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Dietary Immune Nutritive Effect of Chitosan/Chitosan Nanoparticles on the Nile Tilapia: Short-term Exposure

Nesreen Hossam-Elden[1], Nermeen Abu-Elala[1, 2], Sara E. Ali[3], Marwa S. Khattab[4], Mohamed S. Marzouk[1]

[1]Department of Aquatic Animal Medicine and Management, Faculty of Veterinary Medicine, Cairo University, Egypt
[2]Faculty of Veterinary Medicine, King Salman International University, South Saini, Egypt
[3]Department of Physiology, Faculty of Veterinary Medicine, Cairo University, Egypt
[4]Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Egypt
*Corresponding Author: nermeen-abuelala@cu.edu.eg

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ABSTRACT

Several studies have pursued using chitosan as a feed additive in aquaculture; however, more research is needed to evaluate its inclusion in nanoform to achieve more effectiveness. Hence, this study aimed to demonstrate the influence of dietary chitosan and chitosan nanoparticles on the Nile tilapia (*Oreochromis niloticus*) growth performance, histological intestinal morphometry, antioxidant status, and immunology. For one month, fish (average weight: 42.10± 0.05g)were fed diets containing chitosan (1.0gCS kg^{-1} food) and different concentrations of chitosan nanoparticles (1, 3, and 5gCNP kg^{-1} diet), with the basal diet serving as the control diet. In comparison to the control group, all CS and CNP groups showed a significant increase in growth indices (weight gain, specific growth rate, and feed intake) and intestinal morphometric analysis (average intestinal villous length, width, and depth), where the CS, CNP 1, and 3 groups had the highest values. The CNP 1 and 3 diets significantly ($P< 0.05$) decreased the levels of intestinal malondialdehyde. Moreover, a significant increase in the levels of serum biochemical biomarkers (total protein, albumin, and globulin), immunological parameters (phagocytic activity/index, and NBT), and intestinal antioxidant parameters (total antioxidant capacity and glutathione reduced) were observed in the CNP 1 and CNP 3 groups, followed by CS. The experimental results demonstrated that dietary inclusion of CNP at low concentrations can promote the Nile tilapia growth, intestinal morphometry, antioxidant biomarkers, and immunity at an optimal level of 1.0 g kg^{-1} diet.

INTRODUCTION

Aquaculture is a vital contributor to supporting the demand and needs of fish and other aquatic organisms worldwide. Fishery products are the most important sources of high-quality protein for the nutritional benefit of human beings **(FAO, 2020)**. Fish are

considered the fastest-growing food-producing sector and the most efficient in converting food into biomass **(Kaleem and Bio Singou Sabi, 2021; Elvy *et al.*, 2022)**. The Nile tilapia (*Oreochromis niloticus*) is one of the most widely distributed freshwater fish individual all over the world thanks to its fast growth, high FCR, acceptance by consumers, and high marketing value **(El-Sayed, 2019)**.

Intensive high-density fish production is causing an intrusion of a high number of bacterial diseases, which represent the limiting factor for the healthy and sustainable development of the Nile tilapia farming industry **(Abdel Rahman *et al.*, 2022; Cheng *et al.*, 2022)**. Although antibiotic usage has played a pivotal role in medicine, it is considered one of the major challenges facing the aquaculture industry and human health due to its excessive drawbacks, such as its residue in the water environment and aquatic organisms that will have a potentially adverse effect on human health **(Giri *et al.*, 2015; Huang *et al.*, 2019)**. In addition, overuse and inappropriate prescribing of antibiotics may lead to a bacterial resistance crisis, subsequently decreasing the immunity of aquatic animals **(Pamer, 2016; Palanisamy *et al.*, 2019; Algammal *et al.*, 2022)**. Thus, antibiotic usage was globally banned, forcing people to seek alternative, safe methods for disease prevention and management. The most promising alternative methods for disease management that have achieved reasonable degrees of success are immunostimulants, herbal extracts, probiotics, prebiotics, symbiotics, acidifiers, and vaccines **(Abu Elala and Ragaa, 2015; Abu-Elala *et al.*, 2020, 2023)**.

Immunostimulants are considered an effective dietary additive that stimulates the immune status of cultured organisms and disease resistance. Fish & shellfish, in contrast to vertebrates, depend mainly on innate immunity more than adaptive immunity. Thus, in recent years, products that can stimulate host immunity and disease resistance such as immunostimulants have gained considerable interest **(Kumar *et al.*, 2022)**.

Among the effective immunostimulants used in the aquaculture industry is chitosan, which is a linear homopolymer of β-(1,4)-2-deoxy-D-glucose derived from the process of alkaline deacetylation of chitin, a main shell component of crustaceans, such as shrimp, crab, and crawfish, and can also be found in insects and fungi **(Kou *et al.*, 2021)**. Chitosan has desirable biomedical attributes, including low toxicity, biocompatibility, low cost, and good handling properties **(Mohammed *et al.*, 2017)**. The aquaculture industry is currently interested in chitosan due to its strong antibacterial, growth, and immune-stimulating properties, its ability to regulate antioxidant enzyme activities, reduce both lipid peroxidation and water pollution **(Abu-Elala *et al.*, 2015; Aranaz *et al.*, 2021)**. Today, nanotechnology has garnered much interest and promising avenues in biomedical applications, where it is used in myriad sectors, such as nutrition, medicine, the preparation of vaccines, etc. **(Nasr-Eldahan *et al.*, 2021)**.

For the sustainable aquaculture industry, several studies have inspected the use of chitosan in aquaculture as an immunostimulant; however, more research is needed to see the possibility of its use in nanoforms in aquafeeds to achieve considerable degrees of success. In this respect, the purpose of this study was to evaluate the influence of chitosan and chitosan nanoparticles in different dietary levels on the growth performance, immunity, and antioxidant capacity of *O. niloticus.*

MATERIALS AND METHODS

1. Preparation of chitosan nanoparticles

CNPs were formed by using the ionic gelation method of chitosan with polyanion sodium tripolyphosphate (TPP) **(Ahmed *et al.*, 2021)**. Briefly, chitosan purchased from Sigma-Aldrich (St Louis, MO, USA) (0.5%, W/ V) was dissolved in a 1% (W/ V) acetic acid solution, then magnetically stirred at room temperature for 30min until the solution was clear. Sodium tripolyphosphate (TPP) obtained from Sigma-Aldrich (St Louis, MO, USA) solution (0.25%, W/V) was added dropwise into the chitosan solution with a ratio of 1:3 under continuous magnetic stirring for 45min to promote cross-linking interaction between the amine group of chitosan and the negatively charged group of sodium tripolyphosphate and obtain CNPs. Subsequently, the CNP suspension was centrifuged at 1100x*g* for 20min. The supernatants were removed, and the precipitate was washed with distilled water, centrifuged again for CNP purification, and then lyophilized to produce a nanoparticle to be easily used and stored. Fig. (1) shows a flowchart of the ionic gelation procedure for the synthesis of chitosan nanoparticles.

