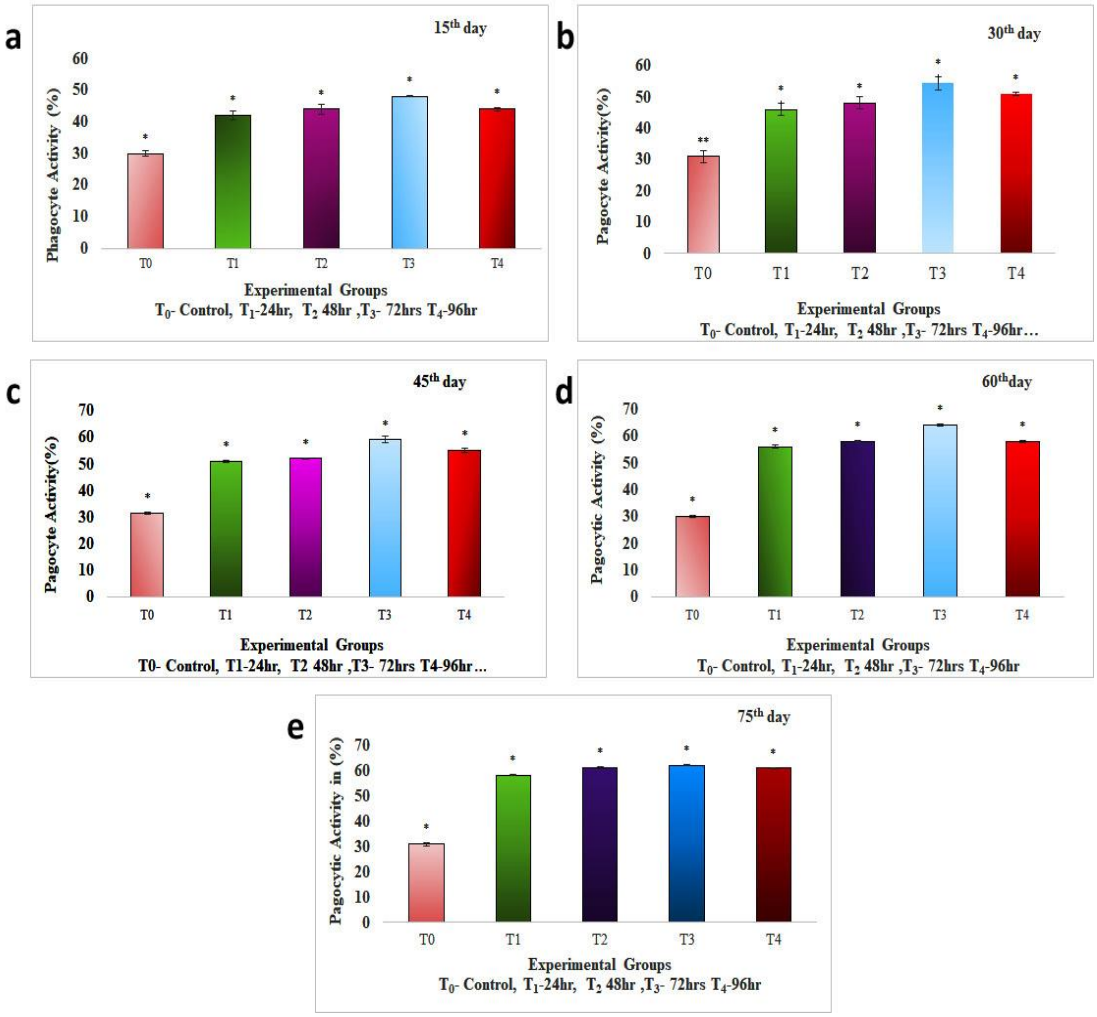


Figure 4: Effect of Phagocytic Activity (%) in *O. mossambicus* fed with (CTKA) for (a) 15th day, (b) 30th day, (c) 45th day, (d) 60th day, and (e) 75th day. Data are presented as mean ± stranded error (SE) of three replicates (n = 10). Within treatment groups, values with different letters are significantly different ($P < 0.05$) as per one-way ANOVA followed by Tukey's test.

