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Comparison between effect of chitosan and Nano-chitosan as feed additives on cultured tilapia nilotica fish (*Oreochromis niloticus*)

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ABSTRACT

Aeromonas hydrophila, a common cause of disease outbreaks in various fish species, was isolated, biochemically and molecularly identified, and subjected to antimicrobial sensitivity tests. The isolates exhibited multiple resistances toward various antibiotics, so searching for an eco-friendly substitute is essential. Fish feed additives are used to combat potential diseases or stressors. Chitosan and its Nano scale materials, which are anticipated to have stronger immune stimulatory effects than conventional materials, are among these feed additives. Two experiments were performed to compare and assess the therapeutic and adverse impacts of chitosan (Ch) and chitosan-nanoparticles (ChNPs) and assess the efficacy of various treatments against *A. hydrophila* infection in *Oreochromis niloticus*. First experiment, *O. niloticus* (n=180; 26±2 g) were distributed into 3 groups each in triplicate (n=60 fish/group); CTR: negative control; Ch: chitosan (10 g/kg diet); and ChNPs: chitosan nanoparticles (100 mg/kg diet) for 4 weeks. No mortalities or significant negative impacts were noted in any groups. The dietary inclusion of Ch and ChNPs positively impacted fish growth performance, feed intake, hematological parameters, immune response, and antioxidant capacity. It significantly reduced the total aerobic bacterial count in fish flesh, demonstrating the immunostimulant and antibacterial effects of chitosan. Second experiment, each group from the 1[first]experiment

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was divided into 2 subgroups of 20 fish each (n=6 groups; 20 fish/group). CTR group subdivided into G1: infected control and G2: infected and treated with levofloxacin (LEV), Ch group subdivided into G3: infected-Ch and G4: infected-Ch+LEV, and ChNPs group subdivided into G5: infected- ChNPs; and G6: infected-ChNPs+LEV for 10 days. The hematological parameters of all the treated groups were closer to the CTR values in the 1st experiment than in the infected CTR group. Liver enzymes and kidney markers were lower in all treated fish. Immune responses were significantly greater ($P \leq 0.05$) in the Ch/ ChNP groups before and after challenge. The mortality rate decreased in the G6 group, followed by the G5 and G4 groups after infection. In conclusion, feeding *O. niloticus* a 10 g Ch or 100 mg ChNPs/kg diet improved growth performance and enhanced antioxidant capacity.

INTRODUCTION

Aquaculture is a rapidly growing industry for food production that seeks to meet the protein demand for human consumption and contributes to meeting the growing global food demand. Fish culture intensification is very important for fulfilling the fish demand as a cheap and high-quality protein source, thus resulting in increased disease incidence due to high stocking densities in limited areas **(Ibrahim et al. 2021)**. However, several diseases have been identified in fish farming, *Aeromonas hydrophila* is considered one of the most important causes of disease outbreaks and is responsible for large economic losses. **Chikwendu et al. (2020)**, demonstrated the highest rates of resistance of *A. hydrophila* to common antibiotics. This bacterium (motile *Aeromonas* septicemia, MAS) can also behave as a secondary opportunistic pathogen by attacking already compromised or stressed hosts, causing severe mortality **(Pauzi et al. 2020)**.

Nanotechnology in aquaculture has several important roles that significantly fund its progress. Moreover, these findings will aid in the control of infections The uses of nanotechnology include disinfection of ponds, treatment of water **(Ajith et al. 2021)**, identification and elimination of water-borne diseases, competent supply of drugs and nutrients, and increased absorption of these substances by fish. Since chitosan (Ch) is a biodegradable carbohydrate with numerous applications in many sectors, it is one of the most commonly used substances in the nanotechnology field **(Harugade, Sherje & Pethe 2023)**. It is abundant in the arthropod exoskeletons, for instance, in crab and shrimp exoskeletons, in addition to walls of the yeast and cuticles of insects **(Abo Elsoud et al. 2019)**. Ch and ChNPs nanoparticles have been used for medical purposes since ancient times because of their antibacterial properties**.** An important weapon in the fight against antibiotic resistance is the use of natural antimicrobial alternatives to conventional antibiotics. The highly reactive surfaces of nanoparticles (NPs) suggest that they might have antimicrobial effects.

Oreochromis niloticus is a highly valuable freshwater-cultured fish in Egypt that provides more than 67% of the total fish production. Because of its adaptability to commercial feed components, rapid growth, and tolerance to laboratory conditions, it has been widely employed for laboratory research **(Abdel-Latif et al. 2020)**.

Three counterparts share the same life circle: fish, pathogens, and the environment. The environment must be properly upgraded, and pathogen count/virulence should be monitored to maintain ideal health. The hypothesis under consideration is the use of bioactive immunostimulants to attain these goals. Therefore, this work is aimed to assess the antibacterial and immune-modulatory properties of Ch and ChNPs and compare their bio-therapeutic effects with those of antibiotics for the control of *A. hydrophila* infection in *O. niloticus,* a particularly hardy strain that causes problems in Egyptian fish farms. Moreover, this trial was conducted to provide additional data on the impacts of Ch and ChNPs on nonspecific immunity, antioxidant enzymes, and hemato-

biochemical parameters in non-infected and infected fish and to evaluate their effects on the total aerobic bacterial count of fish flesh and whole fish body composition in the non-infected groups.

MATERIALS and METHODS

Naturally Infected Fish

O. niloticus were collected from private fish farms in Kafrelsheikh governorate, Egypt (n =100, ten fish per farm) during the summer season, The fish died during the summer season and exhibited specific clinical symptoms of hemorrhagic septicemia, including skin ulceration, pale gills, and hemorrhages. In an oxygen-supported tank, fish were transported to the Kafrelsheikh Province Laboratory, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Egypt; and the fish samples were examined within 1–2 hrs. The average body weight (BW) of diseased fish was 30±5 g.

Bacteriological examination

Swaps were taken from the brain, skin, eyes, and internal organs (spleen, liver, and kidney), and the tissues were enriched in tryptic soya broth TSB (Oxoid, USA) and incubated at 25°C for 24h before being streaked on tryptic soya agar TSA. Aeromonas Medium Base was supplemented with 2.5mg/ 500ml ampicillin in the diluted media and incubated at 25°C for 24-48h. Purified colonies (4-5 tiny dark green convex with a dark green center) were biochemically identified for the following tests: catalase and oxidase (Oxoid, USA) according to the methods of Holt et al. (1994). Additional characterization of the colonies was performed using an API-20E kit (BioMérieux Marcyl'Étoile, France), which performs 20 biochemical tests. Purified biochemically identified isolates were kept at -20°C in brain heart infusion broth (BHI) supplemented with 15% glycerol for subsequent molecular characterization **(Nagarajan et al. 2008)**.

Molecular characterization of the virulence genes of *A. hydrophila* strains

Using specific standard strain *A. hydrophila* ATCC 7966^T (as positive control) Oligonucleotide Primer (Metabion, Germany), three virulence genes, aerolysin (*aer*), lipase (*lip*), and hemolysin (*hly*) of isolated field strains were examined. Table 1 lists the primer sequences, target genes, amplicon sizes, and cycling conditions for the primers.

DNA extraction: DNA was extracted from overnight cultures on TSA (Oxoid®, USA) following the manufacturer's instructions for the QIAamp DNA Mini Kit (Qiagen, Germany, GmbH) with some changes.