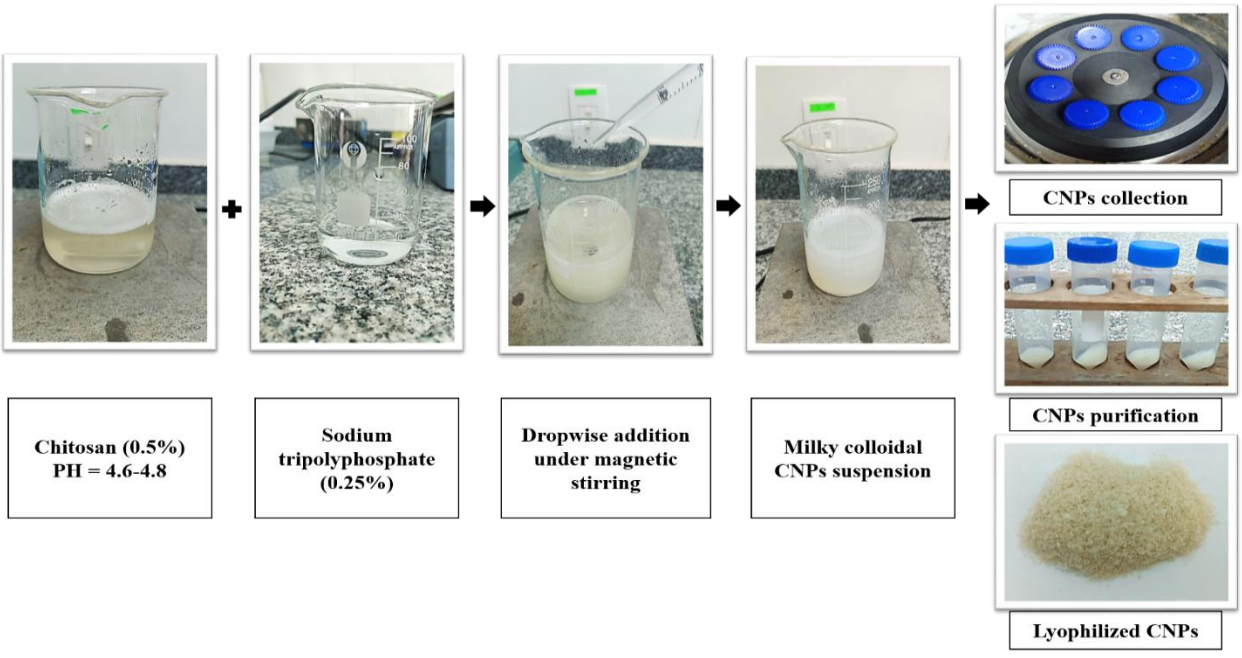

Fig. 1. A flowchart of the ionic gelation procedure for the synthesis of chitosan nanoparticles

2. Characterization of chitosan nanoparticles

Electron microscopy

Morphological examination and the average diameter of CNPs were assessed by transmission electron microscopy (TEM). All samples for TEM analysis were prepared by sonication of dilute suspensions for 20min on an ultrasonicator (Crest Ultrasonics Corp., New Jersey, USA). A few drops of nanoparticle suspension were deposited on a carbon-coated copper grid and left to dry. Subsequently, a few drops of negative staining material, such as phosphotungestic acid (1%), were loaded on the grid to stain the sample. The sample on the grid was left to dry and examined by HR-TEM (JEOL, JEM-2100, Tokyo, Japan), operated at 200kV.

Dynamic light scattering

Particle size and surface charge of CNPs were estimated by using a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). A beam of monochromatic light irradiates a diluted sample that was placed in polystyrene cuvettes, and the scatter light was determined at 25ºĊ. Size values were recorded as mean diameter (MD), and polydispersity index (PI). The force of zeta potential controls the potential stability of suspension. If the particles have high zeta potential values, they will repel each other and become suspended separately, however if the particles in suspension have low zeta potential values, then there is no magnitude of repulsion to prevent the particles from aggregation and there is instability (Fourier transform – infrared (FT-IR) spectroscopy).

Functional groups and structure elucidation of CNPs were identified by infrared spectroscopy (IR). ATR-FTIR was measured using Bruker VERTEX 80 (Germany) combined Platinum Diamond ATR, comprising a diamond disk as that of an internal reflector in the range of 4000- 400cm^{-1}, with resolution of 4cm^{-1} and refractive index of 2.4.

3. Fish samples

Two hundred and twenty-five healthy *O. niloticus* (mean individual weight of 42.10± 0.05g) were obtained from a private fish farm. Fish were maintained for two weeks for acclimatization to the laboratory conditions. Fish were kept in 9 glass aquaria (80 × 30 × 40cm) with 15 fish per aquarium that was provided with aerated de-chlorinated tap water and subjected to a photoperiod of 12h light/ 12h dark. The water temperature was kept at 23– 25°C. Fish were fed till satiation twice a day at 8:00am and 1:00pm. The waste of fish was daily siphoned, and half of the glass aquarium's water was replaced by clean de-chlorinated tap water.

4. Fish diet and experimental design

The experimental diet (30% protein) was obtained from a private company (Aqua International Com. Egypt) for food industries (Table 1). Fish pelletized food was covered with 1.0% gelatin and mixed with chitosan or chitosan nanoparticles in a treated aquarium and 0% chitosan or chitosan nanoparticles in a controlled aquarium **(Abu-Elala *et al.*, 2015)**. Experimental fish were randomly divided into five groups (forty-five fish/ group, three replicates/ tank). The control group was fed a basal diet twice daily; the chitosan (1.0gCS kg^{-1} diet) was fed with chitosan twice daily; and the other three groups fed on chitosan nanoparticles at concentrations of 1, 3, and 5gCNP kg^{-1} diet twice daily. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) Vet CU 08072023712 and followed all national and institutional guidelines for the care and management of aquatic animals.

Table 1. Analytical qualities of the basal diet

Analytical quality	%
Crude protein	Not less than 30%
Total energy	Not less than 4100 Kcal/ Kg
Crude fat	Not less than 5.80%
Crude fiber	Not less than 7.50%
Ingredient	
Soybean meal (44%), yellow corn, sunflower (28%), soybean oil, wheat bran, rice bran non-extraction, fish meal low fat (65%), Di calcium phosphate, limestone, salt, D.l methionine, l. Lysine hydrochloride, sodium bicarbonate.	

5. Growth parameters, feed utilization, and body indices

After one month of feeding trials, growth performance, feed utilization, and body indices were estimated according to the guidelines of **Abu-Elala *et al.* (2013)** and **Jang *et al.* (2023)**, as follows:

A) Body weight gain (WG) = $W_2 - W_1$; where: W_2 is the final fish weight, and W_1 is the initial fish weight.

B) Specific growth rate (SGR, %) = $\frac{100\ [Ln\ W_2(g) - Ln\ W_1(g)]}{T}$; Where: W_2 is the final fish weight; W_1 is the initial fish weight; T is the experimental period (day), and Ln is the natural logarithm.

C) Feed intake (FI/g fish) = $\frac{total\ feed\ consumed\ through\ out\ the\ exp\ eriment\ (g)}{the\ number\ of\ fish}$

D) Feed conversion ratio (FCR) = $\frac{total\ feed\ intake\ (g)}{total\ weight\ gain\ (g)}$

E) Condition factor (CF, %) = $100\ \left(\frac{body\ weight}{total\ body\ length}\right)$

F) Hepatosomatic index (HSI, %) = $100\ \left(\frac{liver\ weight}{body\ weight}\right)$

G) Spleen somatic index (SSI, %) = $100\left(\frac{spleen\,weight}{body\,weight}\right)$

H) Viscerosomatic index (VSI, %) = $100\left(\frac{visceral\,weight}{body\,weight}\right)$

6. Evaluation of intestinal morphometric indices

After one month of the feeding trial and at the end of the experimental period, from each group, the proximal intestinal sections were taken (about 1cm-long piece) and fixed in 10% neutral buffered formalin to preserve the tissue. These specimens were dehydrated by immersing in ascending grades of ethyl alcohol (70- 100%) to remove formalin and water, cleared using xylene to remove alcohol to permit infiltration, and embedded in paraffin wax. Sectioning the specimen at 5µm thick was done by using a rotary microtome (Leica®, Wetzlar, Germany) and stained with hematoxylin and eosin (H&E) stain to give color and contrast to tissue structure, where hematoxylin stains nuclei blue and Eosin stains cytoplasm, muscle fiber, and collagen red. Slides were examined for intestinal morphometric analysis and photographed for histological study by using the Ceti England microscope with an attached AmScope digital camera. Using AmScope Toup View 3.7 software (AmScope, Irvine, CA, USA), 20 images per fish at 40X were required to perform morphometric analysis by determination of average intestinal villus length (AIVL), villus width (AIVW), and villus depth (AIVD) **(Suvarna Kim *et al.*, 2018)**.