Growth performance parameters

The experimental groups growth performance metrics, including weight gain (WG) in %, specific growth rate (SGR), and feed conversion ratio (FCR), are shown in (**Table2**). The *O. mossambicus* growth performance and Specific growth rate (SGR) have been enhanced significantly (P<0.05) at same time Feed Conversion Ratio (FCR) has been declined. Data are shown as the mean and standard error (SE) of three replicates (n = 10). According to a one-way ANOVA followed by a Tukey's test, values with different letters within treatment groups are statistically different (P<0.05).

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Table 2: Growth performance parameters (Feed for 2% Body Weight Twice daily) on 30th -day analysis. (Mean ± S.E).

Experiment	T_0	T_1	T_2	T_3	T_4
Initial weight (gm)	30 ± 0.47*	30 ± 0.47*	30 ± 0.04*	30 ± 0.47*	30 ± 0.47*
Final weight (gm)	32 ± 0.57	40 ± 0.57**	55 ± 0.76*	68 ± 0.50	58 ± 1.00*
Weight gain (gm)	2 ± 0.57	10 ± 0.76	25 ± 0.32	38 ± 0.02	28 ± 0.05
Weight gain %	6.6 ± 0.5	33.3±0.57	83.3±0.57	126.6 ±0.95	93.0 ±0.15
SGR	0.06 ± 0.01	0.33 ± 0.015	0.83 ± 0.01	1.26 ±0.005	0.93 ±0.01
FCR	0.93 ± 0.02	3.00 ± 0.186	1.6 ± 0.361	1.15 ± 0.020	1.5 ± 0.173

Table 3: Growth performance parameters (Feed for 2% Body Weight Twice daily) on 60th-day analysis. (Mean ± S.E).

Experiment	T_0	T_1	T_2	T_3	T_4
Initial weight (gm)	32 ± 0.47*	40 ± 1.04*	55 ± 0.87*	68 ± 0.07*	58 ± 0.47*
Final weight (gm)	38 ± 0.89*	62 ± 0.88*	85 ± 0.29*	115 ± 1.45*	90± 0.88*
Weight gain (gm)	6 ± 0.28	22 ± 0.28	30 ±0.57	47 ±0.65	32 ± 0.76
Weight gain (%)	18.7± 0.15	35.4 ± 0.95	54.5 ± 1.04	69.1 ± 0.37	55.1± 0.72
SGR	0.1± 0.05	0.36 ± 0.005	0.5 ± 0.05	0.78 ± 0.01	0.53 ± 0.005
FCR	2.75± 0.104	1.9± 0.176	1.5± 0.088	1.06± 0.180	1.32 ± 0.49

Table 4: Digestive enzymes activity of Tilapia fed five experimental diets after 60 days. Values are presented as the mean ± SE

Digestive enzymes	Control(T0)	T1	T2	T3	T4
Amylase U/ml	2.16±0.05	2.32±0.03	2.46±0.64	2.84±0.07	2.65±0.64
Lipase U/ml	1.12±0.02	1.34±0.011	1.53±0.01	1.86±0.02	1.73±0.01
Protease U/ml	18.04±0.025	19.24±0.025	20.38±0.02	23.06±.0.01	21.34±0.02
Trypsin U/mg	6.34±0.02	7.42±0.02	7.26±0.02	8.56±0.011	7.66±0.02

Table 5: Liver specific enzymes activity of Tilapia fed five experimental diets after 60 days. Values are presented as the mean ± SE.

Liver specific enzymes enzymes	Control(T0)	T1	T2	T3	T4
Aspartate amino transferase (AST)U/min/mgprotein	9.7±0.15	11.3±0.15	24.7±0.1	26.8±0.15	25.7±0.1
Alanine amino transferase (ALT) U/min/mg protein	11.2±0.15	15.8±0.20	20.8±0.1	23.4±0.15	21.8±0.1
Alkaline phasphatase (ALP) U/min/mg	36.2±0.35	40.4±0.11	41.0±0.15	52.3±0.25	42.6±0.15

