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Immunostimulatory effect of dietary chitosan nanoparticles on the performance of Nile tilapia, *Oreochromis niloticus* (L.)

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ABSTRACT

Feed supplements to fish are generally used to overcome any expected diseases and stressors and to sustain eco-friendly fish farming. One of these feed supplements is chitosan, which stimulated growth and immune properties for many aquatic organisms. It is expected that the nano-sized materials may have stronger immune activation in fish than the ordinary size. Therefore, the current study was conducted to evaluate the effect of dietary chitosan nanoparticles (CNP) on growth performance, antioxidant activity, and innate immunity of Nile tilapia, *Oreochromis niloticus* (L.). Fish (19.8 ± 0.59 g) were fed on diets enriched with 0.0, 0.25, 0.5, 1.0, and 2.0 g CNP/kg diet for 45 days. Fish performance was significantly improved with increasing CNP levels over the control diet with optimum level of 1.0 g CNP/kg diet. Antioxidant-stimulated activity was observed due to dietary CNP supplementation over the control diet in a dose-dependent manner. However, malondialdehyde level decreased significantly, whereas activities of catalase, superoxide dismutase, lysozyme, and respiratory burst increased significantly due to CNP supplementation in a dose-dependent manner. The current study evoked that dietary CNP showed strong immune modulatory properties and enhanced significantly the performance and health of Nile tilapia with optimum level of 1.0 g CNP/kg diet.

1. Introduction

Nile tilapia, *Oreochromis niloticus* (L.), is one of the popular fish species among fish farmers all over the world due to its good growth and high-marketing value [1]. The intensive fish culture is regarded as the most appropriate approach in modern aquaculture although intensified fish may be stressed by deteriorated water quality, hypoxia, bacterial infection, and so on. This, in turn, would suppress immune system and increase the risk of fish farming. Therefore, there is a great interest in developing natural feed additives to overcome any expected difficulties and sustain eco-friendly fish farming.

It is imperative to enhance the feed quality via using natural feed additives to support fish growth, health, immunity, and productivity [2–10]. One of these feed additives is chitosan, which is a linear homopolymer of β-(1,4)-2-amino-2-deoxy-D-glucose prepared by alkaline deacetylation of chitin obtained from crab, shrimp, and crawfish [11]. Chitosan showed unique properties, including low-toxicity, biocompatibility, low-cost, and good handling properties [12]. Currently, chitosan has attracted interest of the aquaculture sector because of its marked anti-bacterial activities for many aquatic organisms against wide range of pathogenic bacteria [11,13–15]. Previous studies showed that chitosan has growth and immune stimulating properties for many aquatic animals [11,15–20].

Nanotechnology is one of the fastest developing fields in biomedical applications, where it is widely used now-a-days in many sectors such as nutrition, therapy, preparations of vaccines, and so on. In this respect, Sasson et al. [21] stated that lowering the particles size into nano-scale improved their solubility, mobility, and efficacy of bioactive agents over larger ones. Hence, nano-sized materials would exhibit novel properties [22] and remain in the blood stream for long period facilitating their good bioavailability [23]. Udayangani et al. [24] reported that smaller size β-glucan particles have stronger immune activation in fish and thereby higher disease resistant capacity. Additionally, nano-sized materials have been used in aquaculture as antimicrobial agents [25]. Some researchers have proposed that chitosan nano-particles (CNP) are more efficient in inhibiting the growth of *Staphylococcus aureus, Candida albicans, Saccharomyces cerevisiae, Escherichia coli,* and *Fusarium culmorum* than ordinary chitosan particles [26,27]. The antibacterial activity of CNP could be attributed to its polycationic nature owing to the presence of primary amine groups in their repeated units. These amine groups bind to the negatively charged bacterial cell wall, altering the membrane permeability, disrupting the

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cell, and subsequently binding to the DNA leading to the inhibition of DNA replication and cell death [28,29]. The use of nano-sized materials with stronger immune stimulatory properties would be a novel technique in aquaculture industry for improving fish growth, health, and immunity by replacing the existing conventional products. Hence, it is hypothesized that the use of CNP may improve feeds characteristics, and in turn, may improve fish performance, health, and immunity. Therefore, the present study was conducted to evaluate the effect of dietary CNP on growth performance, antioxidant activity, and innate immunity of Nile tilapia.