Amplification via PCR A 25-µl reaction mixture containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer at a concentration of 20 pmol, 5.5 µl of nuclease-free water, and 6 µl of DNA template was used to test the primers. Each run included a negative control (PCR devoid of DNA template) and a positive control (standard strain *A. hydrophila* ATCC 7966^T). The reaction was conducted using a heat cycler (Applied Biosystems, model 2720).

Analysis of the PCR products: At room temperature, the PCR products were electrophoresed on a 1.5% agarose gel in 1x TBE buffer (Applichem, Germany, GmbH). The sizes of the fragments were determined using a ruler with a 100 bp ladder (Fermentas, Germany). Photographs of the gel were taken using a gel documentation system (Alpha Innotech, Biometra), and the information was analyzed using software.

Table 1. Primer sequences, target genes, amplicon sizes, and cycling conditions

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
aerolysin	CACAGCCAATATGTCGGTGAAG GTCACCTTCTCGCTCAGGC	326	94˚C 5 min.	94˚C 30 sec.	52˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	(Singh et al. 2008)
Lipase	ATCTTCTCCGACTGGTTCGG CCGTGCCAGGACTGGGTCTT	382	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	(Sen and Rodgers 2004)
hemolysin	GGCCGGTGGCCCGAAGATACGGG GGCGGCGCCGGACGAGACGGGG	592	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	(Rozi et al. 2018)

Antimicrobial Susceptibility of *A. hydrophila*

The isolated identified 37 field strain isolates of *A. hydrophila* were examined for susceptibility to antimicrobial agents using the disc diffusion method against 12 commonly used antimicrobial agents on Mueller–Hinton agar (Oxoid, USA) following the guidelines of CLSI (2020) for Aeromonas species. The 12 antibiotic discs (Oxoid, Waltham, MA, USA) used were Levofloxacin (LEV; 5µg), Fosfomycin (200 µg), Spectinomycin (SPT; 10 µg), Florfenicol (KF; 10 µg), Cefotaxime (CTX; 30 µg), Ampicillin (AMP; 10 µg), Amoxicillin (AX; 30 µg) Doxycycline (DO; 30 µg), Oxytetracycline (OTC; 30 µg), Sulfadimethexine (200 µg), Tylosin (30 µg), and Erythromycin (E; 15 µg). The inhibition zone diameters were measured, and the results were categorized and interpreted following previous methods of **CLSI (2020)**.

Tested chemicals

Chitosan was purchased from Chitosan Egypt Company, and 1% ChNP suspension (10 mg/ml) was purchased from Nano-Tech Egypt.

ChNPs characterization and in vitro antibacterial activity

The morphology and particle size were detected using scanning electron microscopy (SEM) (JUMP UP).

The antibacterial activity of ChNPs was investigated by SEM on *A. hydrophila* cultures on media supplemented with 1.25 mg/ml ChNPs **(El-Sherbeny et al. 2022)** and kept at 37°C overnight. The cultures were then placed in a Pelco Biowave (Ted Pella, Inc., Redding, CA, US) at 120 W under vacuum in the primary fixative and microwave. Spurr's resin was applied to the bacterial samples, which were polymerized for 24 h at 60°C. The sample blocks were sliced into thin sections of 85 nm using Leica Ultra cut microtomes (Leica Microsystems GmbH, Wetzlar, Germany). The sections were placed on thin bar grids and stained for 20 minutes with 5% uranyl acetate and 10 minutes with Sato's triple lead stain.

The stained sections were examined using SEM (JUMP UP) **(Tahmasebi et al. 2015)**.

Experimental fish and culture facilities

All the experiments methodology, protocols, and animal care were conducted in accordance with relevant guidelines and regulations approved of the Committee for Aquatic Animal Care of Faculty of the Aquatic and Fisheries Sciences at Kafrelsheikh University, Egypt (Approval No. IAACUC-KSU-138-2020). A total of 180 *O. niloticus* (26±2 g) were collected randomly from a local fish farm in Kafrelsheikh governorate. The fish showed normal clinical signs, had no history of disease outbreaks, and were transmitted alive to the Kafrelsheikh provincial lab of the Animal Health Research Institute. The fish were acclimated for 2 weeks to the laboratory conditions and kept in glass tanks filled with aerated, dechlorinated fresh water at 25±2°C. The water was changed by one-third daily and fully once weekly after the debris was removed. During the acclimation period, the fish were fed *ad libitum*. The water parameters were checked daily, and throughout the experiments, the mean values were set as follows: pH (7.09-8.18), DO (7.02 ± 0.12 mg/ l), ammonia (0.02 ± 0.002 mg/L), and water temperature (25 ± 2° C). All water parameters were within the permissible limits for *O. niloticus* rearing.

Preparation of fish diets

Commercial fish feed was purchased from Aller-Aqua Company, Egypt (3100 KJ/kg of digestible energy and 30% crude protein). After grinding the pellet feed, 10 g of Ch and 10 mL of CNPs suspension /kg diet (equivalent to 100 mg CNPs/ kg diet) were thoroughly mixed with the diet for 30 minutes to form a dough. Before being extruded into 1-mm-diameter pellets, each dough was passed through a grinder. Before being held in plastic bags at a temperature of -2°C, the diets dried. The control diet was prepared in the same way without any additives.

Experimental design

Two experiments were carried out in this investigation (Fig.1). In the first experiment, three experimental treatments were designed, each in triplicate (60 fish/group). One group served as a CTR (control) and was fed a control diet, and the other two groups were fed either Ch (10 g/kg diet) or ChNPs (100 mg/kg diet) for four weeks. In the second experiment, each group from the 1st experiment was divided into two subgroups (n=6 groups, 20 fish/ group) and half of each experimental diets was sprayed with levofloxacin (LEV) with a coating solution (1% gelatin and 1% tamarind gum). During the experiment, the fish were fed twice daily at 2% of their BW. The fish were weighed every two weeks, and the feed intake was changed based on the new weight. The dose of LEV (10 mg/kg body weight) was chosen according to **El-Sherbeny et al. (2022)**.