7. Hematological and biochemical assay

The blood samples were collected from the caudal vessels of fish (five fish from each group) and divided into two portions. The first portion was added to tubes containing anticoagulant as dipotassium salt of EDTA (0.5mg ml^{-1} blood), which is used for determination of hematological parameters, while the other portion was left without anticoagulant to clot and separate serum by centrifugation at 1100xg for 15min that can be used in biochemical analysis.

7.1. Hemogram

The total count of erythrocytes and leukocytes was determined by a Neubauer hemocytometer and Natt-Herrick solution according to **Martins *et al.* (2004)**. Hemoglobin concentration was calculated using the cyanomethemoglobin process by mixing 20µl of freshly obtained whole blood with 5ml of Drabkin's solution and waiting for 10min at room temperature, then the optical density (O.D) was estimated using a spectrophotometer at 540nm **(Blaxhall and Daisley, 1973)**. Packed cell volume (PCV) was determined using the microhematocrit method, the non-coagulant blood was withdrawn by a capillary tube, and one of its tips was sealed and centrifuged in a hematocrit centrifuge (3100xg for 5min), then the percentage of PCV was estimated with hematocrit reader **(Blaxhall and Daisley, 1973)**.

7.2. Biochemical analysis

Activities of serum transaminases ALT and AST were calorimetrically measured, as described by **Reitman and Frankel (1957)**. Creatinine was determined using the method of **Husdan and Rapoport (1968)**. While, blood urea nitrogen (BUN) was determined by the Bertholot reaction **(Fawcett and Scott, 1960)**.

8. Cellular innate immune response

8.1. Phagocytic assay

Sodium heparinized 3ml blood samples collected from the caudal vessels were carefully overlaid on equal volumes of a histo-paque medium (1.077g/ ml, Sigma – Aldrich, MO, USA) in 15cm falcon tube and centrifuge at 6000rpm for 15min for the separation of peripheral blood leucocytes. The white blood cells at the interface layer were withdrawn with a Pasteur pipette, washed twice, and suspended in RPMI – 1640 supplemented with 0.22µm, filtered 3% (v/v) of pooled tilapia serum, 100IU ml $^{-1}$ penicillin, and 1mg ml $^{-1}$ streptomycin. The trypan blue exclusion method was used to detect the no. of viable cells, then cells were adjusted to $4\times10^{7}ml^{-1}$ using culture medium. *S. cerevisiae* was suspended at RPMI -1640 and adjusted to $1\times10^{6}ml^{-1}$. They were placed onto the cell suspension and incubated for 1h at 37ċ. Ten µl of the mixture was smeared on a clean slide, air dried, fixed with methanol for 10min, drained then stained with Giemsa stain and observed under the oil immersion lens of the ordinary light microscope. Approximately, 200 phagocytic cells were counted and differentiated using the equations as described by **Abu-Elala *et al.* (2019)**, as follows:

$$\text{The percentage of phagocytic activity} = \frac{No.of\ ingested\ phagocytes}{Total\ No.of\ phagocytes}$$

$$\text{The phagocytic index} = \frac{No.of\ ingested\ yeast\ cells}{No.of\ ingested\ phagocytes}$$

8.2. Respiratory burst activity (NBT reduction test)

To measure the NBT, blood suspension (100µl/ well) with 1×10^{6} cells was added to a 96 well plate and incubated at 25ċ for 120min to allow adherence of the cells. The supernatants were removed, and the remaining cells were washed with (RPMI 1640) medium supplemented with 3% tilapia serum. The NBT solution (100µl) (1mg/ ml) directly or in addition to stimulant phorbol 12 – myristate 13 – acetate (PMA, Sigma Aldrich; 1mg/ ml) was added to each well and incubated at 25ċ for 60min. The supernatants were decanted, and cells were fixed by the addition of absolute ethanol for 10min and washed with 70% methanol. After drying, the formazan crystals were dissolved by adding dimethyl sulfoxide (DMSO), and the optical density was determined at an absorbance of 630nm **(Abdelkhalek *et al.*, 2020**).

9. Determination of oxidant and antioxidant biomarkers

At the end of the experimental period, intestinal tissues were collected (five fish from each group) and stored at –80°C till used for the estimation of antioxidant biomarkers including total antioxidant capacity (TAC) and malondialdehyde (MDA). The intestine was rinsed in phosphate buffer saline (PBS) solution, pH 7.4 then homogenized using tissue homogenizer in cold buffer (I, e,50 mM potassium phosphate, pH 7.5.1mM EDTA)/ gram tissue and centrifuged at 4°C for 15min at 4000rpm. The supernatant was removed for assay using commercial test kits (Bio- diagnostic, Egypt). TAC was estimated using commercial diagnostic test kits with CAT. No. TA 25 13 (Biodiagnostic Co., Egypt) according to **Koracevic *et al.* (2001)**. MDA level was assessed by thiobarbituric acid method **(Uchiyama and Mihara, 1978)** using a commercial test kit of Biodiagnostic Co. (CAT. No. MDA 25 29).

10. Histological appraisals

Tissue specimens of liver and intestine from all groups were fixed in 10% neutral buffered formalin followed by processing in ascending concentration of alcohol and xylene. Processed tissue was embedded in paraffin wax for sectioning by a rotary microtome (Leica 2135, Germany) into 4µm tissue sections, which were stained by hematoxylin and eosin stain. Intestinal morphometry including length, width, and depth of intestinal villi was measured by TS View software in 3 micrographs of intestinal sections at 40X magnification power per fish to calculate an average.

11. Statistical analysis

The obtained data were analyzed using one-way ANOVA through SPSS program version 20 (SPSS, Richmond, VA, USA) to evaluate the effect of dietary CS and CNP supplementation. The significance of difference among the experimental groups was tested by LSD multiple range tests while considering the significance differences at $P< 0.05$.

RESULTS

1. Characterization of CNPs

The transmission electron microscope (TEM) micrograph of the CNPs in Fig. 2A shows that the particles have spherical morphology and a size range of around 50- 80nm. The size and size distribution of the CNPs in Fig. (2B) shows an average diameter of 263.1nm at 30Ċ that was measured using dynamic laser scattering (DLS). The zeta potential of CNPs was 39.70mV, showing that the particles are stable in solution (Fig. 2D). The FT-IR spectra of the CNP solution in Fig. (2C) show functional groups of chitosan and TPP.