2. Materials and methods

2.1. Perpetration of chitosan nanoparticles

A CNP was prepared based on ionic gelation mechanism of chitosan with tripolyphosphate (PTT) anions according to Zhang et al. [30]. Briefly, chitosan was dissolved in 1% (w/v) acetic acid solution and stirred for 1 h until the solution was transparent. Sodium tripolyphosphate (0.5 mg/ml) was dissolved in deionized water. Under magnetic stirring at room temperature, cross linking was promoted by adding 1 ml of TPP drop wise to 100 ml of chitosan solution. The mixture was stirred for a further 20 min followed by sonication. The resulted suspension was subsequently centrifuged at 10,000 × *g* for 20 min. Supernatants were discarded and the precipitate was suspended in distilled water, centrifuged again to remove any sodium hydroxide and then freeze-dried (Virtis Advantage EL, SP Industries Company, USA) before further use. The morphological examination of CNP was performed by transmission electron microscopy (TEM). Transmission electron micrographs were captured using a JEM-1400 plus (USA) at 120 kV. Particle size and zeta potential of CNP were measured by Zeta sizer® (Nano-ZS90, Malvern instruments Ltd., Malvern, UK) in triplicates and its range was 10.59–41 nm with an average size of 35 nm.

2.2. Diet preparation and fish culture

Five diets containing 0.0, 0.25, 0.5, 0.1, and 2.0 g CNP/kg diet were formulated to contain 30% crude protein (Table 1). However, CNP of each diet was suspended in 100 ml per kg diet and blended with the other ingredients for 30 min to make a paste of each diet. The pastes were separately passed through a grinder and pelleted through 1-mm diameter paste extruder. The diets were oven-dried at 55 °C for 24 h and stored in plastic bags at – 2 °C for further use.

Nile tilapia, *O. niloticus* (L.), fingerlings were obtained from nursery ponds, Central Laboratory for Aquaculture Research (CLAR), Abbassa, Abo-Hammad, Sharqia, Egypt. Fish were kept in an indoor aerated fiberglass tank for two weeks for adaptation to the laboratory condition and light-dark regime was maintained at 12 - 12 using fluorescent tubes as a light source. Fish (19.8 ± 0.59 g) were randomly distributed at a density of 20 fish per 100-L aquarium in quadruplicates. Each aquarium was supplied with compressed air via air-stones using aquarium's air pump. Fish were fed on one of the tested diets up to apparent satiation twice daily at 9:00 and 14:00 h for 45 days. Settled fish waste along with a half of an aquarium's water was siphoned daily and replaced by clean and well-aerated tap-water from a storage tank. Fish mortality was recorded daily and dead fish were removed.

2.3. Water quality parameters

Water samples were collected weekly from each aquarium to monitor different parameters of water quality. Water temperature and dissolved oxygen were measured in site using a portable oxygen meter (Jenway, London, UK). The pH value was measured using a pH-meter (Digital Mini-pH Meter, model 55, Fisher Scientific, Denver, CO, USA). The unionized ammonia (NH_3) was measured using a Multi-parameters Ion Analyzer (HANNA Instruments, Rhode Island, USA). The parameters of water quality did not show any significant differences due to dietary CNP supplementation and the range of water temperature was 26.8–27.8 °C, dissolved oxygen was 5.8–6.3 mg/L, pH was 7.7–7.8, and unionized ammonia concentration was 0.14–0.32 mg/L. All these ranges are within the acceptable ranges for fish farming [31].

Table 1
Ingredients and proximate chemical composition (% on dry matter basis) of diets containing different levels of chitosan nanoparticles.