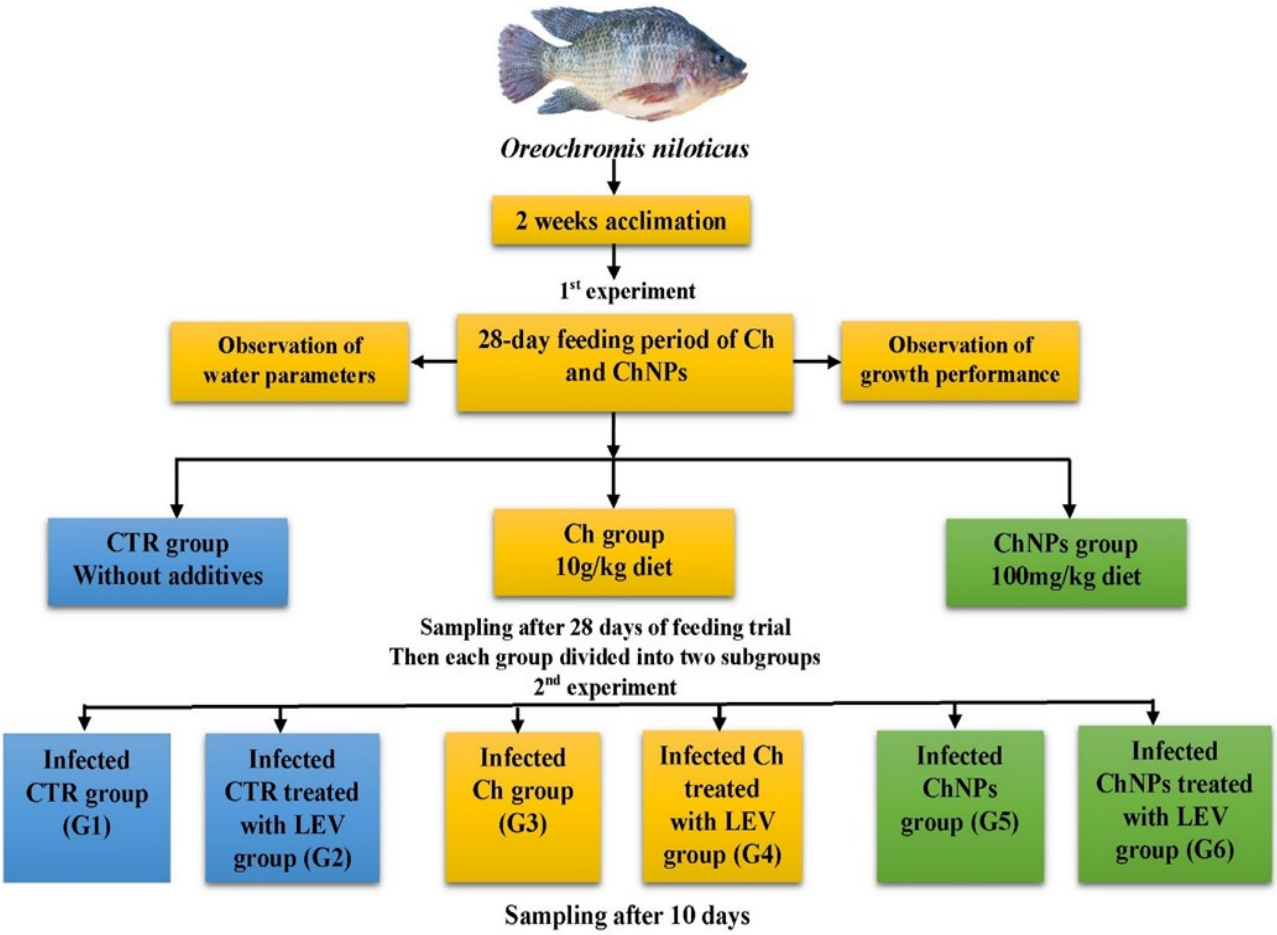

Fig. 1 Experimental design (n= 180 for 1st experiment and 120 for 2nd experiment). CTR=control; Ch= chitosan; ChNPs= chitosan nanoparticles; LEV=levofloxacin

Growth performance

The total live BW of each aquarium was measured every two weeks to assess the fish growth parameters. *O. niloticus* growth parameters were assessed at the end of the 1st experiment (28 days) with the following formulas **(Abu-Zahra et al. 2024)**:

$$\text{WG (weight gain, g)} = \text{W2 (Final BW)} - \text{W1 (Initial BW)}$$

$$\text{ADG (average daily gain, g)} = \frac{\text{W2 - W1}}{\text{Number of days of the feeding trial}}$$

$$\text{Feed efficiency} = \frac{\text{Total BW gain for each aquarium}}{\text{Total feed intake in that aquarium}}$$

$$\text{Survival rate (\%)} = \frac{\text{No. of live fish at the end of the experiment}}{\text{No. of fish at the start of the experiment}} \times 100$$

Chemical analysis of body composition pre-challenge

Nine fish were collected from each treatment at the end of the 1st trial (28 days). After deep anesthesia using 40% ethyl alcohol, the fish were autoclaved (110°C, 3 h), diced into sections of the same size, homogenized with a lab mixer, and stored at -20°C before being dried in a hot air oven (60°C, 48 h). Estimates of moisture, crude protein, crude lipids, crude fiber, the total amount of ash, the total amount of carbs, and the total amount of energy were made following the procedures outlined by the

AOAC (2019). All of the samples were transported in ice boxes to the laboratory within 3 h after sampling.

Detection of total aerobic bacteria count in fish flesh

For counting all aerobic bacteria **(Maturin and Peeler 2001)**, Fish fillet samples (25 g) were homogenized with sterile buffered peptone water (225 mL) to create the initial tenfold serial dilution. One milliliter from each preceding serial dilution was aseptically transferred to every additional sterilized Petri dish. A total of 12 ml of sterilized, melted, and well-combined standard plate count agar medium was added to each plate at a temperature of (45 ± 1°C). Following solidification, the infected plates and the control plate (inoculated with sterile distilled water) were incubated at 32 ± 1°C for 48 ± 3 h **(APHA 2001)**. Each colony represents a single bacterial cell that was present in the sample. The total aerobic bacteria count is usually expressed as colony-forming units per gram (CFU/g) of fish flesh.

Blood sampling

On the final day of the first trial, whole blood samples were taken from the caudal veins (9 fish/group) using syringes with EDTA in EDTA tubes and kept at 4°C for hematological and immunological assessment (phagocytic activity and index). The other biochemical parameters were estimated using the serum samples. After the 2nd experiment, another set of blood and serum samples was collected.

Hemato-biochemistry analysis

A modified Neubauer hemocytometer was used to count RBCs and WBCs according to **(Shah & Altindag 2004)**. Cyanmethemoglobin and microhematocrit analyses were used to determine Hb (hemoglobin) and Ht (hematocrit) levels **(Blaxhall and Daisley 1973)**, respectively. The Briggs and Bain formulas were used for estimating erythrocyte indices:

$$MCV = \frac{Ht}{RBC} \times 10$$

$$MCH = \frac{Hb}{RBC} \times 10$$

For differential leucocyte counts, smears were stained with May-Grunwald-Giemsa-Wright **(Ishikawa et al. 2008)**.

Commercial kits (Biodiagnostic and Spectrum Companies, Egypt) were used to estimate the total serum protein and albumin levels **(Tietz 1995)**, and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined using the International Federation of Clinical Chemistry Kinetic technique **(Bergmeyer et al. 1986)**, urea **(Patton and Crouch 1977)** and creatinine **(Sax 1975)**. Albumin was subtracted from total protein to get the serum globulin concentration, and the albumen/globulin (A/G) ratio was calculated by dividing the albumin concentration by the globulin concentration.

Immune response and antioxidant capacity

The phagocytic function of leukocytes was examined according to **Platt & Fineran (2015)**. The test was conducted on a 100 µl blood sample using foetal bovine serum and *Candida albicans* (1×10^6). In which phagocytic activity equals the percentage of macrophages that engulfed yeast and the phagocytic index equals the total number of engulfed cells phagocytized divided by the total number of phagocytic cells.

Serum lysozyme activity was estimated using a turbidimetric assay based on the lysis of *Micrococcus lysodeikticus* (Sigma, USA), according to **Ellis (1990)**. Serum SOD (superoxide dismutase) and CAT (catalase) (Bio diagnostic Co. Egypt) levels were determined calorimetrically at wavelengths of 560 and 510 nm following **Nishikimi et al. (1972)** and **Aebi (1984)**, respectively, and MDA (malonaldehyde) levels were assessed using

diagnostic reagent kits (Cusabio Biotech Co., Ltd.; China) according to the manufacturer's instructions.