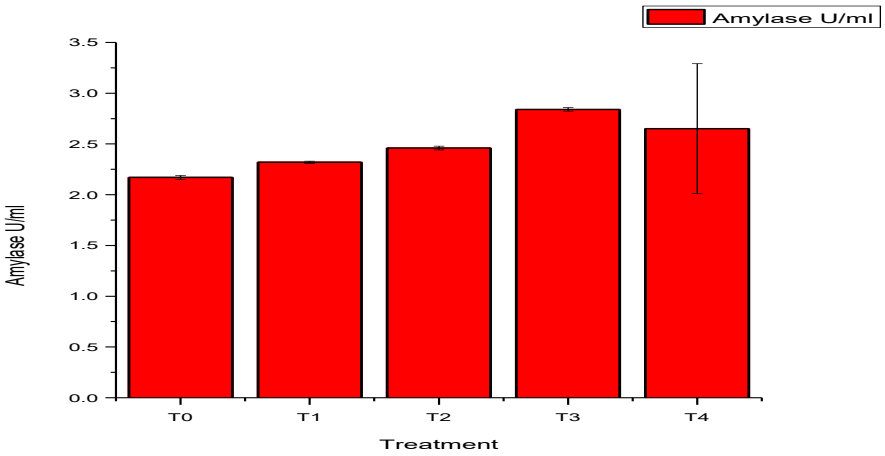
Table 6: Liver antioxidant activity of Tilapia fed five experimental diets after 60 days. Values are presented as the mean ± SE.

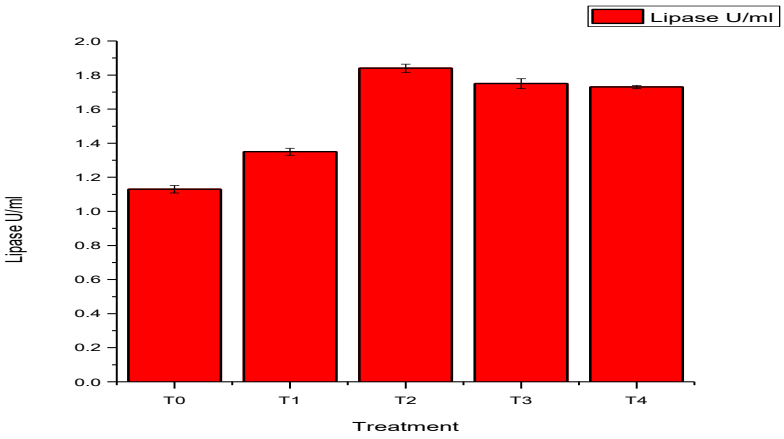
Liver antioxidant parameters	T0	T1	T2	T3	T4
SOD(unit mg^{-1})	2.02±0.02	3.16± 0.02	3.06±0.025	4.23±0.02	3.34±0.025
CAT (nmol/min/mg)	5.23±0.025	6.38±0.01	6.16±0.02	7.86±0.01	6.63±0.02
GP_X (nmol/min/mg)	3.26±0.01	3.64±0.02	3.72±0.02	4.92±0.02	3.82±0.02
GR (µgmg^{-1})	29.08±0.57	30.2±0.20	3i.2±0.02	33.09±0.015	32.5±0.02
LDH (mmol mg^{-1})	13.4±0.2	14.8±0.11	15.6±0.2	17.8±0.2	16.5±0.2
MDA (nmol /mg^{-1})	2.6 ± 0.178	1.7 ± 0.167	1,0±0.1	0.9 ± 0.089	1.1 ± 0.13

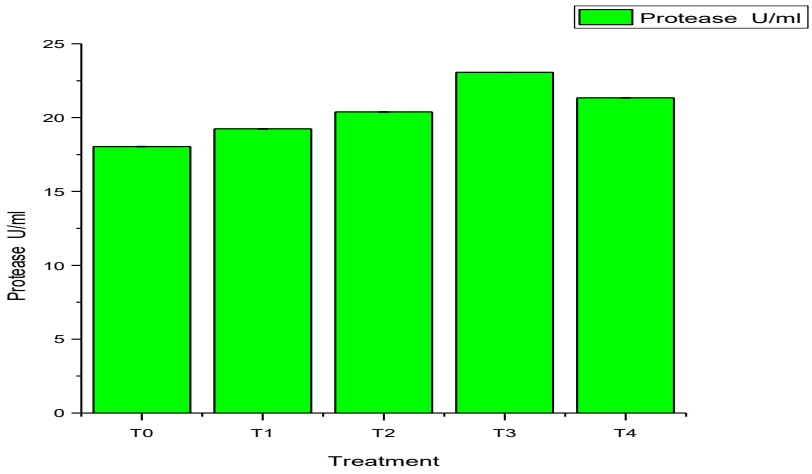
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Table 2: Digestive enzymes activity of Tilapia fed five experimental diets after 60 days. Values are presented as the mean ± SE.