Ingredients	Chitosan nanoparticles (g/kg diet)				
	0.0 (control)	0.25	0.5	1.0	2.0
Fish meal (72% CP)	104	104	104	104	104
Soybean meal (44% CP)	430	430	430	430	430
Ground corn	203	203	203	203	203
Wheat bran	155	154.75	154.5	154	153
Cod fish oil	23	23	23	23	23
Corn oil	15	15	15	15	15
Vitamins premix[a]	10	10	10	10	10
Minerals Premix[b]	20	20	20	20	20
Starch	40	40	40	40	40
Chitosan nanoparticles	0.0	0.25	0.5	1.0	2.0
Total	1000	1000	1000	1000	1000
Proximate chemical analysis (%)					
Dry matter	92.3	91.9	91.8	92.1	91.5
Crude protein	30.8	30.9	31.2	30.2	31.7
Ether extract	7.1	7.3	7.3	7.2	7.2
Total ash	5.2	5.1	5.4	5.9	5.3
Crude fiber	7.2	7.5	7.2	7.6	7.8
Nitrogen free extract[c]	49.7	49.7	49.6	49.6	49.3
Gross energy (Kcal/100g)[d]	445.4	445.8	446.2	440.5	444.4

[a] Vitamin premix (per kg of premix): thiamine, 2.5 g; riboflavin, 2.5 g; pyridoxine, 2.0 g; inositol, 100.0 g; biotin, 0.3 g; pantothenic acid, 100.0 g; folic acid, 0.75 g; *para*-aminobenzoic acid, 2.5 g; choline, 200.0 g; nicotinic acid, 10.0 g; cyanocobalamine, 0.005 g; α-tocopherol acetate, 20.1 g; menadione, 2.0 g; retinol palmitate, 100,000 IU; cholecalciferol, 500,000 IU.

[b] Mineral premix (per kg of premix): $CaHPO_4.2H_2O$, 727.2 g; $MgCO_3.7H_2O$, 127.5 g; KCl 50.0 g; NaCl, 60.0 g; $FeC_6H_5O_7.3H_2O$, 25.0 g; $ZnCO_3$, 5.5 g; $MnCl_2.4H_2O$, 2.5 g; $CuCl_2$, 0.785 g; $CoCl_3.6H_2O$, 0.477 g; $CaIO_3.6H_2O$, 0.295 g; $CrCl_3.6H_2O$, 0.128 g; $AlCl_3.6H_2O$, 0.54 g; Na_2SeO_3, 0.3 g.

[c] Nitrogen free extract (NFE) = 100 – (protein % + lipid % + total ash % + crude fiber %).

[d] Gross energy was calculated according to NRC (1993) as 5.65, 9.45, and 4.11 kcaL/g for protein, lipid, and carbohydrates, respectively.

2.4. Growth and feed utilization parameters

After the feeding trial, fish were collected, counted, and bulk-weighed. Growth performance was determined and feed utilization was calculated as follows:

Weight gain = $W_2 - W_1$;
Weight gain % = 100 ($W_2 - W_1$)/W_1;
Specific growth rate (SGR) = 100 [Ln W_2 (g) – Ln W_1 (g)]/T; where W_2 is final weight, W_1 is initial weight, and T is the experimental period (day);
Feed intake (g feed/fish) = the summation of the diets offered to fish throughout the experiment/fish number;
Feed conversion ratio (FCR) = feed intake/weight gain;
Fish survival (%) = 100 (fish number at final/fish number at initial).

2.5. Assessments of antioxidant and immunity enzymes

At the end of the feeding trial, fish were not fed 24 h immediately prior to blood sampling and blood was collected from the caudal vein via heparinized syringe. The collected blood was centrifuged at 5000 × *g* for 15 min at room temperature. The collected plasma was

stored at −20 °C for further assays. The activities of antioxidant enzymes in fish plasma were measured using the diagnostic reagent kits according to the manufacturer's instructions (MyBioSource Inc., San Diego, California, USA). Malondialdehyde (MDA) level was analyzed by thiobarbituric acid method [32]. Activities of catalase (CAT) and superoxide dismutase (SOD) were measured spectrophotometrically according to methods described by Aebi [33] and McCord and Fridovich [34], respectively. The respiratory burst (RB) activity was determined by assaying the production of superoxide ion by leukocytes via assaying the reduction of Nitro Blue Tetrazolium (NBT, Sigma-Aldrich Chemical, St. Louis, MO, USA) according to Rook et al. [35]. Lysozyme activity of fish plasma was determined by turbidometric assays as described by Caruso et al. [36].