Experimental infection

Briefly, 0.2 ml of 24 hrs old broth culture from the virulent previously identified field *A. hydrophila* strain, from sample number 2 in Table 2 (1×10^7 CFU/ml), according to **Abu-Elala et al. (2015)**, was given by intraperitoneal (I/P) injection. The preparation of the bacterial suspension was performed using McFarland standard tubes. All the inoculated fish were subjected to daily examination for the presence of mortalities and any clinical signs. The cumulative mortality percentage was recorded for each group until the end of the experiment.

Statistical analysis

Two- way ANOVA was used to statistically analyze the collected data (SPSS® version 22, SPSS, Inc., IL, and USA). The results are expressed as the mean ± standard deviation (SD). When there was a statistically significant treatment effect, the means were compared using a Duncan post-hoc test. A substantial level was used to evaluate treatment effects ($P\leq 0.05$).

Quality control & assurance

To guarantee accurate and trustworthy results in microbiological and chemical testing, quality control and quality assurance are crucial. The control samples (normal and abnormal) have been utilized in every set of assays and whenever a fresh bottle of reagent is used. For quality control, commercially available control material with predetermined values may be used often. When the assay of control material fails to yield the desired range of values, it could be a sign of reagent deterioration, equipment malfunction, or procedural errors. The following corrective measures have been employed in such cases: apply the same controls once more, make fresh control serum and run the test again if the results of the repeated control are not within the acceptable ranges. Also, some actions have been conducted to ensure accurate and reliable results, such as frequent equipment calibration and maintenance, use of negative and positive controls in microbiological testing, environmental monitoring to avoid false positive results.

This work has been conducted at the accredited AHRI laboratories (Animal Health Research Institute, Egypt), where the quality control and assurance with good laboratory practices were applied. Also, the most recent measuring methods that included chemical-specific separation and detection were applied.

RESULTS

Phenotypic and biochemical characterization

Aeromonas hydrophila was isolated and identified from diseased *O. niloticus,* in which 37 positive samples (37%) out of 100 tested samples were detected. Colonies exhibited positive catalase and oxidase reactions.

Typical colonies of *Aeromonas* were selected and examined by API 20E kits. All the isolates showed positive reactions with beta-galactosidase, lysine decarboxylase, sodium citrate, arginine dihydrolase, tryptophan deaminase, urea, tryptophane, gelatinase, sodium pyruvate, mannitol, glucose, sucrose, amygdalin, melibiose, and arabinose. However, negative results were displayed for H2S production, ornithine decarboxylase, sorbitol, inositol, and rhamnose.

Molecular characterization

The molecular identification of 10 isolates of field strain *A. hydrophila* was performed (Fig. 2 and Table 2) by agarose gel electrophoresis of PCR for virulence genes with the using specific standard primer of the

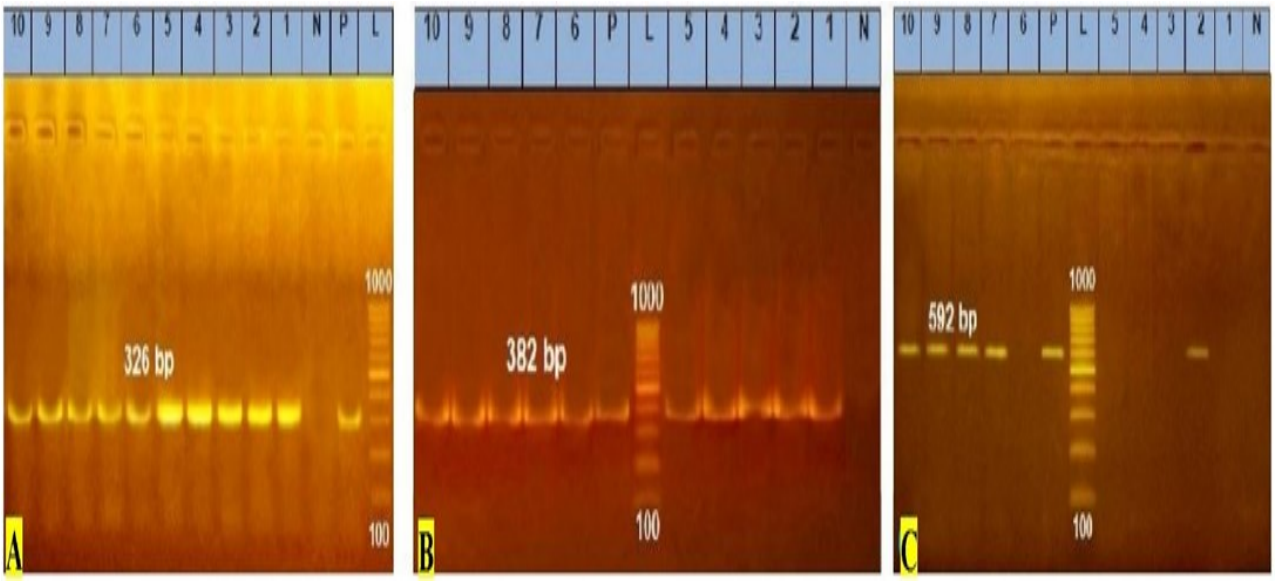

Fig. 2 Agarose gel electrophoresis of PCR amplification products of *A. hydrophila* virulence genes; (A) *aer* (326 bp), Lanes 1 to 10: were positive, (B) *Lip* (382 bp), Lanes 1 to 10: were positive, (C) *hly* (592 bp), Lanes 2, 7, 8, 9, and 10: were positive, Lanes 1, 3, 4, 5, and 6: were negative. Lane L: 100 bp ladder serving as a molecular size DNA marker; lanes P and N represent control genes that are positive and negative, respectively. The samples were derived from the same experiment and the gels were processed in parallel. The figure is a cropped gel

Table 2. Results of PCR for the virulence genes *aer, lip*, and *hyl* of *A. hydrophila* (n=10).

Sample	*aer*	*lip*	*hly*
1	+	+	-
2	+	+	+
3	+	+	-
4	+	+	-
5	+	+	-
6	+	+	-
7	+	+	+
8	+	+	+
9	+	+	+
10	+	+	+
Total	10 (100%)	10 (100%)	5 (50%)

aer: aerolysin; *lip*: lipase; *hly*: hemolysin

Antibiotic Resistance Patterns

Table 3 displays the antibiogram profiles of the identified 37 field strain isolates of *A. hydrophila* susceptibility to 12 antimicrobial agents. Our results revealed that Aeromonas spp. were highly sensitive (94.59%) to Levofloxacin, followed by 83.78%, 75.68%, and 67.57% of the isolates exhibiting sensitivity to Fosfomycin, Spectinomycin, and Florfenicol, respectively. Approximately 91.89% and 83.78% of the isolates exhibited resistance to erythromycin and tylosin respectively. Approximately, 78.38 and 64.86% of isolates were resistant to Sulfadimethexine and oxytetracycline. Moderate resistance levels of 59.46%, 54.05%, 51.35%, and 40.54% were detected between the examined isolates to doxycycline, amoxicillin, ampicillin, and cefotaxime, respectively.