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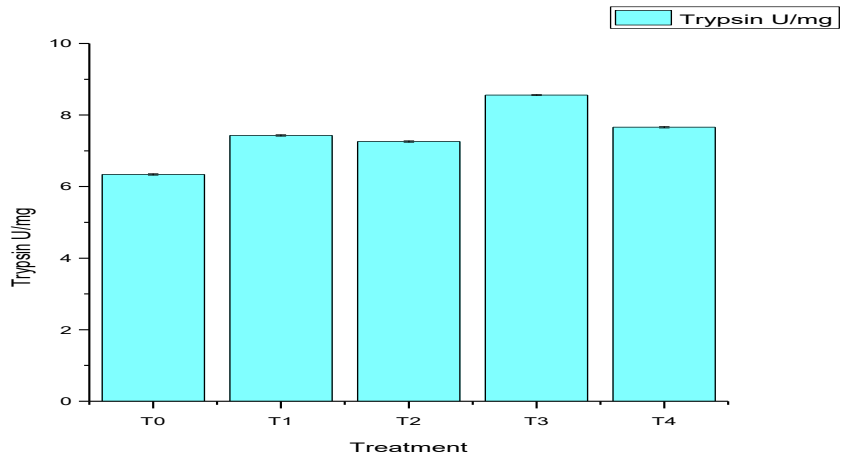
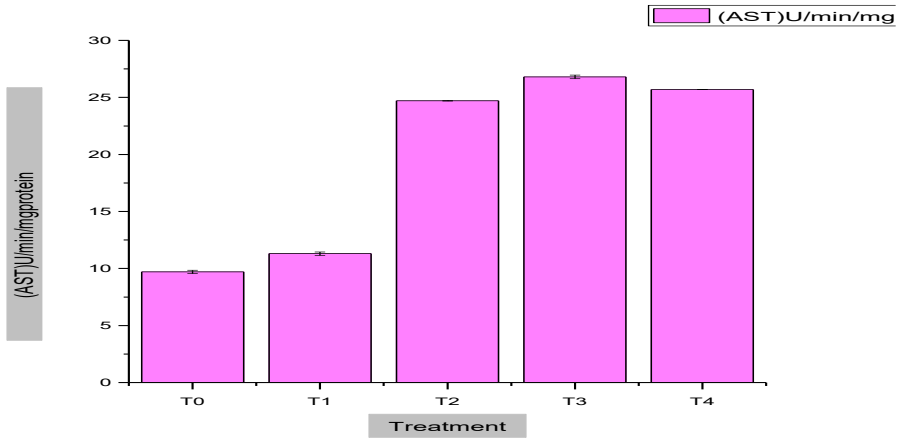
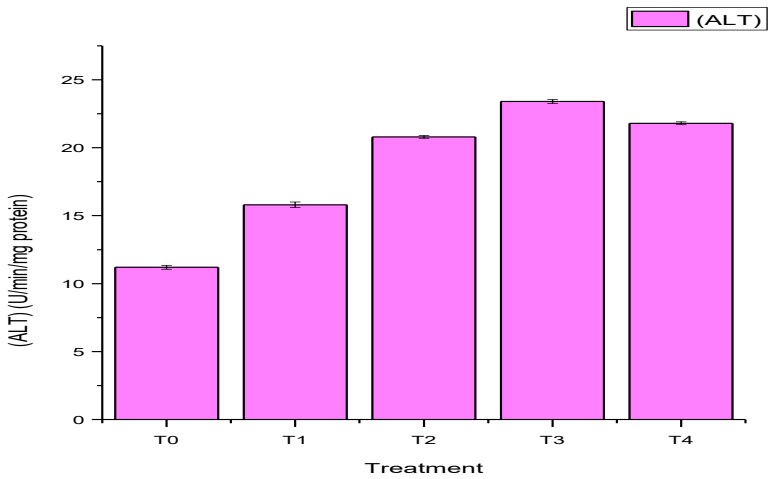


Table 3: Liver specific enzymes activity of Tilapia fed five experimental diets after 60 days. Values are presented as the mean ± SE.