2.6. Statistical analysis

The obtained data were subjected to one-way ANOVA to evaluate the effect of dietary CNP supplementation. Differences between means were tested at 5% probability level using Duncan test as a post-hoc test. The optimum CNP level was determined using a polynomial regression analysis. All the statistical analyses were done using SPSS program version 20 (SPSS, Richmond, VA, USA) as described by Dytham [37].

3. Results

It was noticed that dietary CNP has a positive effect on fish growth and feed intake, which were significantly higher when fish fed CNP-enriched diets as compared to those fed the control diet ($P < 0.05$; Table 2). The relationship between final weight and CNP levels (Fig. 1) was best expressed by the second-order polynomial regression equations as follows:

$$Y = -0.75\ X^2 + 6.45\ X + 25.34\ (R^2 = 0.989)$$

This figure showed that the optimum CNP level for Nile tilapia was 1.0 g/kg diet. Moreover, fish fed on diets containing 1.0–2.0 g CNP/kg diet consumed more diet (30.6–30.7 g feed/fish, respectively) than the control diet (15.1 g feed/fish); meanwhile no significant ($P > 0.05$) change in FCR values was observed and its range was 1.41–1.45. Throughout the feeding period, fish in all experimental groups were in good health as observed from their general activity. Fish survival ranged from 96.7 to 100% with no significant ($P > 0.05$) difference among the different fish groups ($P > 0.05$; Table 2). This result suggests that dietary CNP has no toxic effect on fish.

Regarding the antioxidant activity, it was noticed that plasmatic MDA was significantly ($P < 0.05$) prohibited, whereas CAT and SOD activities were significantly induced with increasing dietary CNP levels ($P < 0.05$; Table 3). Likewise, dietary CNP promoted the innate immunity of Nile tilapia where lysozyme and respiratory burst activities were significantly maximized in fish fed 2.0 g CNP/kg diet as compared with the control group (19.7 unit/mg protein and 3.40 mg/mL, respectively).

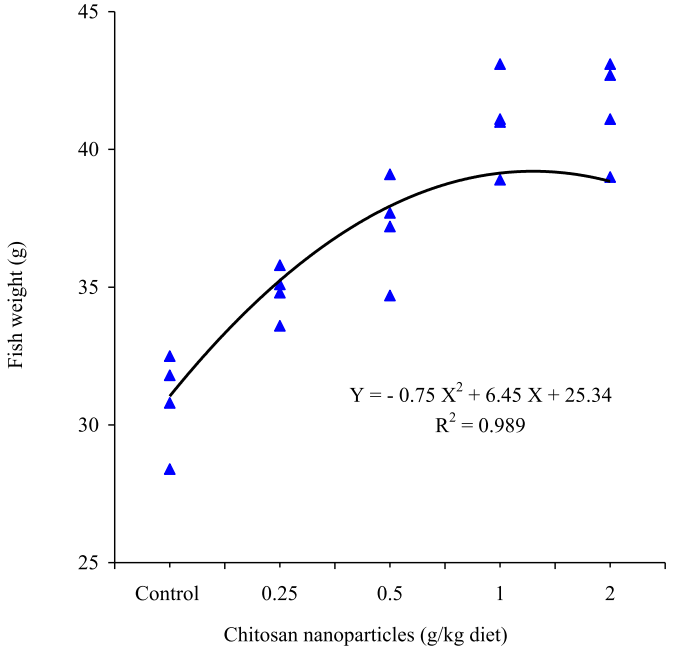

Fig. 1. The relationship between final weight (g) of Nile tilapia and different levels of chitosan nanoparticles for 45 days.