Table 3. Susceptibility of isolated *Aeromonas hydrophila* (n=37 samples) to antimicrobial agents

Antimicrobial agent	(isolates)			
	Sensitive		Resistant	
	No.	%	No.	%
Levofloxacin	35	94.59	2	5.41
Fosfomycin	31	83.78	6	16.22
Spectinomycin	28	75.68	9	24.32
Florfenicol	25	67.57	12	32.43
Cefotaxime	22	59.46	15	40.54
Ampicillin	18	48.65	19	51.35
Amoxicillin	17	45.95	20	54.05
Doxycycline	15	40.54	22	59.46
Oxytetracycline	13	35.14	24	64.86
Sulfadimethexine	8	21.62	29	78.38
Tylosin	6	16.22	31	83.78
Erythromycin	3	8.11	34	91.89

ChNPs characterization and in vitro antibacterial activity

SEM imaging of ChNPs revealed aggregating semi-spherical particles with sizes between 80 and 100 nm (Fig. 3C) and that of *A. hydrophila* cells cultured in medium supplemented with 1.25 mg/ml ChNPs attacked and adhered to the bacterial cell wall causing atypical cell sizes and shapes, leakage through the cell membrane, cytoplasmic lysis, and cell damage (Fig. 3A, B).

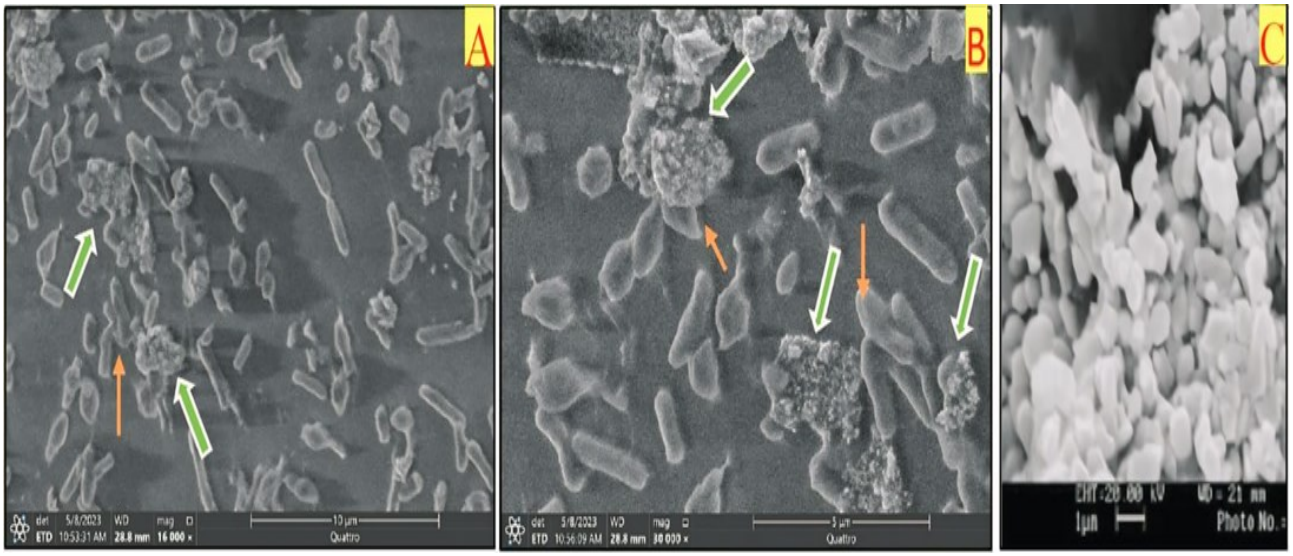

Fig. 3 (A, B) SEM image of *A. hydrophila* cells cultured in medium supplemented with 1.25 mg/ml ChNPs revealing nanoparticle attack and adherence to the bacterial cell wall, blue arrow refers to ChNPs and orange arrow refer to bacteria, Scale bar = (A) 10 µm and (B) 5 µm, (C) SEM image of ChNPs revealing aggregating semispherical particles with sizes between 80 and 100 nm, Scale bar= 1 µm

Growth performance

Dietary Ch and ChNPs positively impacted fish growth performance and feed intake, which was significantly greater in the fish-fed Ch and ChNPs-supplemented diets than in the CTR diet ($P \leq 0.05$; Table 4). The final body weight, weight gain, average daily gain, and feed efficiency of the fish in the Ch and ChNP groups were significantly ($P \leq 0.05$) greater than those in the CTR group, and the fish in the ChNPs group demonstrated the greatest growth performance. These findings imply that dietary Ch and ChNPs have no harmful effects on fish. All the fish were in good health during the feeding period, as evidenced by their general activity. Fish survival was 100% in all groups from pre-challenge until the end of the 1st trial, and all groups had 0% mortality (Table 4).

Table 4. Growth performance of *O. niloticus*-fed Ch and ChNPs

Parameter	CTR	Ch	ChNPs
Initial Body Weight (g)	26.17 ± 0.6	26.10 ± 2.07	26.80 ± 0.42
Final Body Weight (g)	43.23 ± 0.59^{c}	49.10±0.50^{b}	53.30± 0.35^{a}
Weight Gain	17.05 ± 0.22^{c}	22.98 ± 0.66^{b}	26.51±7.42^{a}
Average Daily Gain	0.61 ± 0.001^{c}	0.82 ± 0.023^{b}	0.95±0.027^{a}
Feed Intake (g)	16.91 ± 0.40^{b}	17.63 ± 0.083^{b}	18.41 ± 0.17^{a}
Feed Efficiency	1.01 ± 0.025^{c}	1.30 ± 0.031^{b}	1.44 ± 0.029^{a}
Survival Rate	100% ± 0.00	100% ± 0.00	100% ± 0.00

The values are the means ± SD (n = 9/ group). Different letters in the same row indicate significant differences at $P \leq 0.05$. CTR=control; Ch= chitosan; ChNPs= chitosan nanoparticles

Chemical analysis of fish body and bacteriological examination of fish flesh

The results of bacteriological examination of the fish flesh before bacterial challenge are presented in Table 5. Compared with those in the CTR group, the total aerobic bacterial count in the Ch/ChNPs groups substantially decreased. Chemical analysis of the fish body revealed a significant increase ($P \leq 0.05$) in crude protein in the Ch/ChNPs groups compared with the control group. Moreover, moisture, crude lipids, carbohydrates, and total energy were significantly lower in the Ch/ChNPs groups than in the CTR group. Moreover, crude fiber and crude ash showed insignificant ($P>0.05$) increases in the Ch/ChNPs groups (Table 5).

Table 5. Bacteriological and proximate body chemical compositions (%) of fish in the groups supplemented with Ch and ChNPs

Parameter	CTR	Ch	ChNPs
Total aerobic bacterial count (CFU/gm.)	$5.82\times10^{4}\pm 4.52\times10^{3a}$	$4.96\times10^{3} \pm 5.81\times10^{2b}$	$3.87\times10^{3}\pm 4.31\times10^{2b}$
Moisture%	75.65 ± 0.19^{a}	74.82 ± 0.09^{ab}	74.25 ± 0.14^{b}
Crude protein%	57.27 ± 0.27^{b}	60.21 ± 0.35^{a}	60.04 ± 0.35^{a}
Crude lipids%	20.26 ± 0.02^{a}	18.84 ± 0.45^{b}	17.69 ± 0.57^{b}
Crude fiber%	2.44 ± 0.01	2.53 ± 0.02	2.53 ± 0.09
Crude ash%	13.79± 0.60	14.21 ± 0.69	14.22 ± 0.34
Carbohydrates%	3.25 ± 0.17^{a}	2.45 ± 0.25^{b}	2.35 ± 0.17^{ab}
Total energy%	22.64 ± 0.20^{a}	21.74 ± 0.18^{b}	21.54 ± 0.09^{b}

TABC is the total aerobic bacterial count, while CFU/gm. represents the number of colonies forming units per gram. The values are presented as the means ± SDs (n = 9/group). There is a significant difference between letters in the same row at ($P\leq$05). CTR=control; Ch= chitosan; ChNPs= chitosan nanoparticles.