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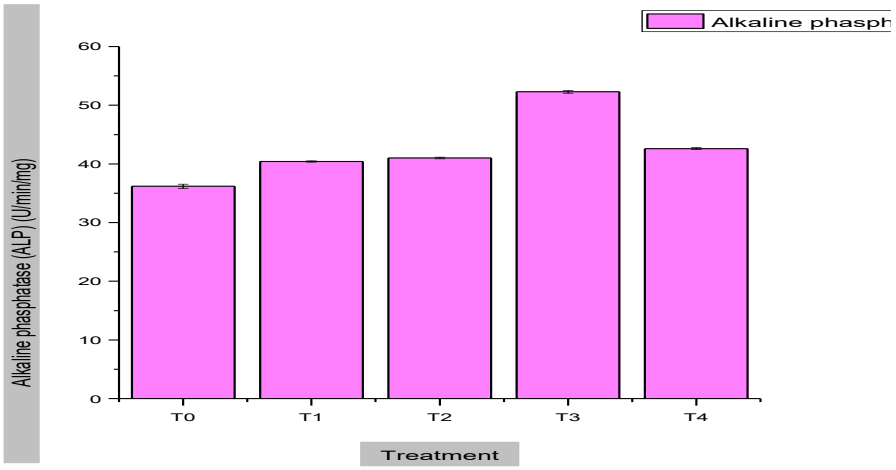
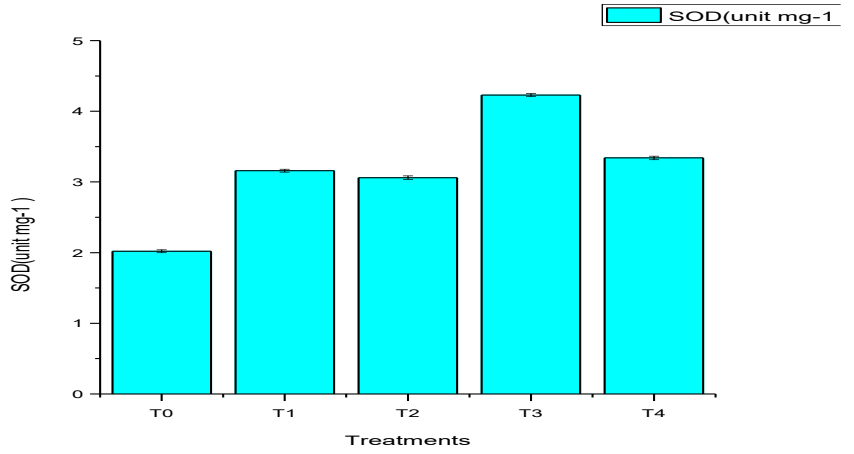
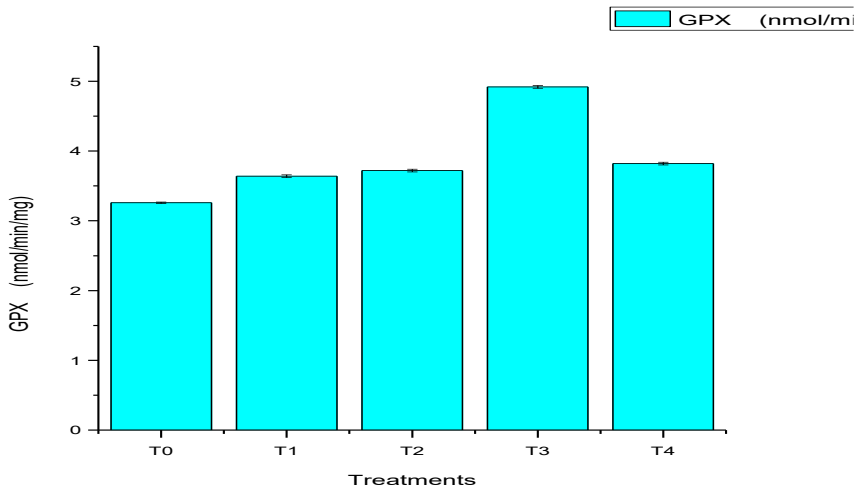