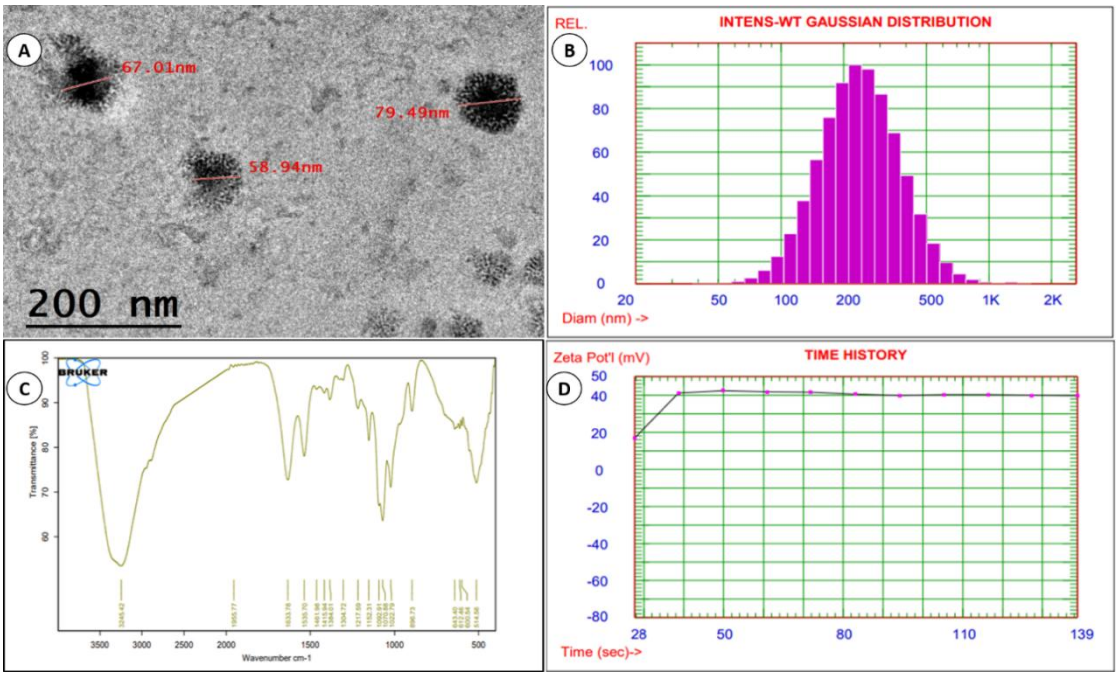

Fig. 2. Characterization techniques of the chitosan nanoparticles: **(A)** TEM image, **(B)** nanoparticle size was measured by dynamic laser scattering (DLS), where the size of CNPs was 263.1 nm, **(C)** FT-IR spectrum, and **(D)** Zeta potential of CNPs was 39.70mV

2. Growth performance

The Growth parameters and feed utilization of *O. niloticus* fed diets supplemented with chitosan and chitosan nanoparticles at various doses are summarized in Table (2). In comparison to the control group, CS, CNPs 1, and CNPs 3 groups exhibited the highest and optimal weight gain (WG), specific growth rate (SGR, %), and decreasing FCR, while the somatic index didn't differ between the experimental groups (Fig. 3).

3. Effect of dietary CS and CNPs on intestinal morphometric parameters

Chitosan and chitosan nanoparticles were found to have a significant effect ($P< 0.05$) on the intestinal histomorphometry of *O. niloticus* in the present study. Table (3) shows that the AIVL, AIVW, and AIVD were significantly ($P< 0.05$) greater in the CS, CNPs 1 and 3 groups, compared to the control and CNPs 5 groups.

Table 2. Effects of CS and CNP dietary administration on growth performance and feed utilization of *Oreochromis niloticus*

Parameter	C	CS (1.0g/kg diet)	CNPs (1.0 g/kg diet)	CNPs (3g/kg diet)	CNPs (5 g/kg diet)
IBW (g)	42.10 ± 0.05	42.05 ± 0.03	42.19 ± 0.02	42.14 ± 0.07	42.11 ± 0.06
FBW (g)	66.82 ± 0.04 ^{d}	73.65 ± 0.03 ^{b}	73.80 ± 0.02 ^{a}	73.89 ± 0.02 ^{a}	70.10± 0.06 ^{c}
FI (g/fish)	31.41 ± 0.23 ^{c}	36.93 ± 0.39 ^{a}	36.43 ± 0.31 ^{a}	36.02 ± 0.21 ^{a}	34.44± 0.48 ^{b}
CF (%)	1.68 ± 0.13	1.75 ± 0.10	1.74 ± 0.06	1.67 ± 0.06	1.65 ± 0.03
HIS (%)	3.18 ± 0.24	3.04 ± 0.38	3.04 ± 0.33	3.43 ± 0.35	2.89 ± 0.41
SSI (%)	0.08 ± 0.02	0.16 ± 0.03	0.08 ± 0.03	0.10 ± 0.03	0.13 ± 0.04
VSI (%)	7.35 ± 0.64	6.22 ± 0.46	6.03 ± 0.63	6.02 ± 0.69	5.75 ± 0.64

The mean difference is significant at the 0.05 level. IBW: Initial body weight, FBW: Final body weight, FI: Feed intake, CF: Condition factor, HIS: Hepatosomatic index, SSI: Spleenosomatic index, VSI: Viscerosomatic index.

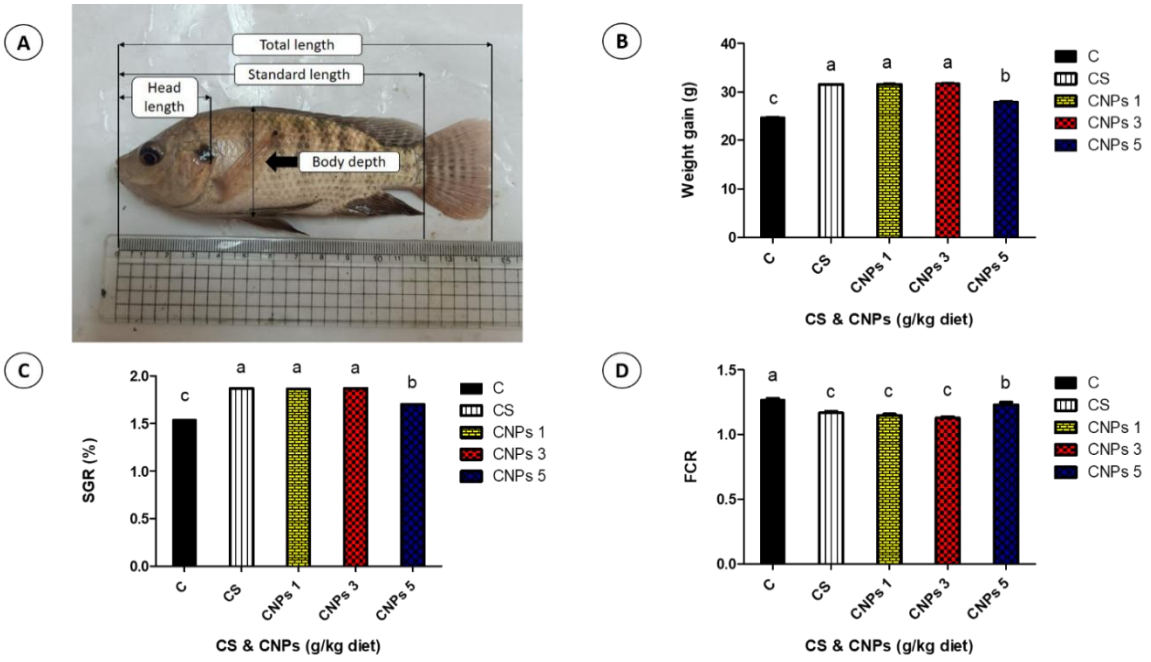

Fig. 3. (A) Different types of length measurement in fish**, (B)** Weight gain, **(C)** Specific growth rate, and **(D)** Feed conversion ratio of *O. niloticus* fed chitosan and varying levels of chitosan nanoparticles. Bars with different letters refer to significant differences between the experimental groups (as mean ± SE, n= 5)

Table 3. Intestinal morphometry indices of *O. niloticus* fed chitosan and different levels of chitosan nanoparticles

Parameter	C	CS (1g/kg diet)	CNPs (1g/kg diet)	CNPs (3g/kg diet)	CNPs (5g/kg diet)
AIVL (µm)	342.70 ± 2.76 ^{c}	422.52 ± 3.17 ^{a}	424.00 ± 4.36 ^{a}	428.33 ± 2.03 ^{a}	384.40 ± 2.60 ^{b}
AIVW (µm)	90.66 ± 1.76 ^{c}	114.95 ± 2.71 ^{ab}	117.43 ± 1.37 ^{a}	118.19 ± 2.86 ^{a}	107.88 ± 3.92 ^{b}
AIVD (µm)	55.38 ± 2.76 ^{b}	66.09 ± 2.56 ^{a}	67.65 ± 2.33 ^{a}	68.00 ± 3.21 ^{a}	60.88 ± 2.19 ^{ab}

Different latter refers to the difference between the experimental groups for each parameter *(P*< 0.05) AIVL: Average intestinal villous length, AIVW: Average intestinal villous width, and AIVD: Average intestinal villous depth.