Hemato-biochemistry profile

Dietary inclusion of Ch or ChNPs resulted in a significant increase in Hb levels, RBC counts, and Ht ($P\leq$ 0.05), but there were no significant differences between the Ch and ChNPs-supplemented groups. MCV and MCH did not significantly ($P>0.05$) decrease in the fish groups fed Ch or ChNPs (Table 6). Total leukocyte counts and heterophils percentages increased significantly in the groups fed Ch and ChNPs compared to those in the CTR group, and the highest values were detected in the fish groups fed ChNPs. Lymphocyte percentage was significantly lower in the Ch and ChNPs groups than in the CTR group, while monocyte, basophil, and eosinophil percentages did not significantly ($P>0.05$) differ among the experimental groups (Table 6).

Dietary inclusion of Ch or ChNPs in *O. niloticus* diets resulted in non-significant *($P>0.05$)* changes in the assessed biochemical parameters except for total protein, globulin, and the A/G ratio (Table 6). Total protein and globulin levels were increased significantly in the groups fed Ch or ChNPs, but the albumen didn't show any significant changes between groups. Thus, the A/G ratio was significantly lower in the same groups than in the CTR group ($P\leq$ 0.05).

Table 6. Hematobiochemical indices of *O. niloticus* groups fed Ch and NPChs

Parameter	CTR	Ch	ChNPs
Hematological indices			
Hb (g/100 ml)	8.22±0.62^{c}	9.49±0.51^{b}	10.53±0.59^{a}
RBCs (x10^{6}/mm^{3})	2.06±0.34^{b}	2.87±0.08^{a}	3.16±0.23^{a}
Ht%	19.04±1.65^{b}	22.09±0.39^{a}	23.9±1.31^{a}
MCV (Fl)	94.75±21.02	76.93±2.63	75.71±2.73
MCH (pg.)	40.42±5.44	34.65±2.34	33.48±3.89
Total Leukocyte Counts (x10^{3}/ mm^{3})	32.10±0.458^{c}	34.19±0.209^{b}	34.38±0.226^{a}
Heterophils %	48.6±0.95 ^{b}	55.99±0.56 ^{a}	56.19±1.74^{a}
Monocyte %	1.34±0.22	1.5±0.1	1.6±0.05
Lymphocyte %	47.08±2.56^{a}	44.07±2.96^{b}	37.88±2.047^{c}
Eosinophil%	3.14±0.31	3.06±0.28	3.51±0.27
Basophil %	0.86±0.095	0.72±0.081	0.82±0.085
Biochemistry indices			
ALT (U/L)	21.01±0.45	20.23±1.15	20.0±1.56
AST (U/L)	70.04±4.07	78.94±3.67	74.09±1.67
Urea(mg/dl)	28.43±4.93	30.5±6.67	28.80±3.44
Creatinine (mg/dl)	0.22±0.012	0.22±0.01	0.20±0.006
Total Protein (g/dl)	5.64±0.30^{b}	6.60±0.40^{a}	6.82±0.07^{a}
Albumin (g/dl)	2.99±0.11	2.73±0.18	2.65±0.08
Globulin (g/dl)	2.65±0.22^{b}	3.87±0.53^{a}	4.17±0.12^{a}
A/G Ratio	1.13±0.075^{a}	0.72±0.133^{b}	0.64±0.035^{b}

The values are the means ± SDs (n = 9/ group). Different letters in the same row indicate significant differences at $P \leq 0.05$. CTR=control; Ch= chitosan; ChNPs= chitosan nanoparticles. Hb: hemoglobin; RBCs: red blood cells; Ht: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin, ALT: alanine aminotransferase; AST: aspartate aminotransferase; A/G: albumin/globulin ratio

Immune response and antioxidant capacity

Dietary supplementation with Ch and ChNPs significantly ($P \leq 0.05$) enhanced the fish immune responses in comparison with those in the control group (Table 7). The highest phagocytic activity, phagocytic index, and lysozyme activity were detected in the groups that received ChNPs followed by Ch, and the lowest values were attained in the CTR.

The oxidative stress indices (SOD, CAT, and MDA) of the *O. niloticus* in the experimental groups are shown in (Table 7). Dietary supplementation of *O. niloticus* with Ch or ChNPs significantly ($P \leq 0.05$) augmented SOD and CAT activity while reducing MDA levels.

Table 7. Immune responses and antioxidant capacity of *O. niloticus* groups fed Ch and NPChs

Parameter	CTR	Ch	ChNPs
PA%	30.35±0.35^{c}	41±0.28^{b}	49.75±1.63^{a}
PI No	2.8±0.14^{b}	3.55±0.07^{a}	3.75±0.21^{a}
Lysozyme (U/ml)	29.8±0.42^{b}	46.55±3.32^{a}	54.4±5.37^{a}
MDA (IU/l)	25.64±0.68^{a}	22.6±0.71^{b}	20.25±0.92^{c}
SOD (IU/l)	12.74±0.26^{b}	14.85±0.07^{a}	15.10±0.28^{a}
CAT (IU/l)	21.15±0.07^{b}	25.15±0.64^{a}	27.14±0.94^{a}

The values are the means ± SDs (n = 9/group). Letters in the same row indicate significant differences at $P \leq 0.05$. CTR=control; Ch= chitosan; ChNPs= chitosan Nanoparticles. PA%: phagocytic activity percent; PI: phagocytic index; MDA: malonaldehyde; SOD: superoxide dismutase; CAT: catalase

Cumulative mortality post infection

The cumulative mortality decreased in the Ch/ ChNP supplemented groups either when Ch/ ChNPs were administered alone or when Ch/ ChNPs were combined with Levofloxacin. The lowest mortality percentage was observed in G6 (ChNPs+levofloxacin; 20%), followed by G5 (ChNPs; 26.67%), while the control positive group (G1) had a mortality rate of 73.33% (Fig. 4)