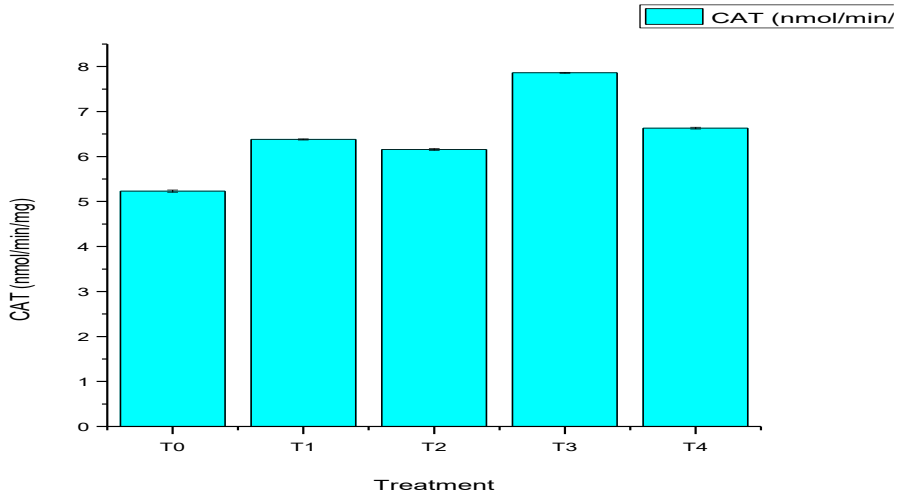
Table 4: Liver antioxidant activity of Tilapia fed five experimental diets after 60 days. Values are presented as the mean ± SE.