4. Haemato-biochemical analysis

Hematological indices of *O. niloticus* were significantly increased in fish fed with chitosan and chitosan nanoparticles. Table (4**)** shows that the values of RBC count, WBC count, and HB concentration were significantly increased in fish fed on 1 and 3g/ kg of chitosan nanoparticles, except for MCH and MCHC. The highest levels of total protein and globulin were noticed in fish fed on 1 and 3g kg^{-1} diet *(P*< 0.05) (Table 5).

Table 4. Hematological indices of *O. niloticus* feed with chitosan and varying levels of chitosan nanoparticles

Parameter	C	CS (1g/kg diet)	CNPs (1g/kg diet)	CNPs (3g/kg diet)	CNPs (5g/kg diet)
RBCs count ($\times 10^6$ μL^{-1})	2.37 ± 0.06 ^{b}	2.54 ± 0.05 ^{b}	2.86 ± 0.12 ^{a}	2.90 ± 0.11 ^{a}	2.62 ± 0.11 ^{ab}
WBCs count ($\times 10^3$ μL^{-1})	80.00 ± 1.73 ^{c}	82.67 ± 2.03 ^{c}	103.67 ± 1.76 ^{b}	113.67 ± 2.03 ^{a}	114.67 ± 2.40 ^{a}
PCV %	37.00 ± 1.15 ^{b}	38.67 ± 0.88 ^{b}	43.67 ± 1.45 ^{a}	42.67 ± 1.20 ^{a}	40.33 ± 1.45 ^{ab}
HB (g/dl)	6.00 ± 0.12 ^{c}	6.36 ± 0.12 ^{bc}	7.03 ± 0.10 ^{a}	7.19 ± 0.16 ^{a}	6.51 ± 0.22 ^{b}
MCV (fl)	156.41 ± 1.42 ^{a}	152.43 ± 1.04 ^{ab}	153.07 ± 3.95 ^{ab}	147.45 ± 1.78 ^{b}	154.21 ± 1.09 ^{a}
MCH (pg)	25.37 ± 0.14	25.07 ± 0.16	24.67 ± 0.70	24.85 ± 0.39	24.91 ± 0.33
MCHC (%)	16.22 ± 0.20	16.45 ± 0.21	16.12 ± 0.36	16.85 ± 0.19	16.16 ± 0.23

Different latter refers to the difference between the experimental groups for each parameter *(P*< 0.05) RBCs: Red blood cells, WBCs: White blood cells, PCV: Packed cell volume, Hb: Hemoglobin, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration.

5. Immunological assay

The effect of dietary CS and CNPs on the cellular innate immune response was measured by phagocytic activity, index, and NBT reduction test. The results indicate that dietary CNPs 1 and CNPs 3 were significantly (*P*< 0.05) augmented with phagocytic activity and NBT reduction compared with the group fed on basal diet and chitosan

macromolecule. The phagocytic index was significantly increased in CNP 3 (1.48), followed by CNPs 1 (1.46) (Fig. 4).

Table 5. Biochemical analysis of *O. niloticus* feed with chitosan and varying levels of chitosan nanoparticles

Parameter	C	CS (1.0 g/kg diet)	CNPs (1.0 g/kg diet)	CNPs (3 g/kg diet)	CNPs (5 g/kg diet)
ALT (U/L)	2.88±0.16	3.24±0.29	3.17±0.25	2.77±0.28	2.95±0.09
AST (U/L)	24.56±2.52	22.07±1.98	20.33±0.88	21.33±2.40	25.00±1.53
BUN (mg/dl)	2.97±0.25	2.51±0.37	2.63±0.39	3.05±0.23	3.11±0.17
Creatinine (mg/dl)	0.41±0.04	0.37±0.01	0.36±0.02	0.37±0.02	0.40±0.01
Total protein (g/dl)	3.01±0.05 ^{c}	3.40±0.08 ^{b}	3.73±0.04 ^{a}	3.77±0.11 ^{a}	3.61±0.05 ^{ab}
Albumin (g/dl)	1.58±0.03 ^{d}	1.82±0.04 ^{c}	1.96±0.04 ^{ab}	1.99±0.06 ^{a}	1.85±0.03 ^{bc}
Globulin	1.44±0.03 ^{c}	1.58±0.04 ^{b}	1.77±0.02 ^{a}	1.78±0.05 ^{a}	1.76±0.02 ^{a}
A/G	1.10±0.00 ^{ab}	1.17±0.03 ^{a}	1.13±0.03 ^{ab}	1.10±0.00 ^{ab}	1.07±0.03 ^{b}

Different latter refers to the difference between the experimental groups for each parameter *(P*< 0.05) ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, BUN: Blood urea nitrogen, and A/G: Albumin/globulin ratio.

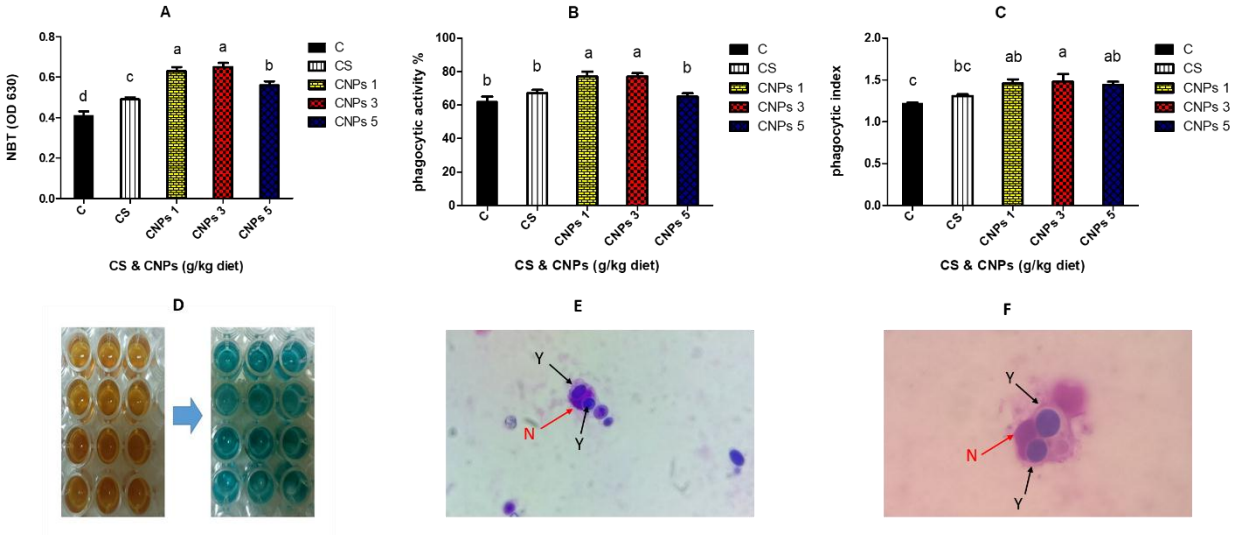

Fig. 4. (A, D): Effect of chitosan and chitosan nanoparticles supplemented diets on NBT; **(B)**: Phagocytic activity; **(C)**: Phagocytic index, and **(E, F)**: Phagocytic cells of CNPs 1.0 g kg^{-1} fish group engulfed more than one Saccharomyces yeast cells (Giemsa stain 1000X, red arrows point to nucleus, black arrows point to yeast cells. A significant difference *(P*< 0.05) between groups was indicated by different letters according to one-way ANOVA and tested by LSD.

6. Oxidant/antioxidant parameters

Table (6) exhibits the total antioxidant capacity (TAC) and glutathione reduced (GSH) that were significantly enhanced by feeding chitosan nanoparticles at 1 and 3g/ kg diet, with the highest being in fish fed 1g/ kg *(P*< 0.05). Otherwise, the level of malondialdehyde (MDA) was reduced by feeding chitosan nanoparticles at 1 and 3g/ kg, in comparison with fish-fed basal diet and chitosan macromolecules.