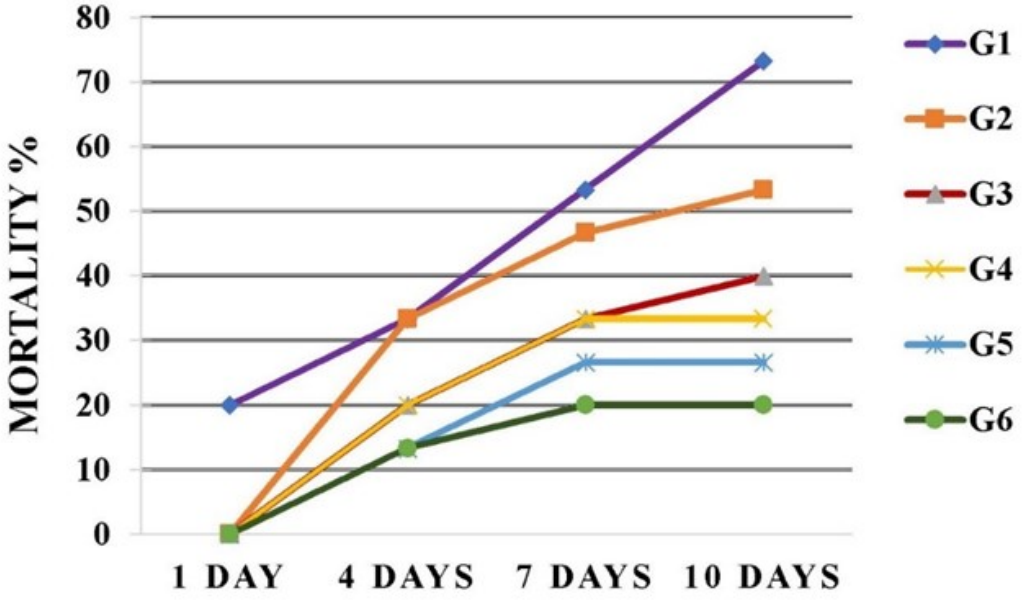

Fig. 4 Effects of the experimental treatments on the mortality percentage of *O. niloticus* infected with *A. hydrophila* (1×10^{7} CFU/fish) after 10 days of feeding. G1: Fish inoculated with *A. hydrophila* and fed a basal diet, G2: fish inoculated with *A. hydrophila* and fed a basal diet with levofloxacin for 10 days, G3: fish inoculated with *A. hydrophila* previously fed 10g Ch/kg diet for 28 days and continuously fed for 10 days post- inoculation, G4: fish inoculated with *A. hydrophila* previously fed 10g Ch/kg diet for 28 days and continuously fed plus levofloxacin for 10 days post- inoculation, G5: fish inoculated with *A. hydrophila* previously fed 100 mg ChNPs/kg diet for 28 days and continuously fed for 10 days post-inoculation, G6: fish inoculated with *A. hydrophila* previously fed 100 mg ChNPs for 28 days and continuously fed plus levofloxacin for 10 days post- inoculation

Clinical and P/M examination post challenge

Clinical examination of the experimentally infected *O. niloticus* revealed signs of *A. hydrophila* infection similar to those detected in naturally diseased fish, which included skin erosion and ulcers, fin erosion, and eye protrusion and hemorrhage (Fig. 5). The severity of these signs varied among the experimental groups, they were more severe in G1, while they were less severe in the Ch/ and ChNPs groups**.**

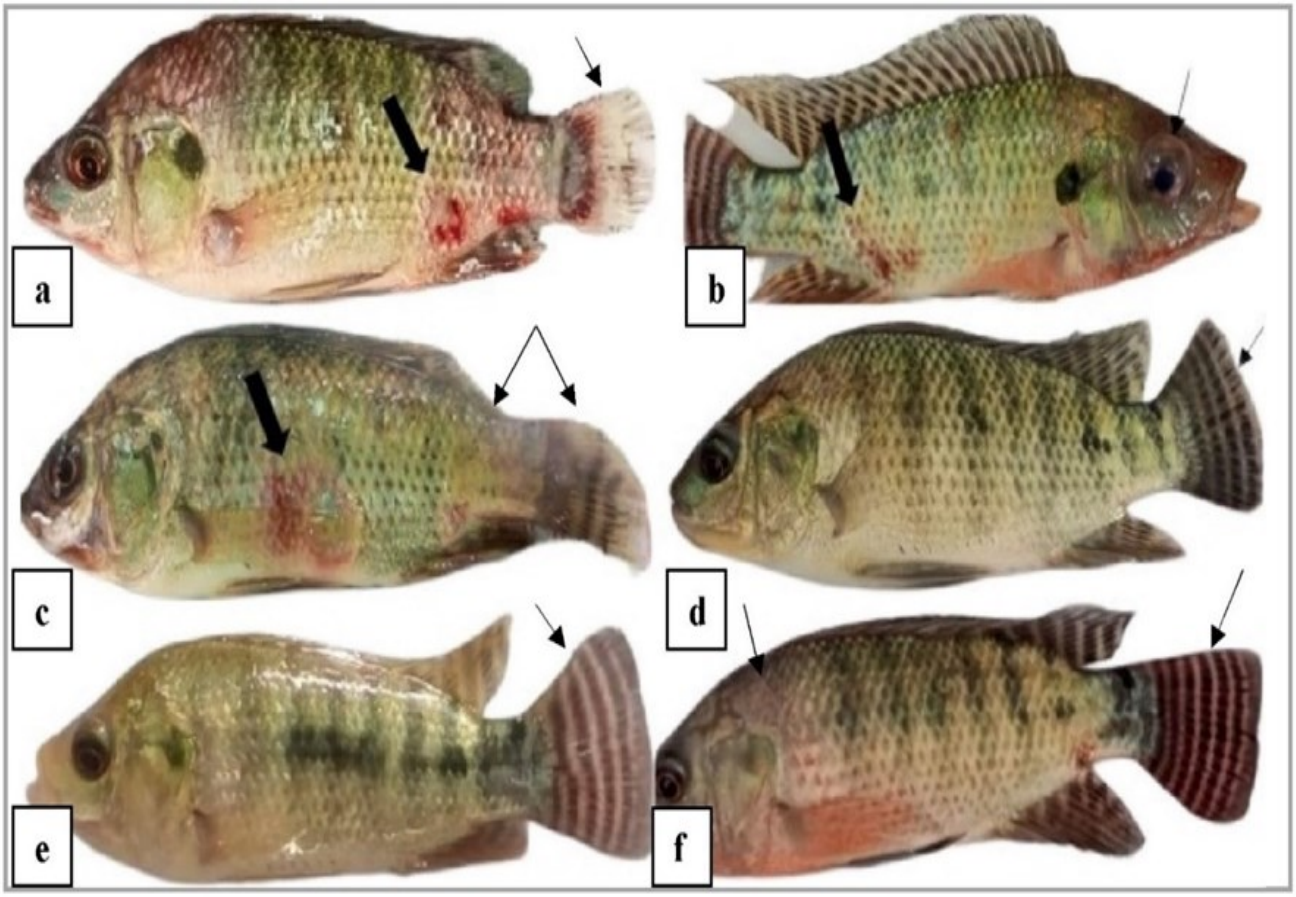

Fig. 5 Clinical signs of experimentally infected *O. niloticus* groups: (a) G1 showed tail erosions and skin erosion, hemorrhage, and ulcers, (b) G2 showed eye protrusion and hemorrhage and some skin ulcers, (c) G3 showed tail erosions and skin hemorrhages and ulcers, (d) G4 showed normal clinical signs with some tail erosion, (e) G5 showed hemorrhagic tail, (f) G6 showed hemorrhagic tail and some hemorrhagic spots on the skin. G1: Fish inoculated with *A. hydrophila* and fed a basal diet, G2: fish inoculated with *A. hydrophila* and fed a basal diet with levofloxacin for 10 days, G3: fish inoculated with *A. hydrophila* previously fed 10g Ch/kg diet for 28 days and continuously fed for 10 days post-inoculation, G4: fish inoculated with *A. hydrophila* previously fed 10g Ch/kg diet for 28 days and continuously fed plus levofloxacin for 10 days post- inoculation, G5: fish inoculated with *A. hydrophila* previously fed 100 mg ChNPs/kg diet for 28 days and continuously fed for 10 days post- inoculation, G6: fish inoculated with *A. hydrophila* previously fed 100 mg ChNPs for 28 days and continuously fed plus levofloxacin for 10 days post- inoculation

Figure 6 showed P/M examination of the internal organs of the experimental treatments. Results demonstrated less severe symptoms (hemorrhagic spots on the liver) than those in the control-infected group.