4. Discussion

The present study indicates that dietary CNP could be used as a potential feed additive to enhance performance and innate immunity of Nile tilapia with an optimum level of 1.0 g CNP/kg diet. Chitosan is an active growth promoter and could be considered as an essential element for the growth of aquatic animals [38]. The improvement in fish growth may be attributed to enhancing the intestinal villi height of the small intestine; that improved nutrients absorption, feed intake, and subsequent fish growth, as has been revealed by Zaki et al. [39] who found that the chitosan supplementation to European sea bass, *Dicentrarchus labrax* at levels of 1.0 and 2.0 g/kg diet enhanced the growth performance and feed utilization. These findings are supported by Shi et al. [40] who found that at a low concentration of dietary chitosan elevated significantly nitrogen utilization and amino acid digestibility in broiler chickens. The obtained results herein may be because CNP supplementation to Nile tilapia enhanced their digestion and nutrient digestibility leading to improved nutrient utilization/assimilation, which in turn improved feed intake and growth. Another hypothesis is that CNP may inhibit potential pathogens, enhance the population of beneficial microorganism, and/or enhance the microbial enzyme activities in fish gut that consequently improve feed digestibility and nutrient absorption/assimilation. In this respect, Abd El-Naby et al. [41] fed Nile tilapia on diets containing 0.0, 1.0, 3.0, and 5.0 g CNP/kg diet for 70 days. They found a concentration-dependent decrease in the total anaerobic and aerobic bacterial count in fish intestines after CNP

Table 2
Growth performance, feed intake, feed conversion ratio (FCR), and survival rate (Mean ± SE) of Nile tilapia, O. niloticus, fed diets containing different levels of chitosan nanoparticles for 45 days.

	Chitosan nanoparticles (g/kg diet)				
	0.0 (control)	0.25	0.5	1.0	2.0
Initial weight (g)	19.8 ± 0.59	19.7 ± 0.32	19.7 ± 0.95	19.7 ± 0.40	19.9 ± 0.09
Final weight (g)	30.6 ± 1.18 d	34.8 ± 0.65 c	37.2 ± 1.29 b	41.0 ± 1.21 a	41.1 ± 1.83 a
Weight gain (g)	10.8 ± 0.76 d	15.1 ± 0.68 c	17.5 ± 0.69 b	21.3 ± 0.88 a	21.2 ± 1.16 a
Weight gain %	54.6 ± 3.22 d	76.6 ± 4.07 c	88.8 ± 4.76 b	108.1 ± 3.12 a	106.5 ± 5.71 a

Means having the same letter in the same row are not significantly different at $P < 0.05$.

Table 3
Changes in malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), lysozyme, and respiratory burst activity (Mean ± SE) of Nile tilapia, *O. niloticus*, fed diets containing different levels of chitosan nanoparticles (CNP) for 45 days.

Respiratory burst activity (mg/mL)	Lysozyme (unit/mg protein)	SOD (IU/L)	CAT (IU/L)	MDA (nmol/L)	CNP (g/kg diet)
1.73 ± 0.096 d	4.0 ± 0.50 d	15.6 ± 0.73 d	16.7 ± 0.41 d	4.6 ± 0.14 a	0.0 (control)
2.15 ± 0.044 c	8.7 ± 0.49 c	21.8 ± 1.71 c	19.4 ± 0.72 c	4.1 ± 0.15 ab	0.25
2.32 ± 0.075 c	9.3 ± 0.36 c	26.8 ± 4.11 bc	20.3 ± 0.64 bc	3.6 ± 0.30 b	0.5
2.94 ± 0.107 b	12.1 ± 1.30 b	28.8 ± 1.45 ab	23.9 ± 1.54 b	2.9 ± 0.20 c	1.0
3.40 ± 0.142 a	19.7 ± 0.57 a	31.5 ± 0.99 a	33.2 ± 2.98 a	1.6 ± 0.21 d	2.0

Means having the same letter in the same column are not significantly different at $P < 0.05$.

supplementation. Li et al. [42] found that the inclusion of chitosan supplementation to crucian carp (*Allogynogenetic crucian*) juvenile at levels of 5 and 7.5 g/kg diet affected significantly the abundance and diversity of detected intestinal bacterial; however, some detected pathogenic bacteria either decreased or totally disappeared. Besides the above mentioned reasons, Abd El-Naby et al. [41] found that dietary CNP increased significantly activities of digestive enzymes leading to subsequent increases in feed intake and fish growth. They also reported that fish fed CNP-supplemented diets resulted in significantly higher fish performance than the control diet with an optimum level of 1.0 g/kg diet.