Table 6. Effect of dietary CS and CNPs on oxidant/antioxidant parameters of *O. niloticus*

Parameter	C	CS (1g/kg diet)	CNPs (1g/kg diet)	CNPs (3g/kg diet)	CNPs (5g/kg diet)
MDA (nM/g)	27.74±0.65 [a]	20.41±0.30 [b]	17.34±0.55 [c]	17.94±0.35 [c]	17.03±0.38 [c]
GSH (mg/g)	38.83±1.14 [c]	39.81±0.75 [c]	49.58±1.02 [a]	46.94±0.70 [a]	43.36±0.45 [b]
TAC (mM/L)	1.04±0.02 [d]	1.39±0.01 [c]	1.61±0.01 [a]	1.51±0.01 [b]	1.37±0.02 [c]

Antioxidant parameters (TAC, GSH, and MDA) in the intestine of *O. niloticus* fed with chitosan and different levels of chitosan nanoparticles. TAC: Total antioxidant capacity, GSH: Glutathione reduced, MDA: Malondialdehyde. A significant difference *(P*< 0.05) between groups was indicated by different letters according to one-way ANOVA and tested by LSD.

7. Histological appraisals

Microscopy of the intestine after one month of the experiment revealed normal histological structure in the control group, CS group, CNPs1, and CNPs3, whereas, in CNPs5, there was slight edema in the wall of the intestinal villi (Fig. 5). Microscopy of the liver of all groups after 1 month revealed normal histological structure (Fig. 6).

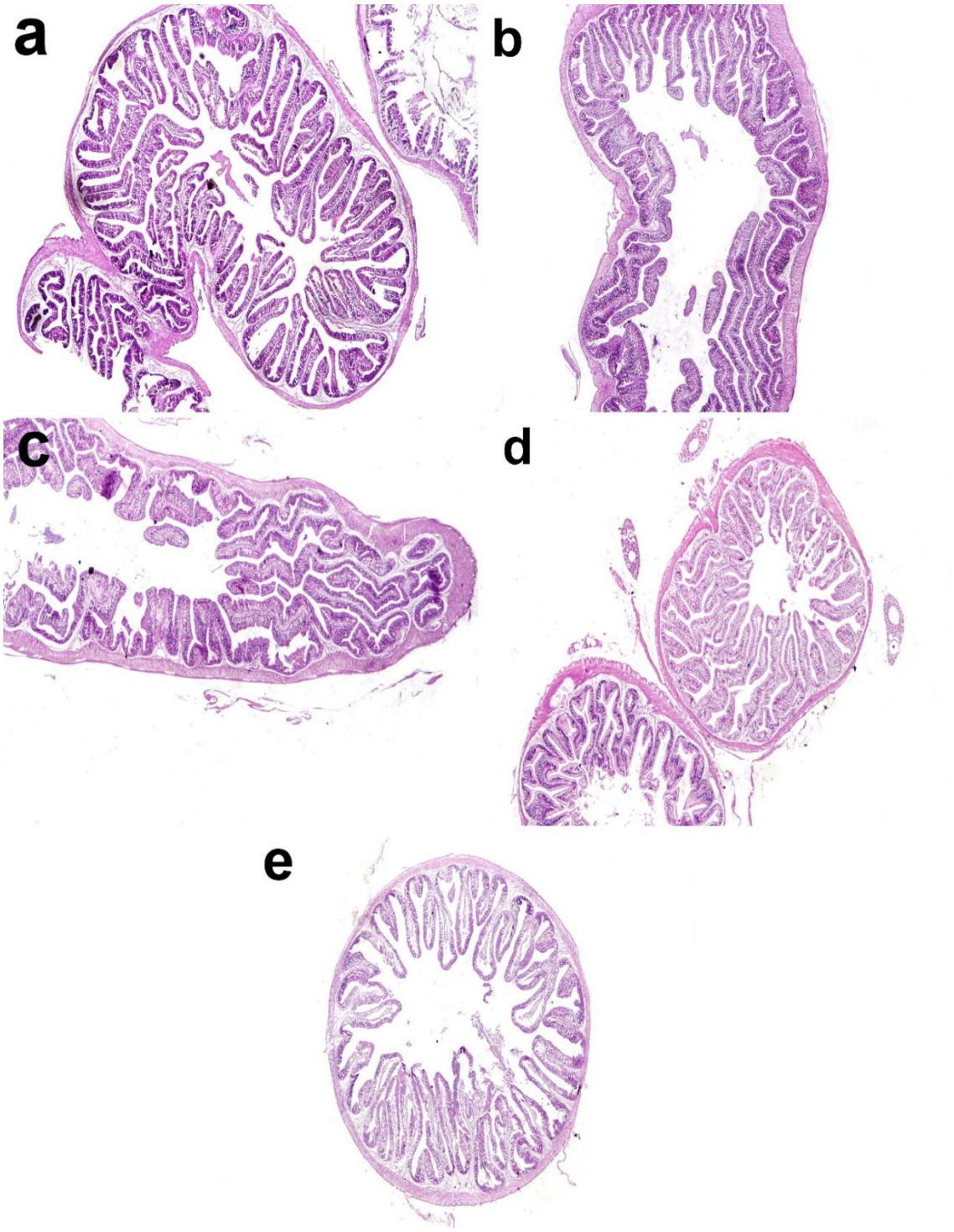

Fig. 5. Histopathological micrographs of the intestine in different groups after one month of experiment showing: **(a)** Normal histological structure in the control group, **(b)** CS group, **(c)** CNPs 1 group, **(d)** CNPs 3 group, and **(e)** mild edema in the lamina propria in CNPs 5 group. Hematoxylin and eosin stain 40X