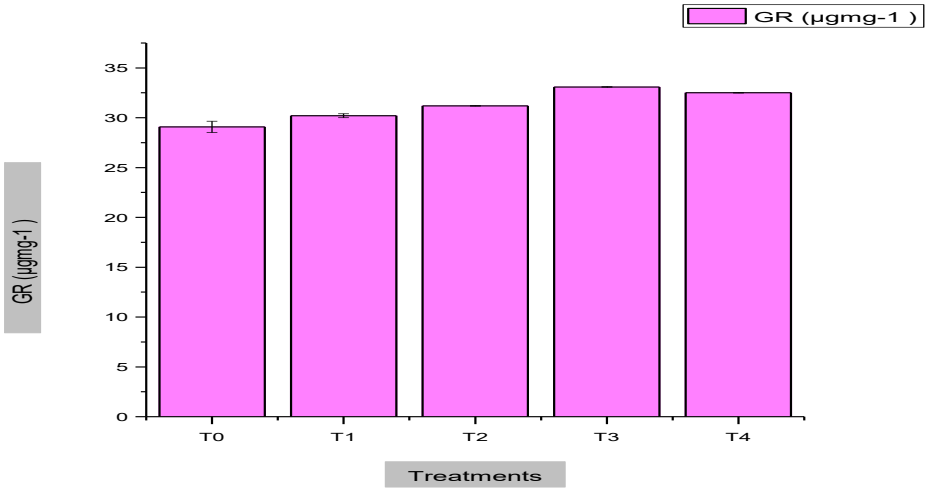


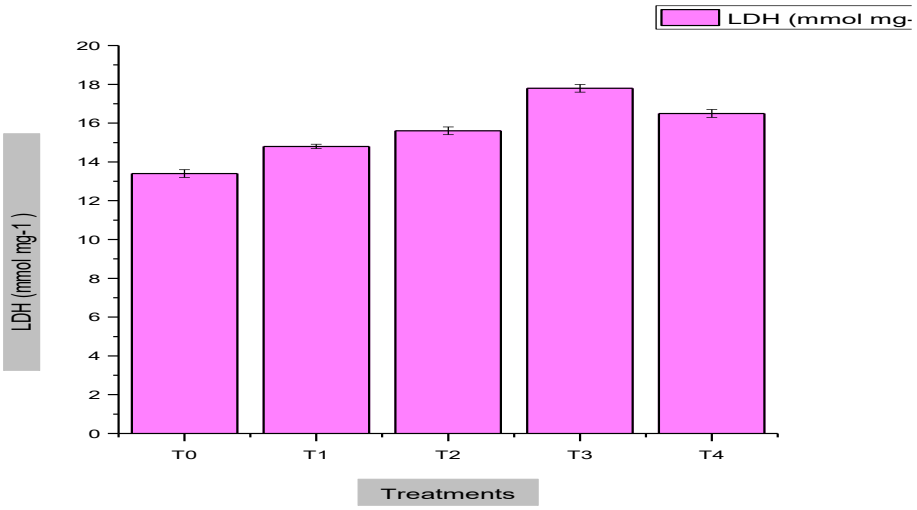


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Challenging study

In the control group [T_0] and all of the experimental groups (T_1, T_2, T_3, and T_4) of fish fed with (CTKA) at different hours (24hr, 48hr, 72hr, and 96hr) for 15 days, challenging analysis with *Aeromonashydrophila* was carried out. Results statement death rates in the treatment groups T_1, T_2, T_3, and T_4 were correspondingly (60.55%), (42%), (21%),and (30.7%) and 80% in the control group. Fish-fed (CTKA) showed a relative percentage of survival (RPS) of (40%) in T_1, (58%) in T_2, (79.17%) in T_3, and (60%) in T_4 following the 15th day. Comparing the T_3experimental group to the control and other experimental groups, the highest RPS (79.17%) %was observed in (**Fig-5**).

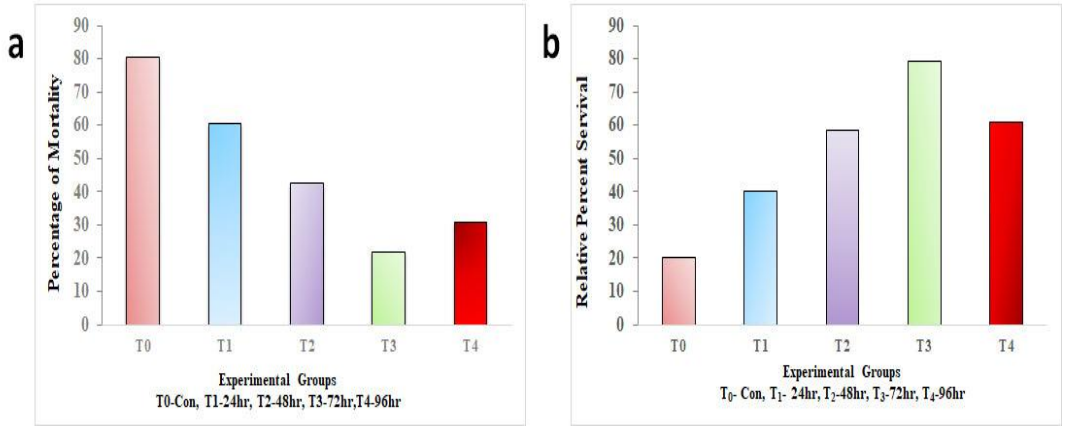


Figure 5: (a) **Percentage of mortality in *O. Mossambicus* challenged with *A. hydrophila* after75 days**. (b) **Relative percent survival in *O. Mossambicus* challenged with *A. hydrophila* after75 days**.

DISCUSSION

Due to unsuitable circumstances for fish raising, the resurgence of dangerous infections, and a lack of fish health monitoring at various stages, large-scale fish hatcheries and aqua farms are still seen as a barrier to further industrializing aquaculture practices (Assefa and Abunna 2018). The predominance of parasitic and pathogenic diseases is the fundamental flaw that degrades the quality and productivity of cultured organisms (Köhl, et al. 2019). To prevent and manage the negative effects of pathogenesis in animals, simple yet novelistic approaches must be designed.

Accordingly, the current study implemented a novel feeding experiment called bio-encapsulation of (CTKA) to lessen and prevent the clinical symptoms of *Aeromonas hydrophila* in freshwater fish called *O. mossabmicus*.In addition, the study shows that feeding *O. mossambicus* with (CLKA) resulted in significant improvements in **total WBC, RBC count, serum protein, phagocytic activity, rate of survival,** and drop in mortality at the end of the experimental period. An indicator of fish's physiological state is the hematological parameter. By concentrating on the main (nonspecific) innate immune system components, such as macrophages, monocytes, granulocytes, and humoral components including the complement system and lysozyme.

The current study demonstrates that feeding fish bio-encapsulated feed for 72 hr increases their non-immune response, particularly on the 60thday in experimental (T_3) groups. Compared to the controls (9.5 x 10^3 mm^3) and (2.4 x 10^3 mm^3), the total WBC and RBC count increased to (22.2 x 10^3 mm^3) and (8.2 x 10^3 mm^3), respectively [Table 1&2].The research's findings support the use of bio-encapsulated artemia in combination with *Lactobacillus plantarum* and *Bacillus subtilis* as immunostimulants in European sea bass *(Dicentrarchus labrax, L)* (Wuertz, et al. 2021). Additionally, it supports the report's findings that European sea bass fed with Persian sturgeon (*Acipenser persicus*) larvae bio-

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encapsulated with Bacillus species, *Oncorhynchus mykiss* supplemented with seaweed, *Cyprinus carpio*, *Oreochromis niloticus*, and *Rainbow Trout* all had elevated RBC and WBC contents(Staykov, et al. 2007).