On the other side, nano-sized chitosan could have played a crucial role in improving the assimilation and absorption of nutrients at lower concentrations in fish as compared to ordinary chitosan [15]. In this respect, Wang and Li [43] compared the growth response of Nile tilapia to dietary chitosan and CNP, where fish fed chitosan and CNP-enriched diets at a level of 5.0 g/kg of diet for 60 days. They found that the dietary CNP improved significantly final weight, daily weight gain, and feed conversion ratio compared to fish fed chitosan-containing diet and the control diet. This comparison indicates that nano-sized chitosan is more efficient than its ordinary form. These results may be because the CNP remained in the blood stream for long period facilitating its good bioavailability. In another study, Korni and Khalil [44] used traditional ginger and its nanoparticle in diets for common carp, *Cyprinus carpio*. They found that fish growth, immunity, and challenge against *Aeromonas* septicemia were more efficient with ginger nanoparticles than the traditional form. Abdel-Tawwab et al. [45] evoked that dietary nano-sized cinnamon enhanced the growth, health, and immunity of Nile tilapia over the traditional form of cinnamon represented by Ahmad et al. [46].

The activity of antioxidant enzymes and lipid peroxidation (indicated by MDA) are indicative biomarkers of oxidative cell damage and examples of the toxic mechanisms of reactive oxygen species (ROS), which are involved in pathological processes and in the aetiology of many fish diseases [47]. Hence, they are commonly used as biomarkers and quick responses to ROS generation [48,49]. The present study evoked that dietary CNP had antioxidant activity where MDA level decreased significantly, while activities of CAT and SOD increased significantly in fish fed CNP-enriched diets in a dose-dependent manner. Antioxidant activity of chitosan could be attributed to free radical-scavenging activities on hydroxyl radicals and its chelating abilities [50,51]. Chitosan could chelate metal ions or scavenge free radicals through the donation of hydrogen or one pair of electrons [52,53]. In this concern, Kim and Thomas [54] found that chitosan-supplemented diets for salmon (*Salmo salar*) showed anti-oxidative activities and reduced lipid oxidation. It has been reported that the antioxidant properties of chitosan increased progressively as its concentration increased [38,55].

Immunostimulants can be defined as natural or synthetic substances capable of activating non-specific and/or specific immune responses [56]. The inclusion of immunostimulants in aquaculture practices is upcoming now. Recently, chitosan has been proved as an effective immunostimulant in different species of fish [38]. In the present study, higher activities of lysozyme and RB were observed in CNP-fed fish in a dose-dependent manner. It is known that lysozyme and RB activities have important roles in the non-specific immune defense system [57,58]. The lysozyme is a cationic enzyme that breaks β-1, 4 glycosidic bonds between N-acetylmuramic acid and N-acetyl glucosamine in the peptidoglycan of bacterial cell walls. It is known to attack mainly the Gram-positive and Gram-negative bacteria [59]. On the other hand, respiratory burst is produced by phagocytes in order to attack invasive pathogens during phagocytosis, and have been widely used to evaluate the defense ability against pathogens. Since superoxide anion is the first product released during the respiratory burst, its concentration has been accepted as an accurate parameter to quantify the intensity of respiratory burst [60]. Hence, the increments in lysozyme and RB activities are indicative to an increased level of fish protection against possible bacterial infection. Harikrishnan et al. [61] reported that the dietary chitosan supplementation stimulated the immunological parameters in kelp grouper, *Epinephelus bruneus*. Similar results demonstrated that the dietary chitosan supplementation to fish showed immune-modulating effects on some nonspecific immune functions [11,15,18–20,62–64]. The mechanism of action of immunostimulation effect of chitosan inside fish body is not fully understood yet. Cha et al. [19] attributed the immune-inducing characteristic of chitosan for olive flounder (*Paralithchys olivaceus*) by inducing non-specific defense mechanisms in the fish body via its amino moieties. Moreover, the immunostimulation effect of chitosan may be via promoting the functions of inflammatory cells such as polymorphonuclear leucocytes, cytokine, and macrophages [65,66].

5. Conclusion

The present study evoked that dietary CNP enhanced significantly the fish performance with an optimum level of 1.0 g/kg diet. Moreover, dietary CNP enhanced significantly the antioxidants and innate immunity response of Nile tilapia.

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