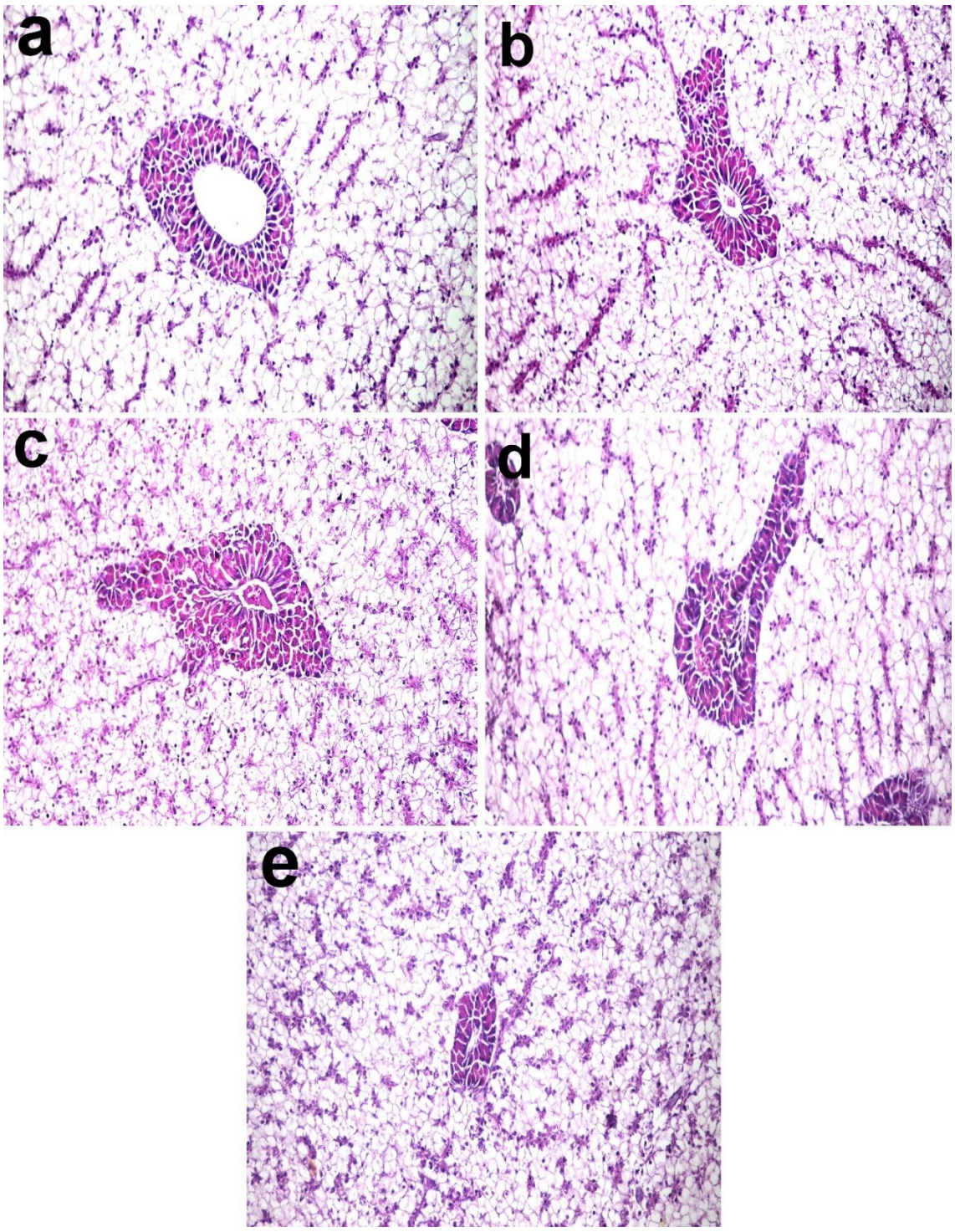

Fig. 6. Histopathological micrographs of liver in different groups after one month of experiment showing: **(a)** Normal histological structure in control group, **(b)** CS group, **(c)** CNPs 1 group, **(d)** CNPs 3 group, and **(e)** CNPs 5 group. Hematoxylin and eosin stain 200X

DISCUSSION

The TEM image of synthesized CNPs showed an average particle size of 50-80nm, and the zeta potential was +39.70mv, respectively (Fig. 2A, D). The particle size of CNPs obtained from dynamic light scattering (DLS) analysis was 263.1nm Fig. (2B) was much bigger than that of the TEM photograph. This may be due to the swollen chitosan hydrogel shell in the aqueous solution, which has shrunk remarkably in the dried state at TEM measurement **(Nikapitiya *et al.*, 2018)**; DLS measurements examined several thousands of particles, while TEM analyzed a limited number of nanoparticles, as well as DLS evaluates the size more quantitatively and gives a larger picture of the whole sample. However, TEM is more qualitative **(Hembram *et al.*, 2016)**. Although the evaluation of the size of nanoparticles by TEM and DLS may be discrepant, complementary methods provide an overall determination of nanoparticle size. FTIR spectrophotometer was used to determine the presence of functional groups of chitosan and TPP in the chitosan nanoparticle solution to ensure the synthesis of the nanoparticle solution. It shows the peak at wavenumbers 3245.42cm^{-1}, indicating the stretching vibration of the hydrogen-bonded (O-H) and N-H stretching from primary amine and type II amide, **Maria Widyasari *et al.* (2019)** reported that a wide peak between 3650-3200cm^{-1} areas was indicated with the stretching vibration of the –NH2 and –OH groups. The peaks formed at 1633.78cm^{-1} indicate the presence of a functional NH amine primary group with a bending vibration band; this finding is supported **by Silverstein *et al.* (2005)** who found that wave number 1650- 1580 cm^{-1} indicates the interaction between NH+3 groups. Wave number 1022.79cm^{-1} indicates a P=O group, **Silverstein *et al.* (2005)** reported that the peak formed at 1040- 910cm^{-1} indicates the functional group P=O, with stretching vibrations that shows the cross-linkage between phosphate groups of TPP and chitosan by an ionic bond.

One effective way to reflect the health state of fish is to measure the growth rate and intestinal morphometric parameters of fish in response to dietary natural supplements **(Abdel Rahman *et al.*, 2021; Abdel Rahman *et al.*, 2022)**. The present study demonstrated that dietary CNPs could be considered as an active growth promoter with immune-modulatory effects on the non-specific immunity of *O. niloticus*. In the present study, growth metrics (WG, SGR, FI, and FCR) were improved in fish given CS and CNPs diets, with a significant improvement at 1g of CS, as well as at 1, 3g of CNPs Kg^{-1} diet when compared to fish fed with basal diet and 5g CNPs kg^{-1}, as well as an increase in villus length and villus width in fish fed a dietary CS and CNPs, without any pathological changes.

This could be explained by improving the intestinal villi height of the small intestine, which enhanced feed intake, nutrient absorption, and subsequent fish growth conditions, as observed in the study of **Zaki *et al.* (2015)**. Furthermore, the potential role of chitosan in enhancing growth performance may be attributed to its vital role in stimulating digestive enzymes by inhibiting potential pathogens and enhancing beneficial

bacteria **(Qin *et al.*, 2014)**. The population of beneficial microorganisms can secrete digestive enzymes to improve feed digestibility and nutrient absorption, and consequently improving feed intake and growth **(Dawood and Koshio, 2020)**. The nano-sized chitosan is characterized by good bioavailability; this could be due to its availability in the bloodstream for an extended period, allowing better feed digestibility, utilization, and absorption and finally higher growth performance. Therefore, **Abdel-Tawwab *et al.* (2019)** recommended that chitosan in nanoscale form at low concentration is more effective than in the ordinary form. Another hypothesis is that nitrogen utilization and amino acid digestibility increased at low concentrations of dietary chitosan **(Shi *et al.*, 2005)**. Furthermore, at high concentrations of dietary chitosan, fish growth is inhibited due to the heavy development in the microvilli of the small intestine to a degree, which resulted in an intestinal blockage; this might hinder food movement, resulting in fish growth regression **(Zaki *et al.*, 2015)**. In this respect, high dietary chitosan levels may increase viscosity and impact gastrointestinal motility, ultimately leading to the suppression of digestive enzymes **(Abdel-Ghany and Salem, 2020)**. Another reason for the observed growth inhibition at high chitosan concentrations may be attributed to the ability of chitosan to reduce fat digestibility by suppressing intestinal absorption of dietary fat leading to hypolipidemia and growth performance inhibition **(Yuan Shi-bin, 2012; Zhang *et al.*, 2012)**.

Several research proved the potential growth-promoting effect of chitosan and chitosan nanoparticles in aqua-feeds. However, the precise inclusion rate and regime for application in aquaculture need further investigation. **Wang and Li (2011)** reported that chitosan in nanoscale has a remarkable enhancement in growth performance as final weight, weight gain, and feed conversion ratio when fortified in the Nile tilapia diet at a concentration of 5.0g Kg^{-1} for 60 days more than ordinary chitosan. Furthermore, **Zaki *et al.* (2015)** noticed that dietary chitosan supplemented to European sea bass (*Dicentrarchus labrax*) at a concentration of 1.0 and 2.0g Kg^{-1} diet has a positive effect on fish growth performance and feed utilization compared to fish fed with 0.5, 3 and 4g kg^{-1}. In another experiment, *O. niloticus* juveniles fed with varying inclusion levels of dietary irradiated CS at concentrations of (10, 20, and 50g kg^{-1}) for 3 weeks revealed no significant effect **(Gonzales *et al.*, 2015)**. Although, **Kamali Najafabad *et al.* (2016)** supplemented the Caspian white fish (*Caspian kutum*) fingerlings diet with different levels of CNP (0.25, 0.5, 1.0, and 2.0g CNP Kg^{-1} diet) for 60 days. The fish group fed with 1.0g kg^{-1} recorded a higher improvement in FCR, intestinal villi length, non-specific immunity, and resistance to some of the environmental stressors. Additionally**, Abdel-Tawwab *et al.* (2019)** supplemented the diet of the Nile tilapia with varying levels of CNP (0.25, 0.5, 1.0, and 2.0g CNP Kg^{-1} diet). After 45 days of feeding experiment, fish fed with CNP had noticeably increased weight gain and feed intake, with an optimal level of 1.0g CNP Kg^{-1} diet. The effects of dietary CS (0, 2, 4, 6, and 8g kg^{-1}) were recently investigated by **Wu (2020)**, who indicated that *O. niloticus* groups fed with CS-