The bio-encapsulation of probiotics and oil emulsion in Artemia parthenogenetic, which provides amino acids for animal growth, is crucial for analyzing the nutritional potential of cultured individuals(Langdon, et al. 2008). Analysis of the [Fig. 3] total protein content following the administration of bio-encapsulated feed, there was a general improvement in the serum protein content (2.9 g/dl) in T_3 groups during the fourth week compared to the control (0.74 g/dl), and these values were relatively greater than those of other groups (T_1, T_2, T_4).

As macrophages are crucial to the host defense system, increased phagocytic activity of neutrophils and macrophages was observed in experimental groups [Fig. 4] demonstrates the effectiveness of our bio-encapsulated meal (72 hr) to alter immunological responses. According to the current study's findings, more neutrophils help to significantly improve the fish's immune system. This means that measuring phagocytosis can be used as a great measure of macrophage effector activity and can replace the key and most important component of the immune defensece system. Additionally, the results of our investigation are consistent with the reports (Hirayama, et al. 2018).

As shown in [Fig. 5], a challenge study against *A. hydrophila* in *O. mossambicus* fed (CTKA) increased disease resistance in treatment groups (T_1, T_2, T_3, and T_4) over the control. However, in the T_3 groups, there was a significant decrease in mortality (21.83%) and an increased survival rate (79.17%). When the fish were fed live bio-encapsulated feed, similar outcomes have been well-documented(Samat, et al. 2020;).Results of the preliminary analysis (non-specific immune parameters), mortality, and survival percent show that T_3 experimental groups have the best values, which advanced the study to the next stage for additional evaluation of growth-related performance in them to demonstrate the effectiveness of the (CTKA). The T_3 group significantly outperformed the other groups in terms of growth performance metrics such as weight gain (48% in grams), specific growth rate (0.78 0.01), and feed conversion ratio (0.01 0.05), as shown in [Table-1],When the fish were fed live bio-encapsulated feed, the same outcomes were observed(Ibrahem 2015). Furthermore, incorporating *S. platensis* into the diet of the Oscar fish *Astronotus ocellatus* enhanced its growth performance and improved feed utilization efficiency (Mohammadiazarm et al., 2021). This explains the nutritional benefits of algal bioconstituents, such as fatty acids, amino acids, vitamins, and minerals. Same results corroborate with the results when Spirulina plentensis incorporated in the diets of nile tilapia Norah (Mobasshsirin Rahman et al 2023)., dietary *Hermetia illucens* (HI) larvae meal fed to gilt head sea bream,(Serena Busti *et al.,* 2024).The current study recommends a formulation of 1 gm of (CTKA) for 72 hr in the bio-encapsulation procedure after demonstrating the immunomodulatory efficacy of bio-encapsulated (CTKA) and by increasing the survival rate in the cultured organisms. Additionally, it suggests giving this unique diet to fish for 60th day to boost their health, growth, and relative percent survival to strengthen their immune defenses against *A. hydrophila*. To the best of our knowledge,*C. transvaalensis* were bio-encapsulated with *K. alvarezii*, increasing the total WBC and RBC count, total serum protein, phagocytic activity, survival rate, and lowering mortality in *O. mossambicus* against *A. hydrophila*. The farmers can be advised to bio-encapsulate (CTKA) for 72 hr on the 60th day to improve the fish's immunological state, growth parameters, and illness resistance, preventing production loss. However, detailed immunological and molecularstudies are needed to strengthen the role of this bio-encapsulated (CTKA) in aquaculture before recommending the supplementation diets as potential immunostimulants in other culturedfish species against pathogens.

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Declarations

Ethical Approval

Not applicable

Competing interests

Not applicable

Authors' Contributions

A.J. and C.M. done the experiment. S.S.B. Wrote the main manuscript text and prepared all figures. All authors reviewed the manuscript.

Funding

Not applicable

Availability of Data and Materials

Not applicable

Conflict of Interest

The authors declare that they have no financial interests or personal relationships that could have appeared to influence the work reported in this article.

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