supplemented diets exhibited higher growth performance and immune response than the control group. However, the higher concentrations of the Chitosan 6g kg^{-1} diet did not further increase growth performance, and the inclusion of 8g kg^{-1} diet negatively affected the activity. In contrast, **Abd El-Naby *et al.* (2019)** observed that dietary supplementation of chitosan nanoparticles to the Nile tilapia, *O. niloticus* at different concentrations (0,1.0,3.0, and 5.0g CNPs Kg^{-1} diet) for 70 days, resulted in a significant increase in feed utilization and growth performance with dose-dependent manner, especially in the group fed with 3 and 5g CNPs kg^{-1}. In another study, **Fadl *et al.* (2020)** reported that dietary inclusion of CS to *O. niloticus* has the potential to enhance the growth rate, and FCR and improve disease resistance against *Streptococcus agalactiae* infection with an optimal concentration of 3 and 5g chitosan kg^{-1} diet.

The equilibrium of reactive oxygen species (ROS) formation and clearance is critical for maintaining metabolic homeostasis in the fish body. Under normal physiological conditions, cells generate ROS. Meanwhile, the body is enclosed by a complex network of antioxidant systems that represent SOD, CAT, and GSH-Px to eliminate excessive ROS that may exert serious impairment of DNA and other macromolecules. Analysis of those enzymes can reflect the antioxidant function of the animals and can serve as an indicator of oxidative stress. The oxidative stress leads to an increase in the production of ROS in cellular components which could induce lipid peroxidation and accumulation of peroxides in cells. MDA is an indicator of lipid peroxidation. The current study illustrated that chitosan and CNP reduced the amount of MDA significantly. However, 1 and 3g kg^{-1} CNPs increased the TAC and GSH. The antioxidant activity of chitosan could be attributed to its scavenging properties to free radicles through the donation of hydrogen or one pair of electrons and chelation of metal ions **(Ngo and Kim 2014)**. Chitosan has been used as an antioxidant in fish diets by numerous researchers, who have noted that the antioxidant activities of chitosan grew with increasing concentration **(Sarbon *et al.*, 2015)**. The impact of chitosan as an antioxidant in fish diets has been the issue of several studies. For example, research has been done on the antioxidant activity of chitosan with various molecular weights in salmon (*Salmo salar*) diets. It was mentioned that all diets supplemented with chitosan showed a strong anti-oxidative property of approximately 85% and decreased lipid oxidation **(Kim and Thomas, 2007)**. In this respect, **Abdel-Tawwab *et al.* (2019)** found that the dietary inclusion of CNPs to *O. niloticus* at levels of (0.25,0.5,1 and 2g kg^{-1} diet), significantly enhanced the antioxidant activity of *O. niloticus* in a dose-dependent manner, where CAT and SOD activities increased significantly, while MDA level decreased. Furthermore, **Dawood *et al.* (2020)** mentioned that SOD, $GP_{X,}$ and CAT activities were significantly enhanced, as well as MDA levels significantly decreased by feeding CNPs with an optimal level of 1.0g kg^{-1} diet. Similarly, **Abd El-Naby *et al.* (2020)** investigated the influence of dietary thymol and CNPs on *O. niloticus*. The results revealed that supplementation of CNPs or thymol alone or in combination with each other enhanced the antioxidant activity of fish. Even though the

results show that chitosan is a potent antioxidant, more research is required to determine the precise mechanism of action of chitosan as an antioxidant in fish, as well as the optimal inclusion level.

The first line of defense in fish immunology, innate immunity, is critical in preventing infectious diseases and activating the adaptive immune response. Immunostimulants have been utilized in the aquaculture sector as feed additives to maintain fish health and boost performance and disease resistance. Recently, many dietary immunostimulants in fish have been used, including herbal extracts, algal derivatives, dietary supplements containing PAMPs (Pathogen Associated Molecular Patterns), probiotics, prebiotics, symbiotics, acidifiers, and polysaccharides-containing diets **(Abu Elala and Ragaa, 2015; Vallejos-Vidal *et al.*, 2016; Abu-Elala *et al.*, 2021)**. Moreover, chitosan has been reported to have immune-modulating properties, including cytokine release stimulation, macrophage activation, and increased antibody responses.

Chitosan-derived nanoparticles have innovative and unique characteristics compared to their bulk chitosan due to the higher surface-to-charge density, larger surface area, and better cellular uptake lead to unexpected and significant properties of nanoparticles compared to conventional chitosan **(Poznanski *et al.*, 2023)**.

To drive phagocytes to eliminate infections, macrophages create nitric oxide, a highly reactive oxygen molecule with antibacterial properties. Furthermore, leucocytes secrete lysozyme, which has a bactericidal effect through the breakdown of the bacterial cell wall. Blood protein levels, especially globulin, are thought to reflect the fish's nutritional status and improved immunological response. During phagocytosis, phagocytes emit a respiratory burst (e.g., superoxide, and hydrogen peroxides) to kill invading microorganisms. The respiratory burst test or nitro blue tetrazolium reduction test is widely used to evaluate the immune system's ability to resist infections. Since superoxide anion concentration is the first product released during a respiratory burst, it has been regarded as a reliable test for determining the strength of a respiratory burst. As a result, improvements in respiratory burst and lysozyme activity suggest improved health status of fish.

The role of chitosan as an immunostimulant in fish is still unclear. **Cha *et al.* (2008)** discovered chitosan triggering non-specific defensive systems in the olive flounder (*Paralithchys olivaceus*) owing to its amino moieties. Furthermore, chitosan may enhance the activities of inflammatory cells, such as macrophages, cytokines, polymorphonuclear leucocytes (PMN), and lysozyme activity **(Abdel-Ghany and Salem, 2020)**. **Harikrishnan *et al.* (2012)** studied the effect of chitin and chitosan-enriched diets on the immunological response of kelp groupers (*Epinephelus bruneus*) to *V. alginolyticus* infection. Chitosan supplementation increased nonspecific immunological response, serum lysozyme activity, and resistance to *V. alginolyticus*. Similarly, the immune

response of the Nile tilapia (*O. niloticus*) was enhanced by dietary chitosan nanoparticle supplementation at different levels (1, 3, and 5g kg^{-1} diet). This was accompanied by a significant increase in the counts of red blood cells and total white blood cells **(Abd El-Naby *et al.*, 2019)**. **Abu-Elala *et al.* (2015)** found that dietary chitosan 1% significantly increased various innate immunological parameters, such as NBT, phagocytic activity/ index, lysozyme activity, and ACH50. *Liza ramada* treated with dietary chitosan nanoparticles has a significant immunological response, including increased phagocytic index and lysozyme activity at a concentration of 1g kg^{-1} **(Dawood *et al.*, 2020)**. In this context, **El-Naggar *et al.* (2021)** investigated the effect of 0.5% dietary chitosan (CS) and chitosan nanoparticles (CNPs) supplementation on the immunity and health of the Nile tilapia. They discovered that dietary CNP significantly increased innate immunity, biochemical indices, and antioxidant activity, as well as improved the nutritional value of dietary plant-based protein greater than fish fed on chitosan-containing diet and the basal diet.

CONCLUSION

In conclusion, the results elucidated that the Nile tilapia fed dietary CNPs had unique properties that can improve fish growth performance andintestinal architecture, and modulate immunological response as well as antioxidant status. These results suggest the possibility of utilizing chitosan nanoparticles as a feed additive for aquatic animals.

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