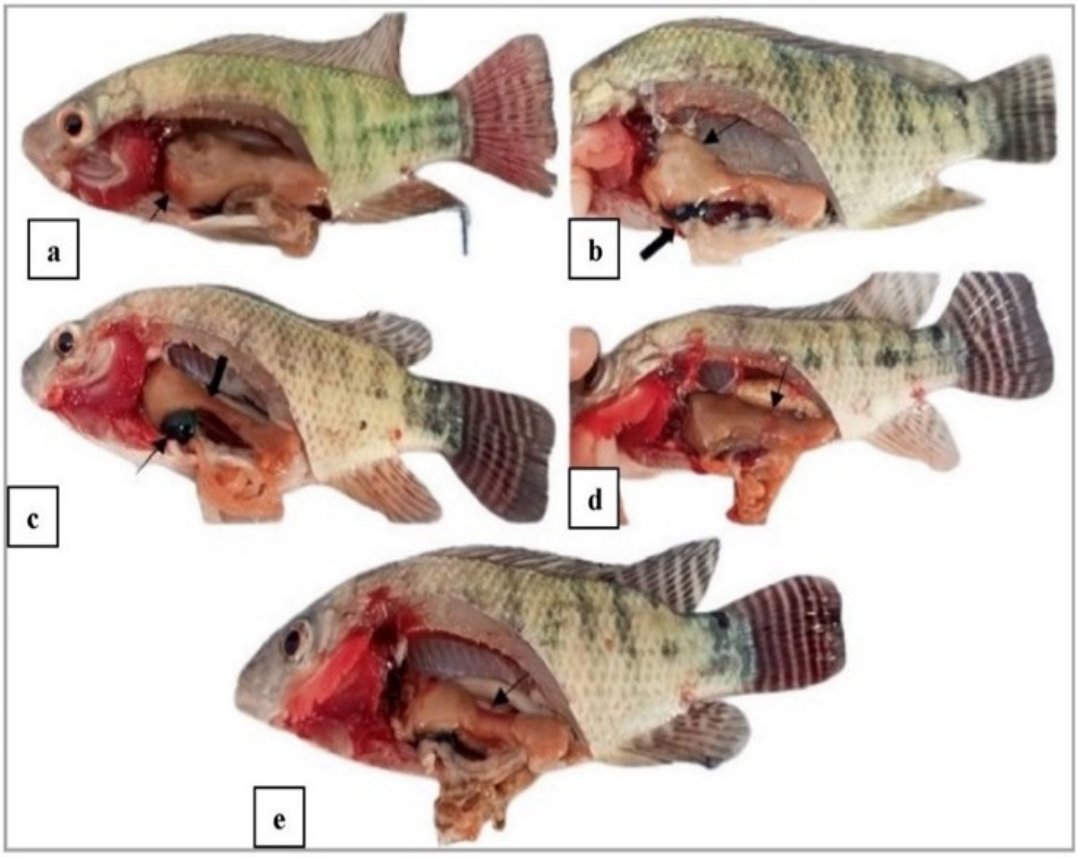

Fig. 6 Showing p/m examination of experimentally infected *O. niloticus* groups: (a) G2 showed enlarged liver with some hemorrhagic spots (arrow), (b) G3 showed a distended gallbladder (bighead arrow), and pale liver (arrow), (c) G4 showed a distended gallbladder (arrow), and hemorrhagic spots on the liver (bighead arrow), (d) G5 showed enlarged liver with some hemorrhagic spots, (e) G6 showed hemorrhagic liver. G1: Fish inoculated with *A. hydrophila* and fed a basal diet, G2: fish inoculated with *A. hydrophila* and fed a basal diet with levofloxacin for 10 days, G3: fish inoculated with *A. hydrophila* previously fed 10g Ch/kg diet for 28 days and continuously fed for 10 days post- inoculation, G4: fish inoculated with *A. hydrophila* previously fed 10g Ch/kg diet for 28 days and continuously fed plus levofloxacin for 10 days post- inoculation, G5: fish inoculated with *A. hydrophila* previously fed 100 mg ChNPs/kg diet for 28 days and continuously fed for 10 days post- inoculation, G6: fish inoculated with *A. hydrophila* previously fed 100 mg ChNPs for 28 days and continuously fed plus levofloxacin for 10 days post- inoculation

Hemato-biochemical profile post infection

Table 8 shows that the hematological parameters (Hb, RBC, and Ht) significantly decreased in *O. niloticus*-infected control group (G1). Dietary supplementation with Ch/ChNPs significantly improved hematological parameters to control values before infection. G6 showed the best results, followed by G5 and G4. MCV and MCH were significantly lower in all the treatment groups than in G1. Total leukocyte counts increased significantly in all groups, and ChNPs+LEV (G6) showed the higher values, followed by ChNPs (G5) and Ch (G3), than other groups. In all groups, heterophils percentage increased significantly, and Ch group had the highest heterophils percentage and the lowest monocyte percentage. Lymphocyte percentages decreased significantly in all groups compared with those in G1 and G2.

As shown in Table 8, *A. hydrophila* infection (G1) resulted in increased serum liver enzymes (ALT and AST) and serum urea and

creatinine. However, supplementation with Ch/ ChNPs or even Levofloxacin resulted in insignificant changes. G1 showed an insignificant decrease in total protein and globulin and, consequently an insignificant increase in the A/G ratio. The groups supplemented with Ch or ChNPs had the highest levels of total protein and globulin, followed by the G2 group (treated with levofloxacin).

Table 8. Hemato-biochemistry indices of experimentally infected *O. niloticus* groups

Parameter	G1	G2	G3	G4	G5	G6
Hematological indices						
Hb (g/100 ml)	6.85±0.31^{d}	7.6±0.22^{c}	7.72±0.1^{c}	8.4±0.1^{b}	8.26±0.13^{b}	8.9±0.09^{a}
RBCs (x10^{6}/ mm^{3})	1.50±0.05^{d}	2.06±0.10^{c}	2.24±0.1^{b}	2.39±0.1^{b}	2.31±0.08^{b}	2.97±0.08^{a}
PCV%	14.33±0.15^{d}	16.63±0.39^{c}	16.87±0.60^{c}	19.27±0.21^{b}	18.53±0.74^{b}	20.37±0.15^{a}
MCV (Fl)	95.61±2.90^{a}	80.81±2.05^{b}	76.09±2.69^{c}	80.80±2.74^{c}	80.48±5.84^{c}	68.69±2.11^{d}
MCH (pg.)	45.67±1.72^{a}	36.94±1.78^{b}	34.48±1.51^{b}	35.23±1.26^{b}	35.81±0.71^{b}	30.02±1.03^{c}
Total Leukocyte Counts (x10^{3}/ mm^{3})	39.46± 0.72^{d}	45.216.33 ±0.32^{cd}	49.41±0.13^{b}	47.53 ±0.32^{c}	49.52±0.24^{b}	51.12 ±0.11^{a}
Heterophils %	39.45±1.12^{c}	42.54±3.25^{c}	45.55±1.8^{a}	47.07±3.21^{a}	44.89±3.45^{b}	44.61±1.25^{b}
Monocyte %	1.95±0.12^{b}	1.83±0.21^{b}	1.78±0.13^{c}	1.74±0.09^{c}	1.89±0.16^{b}	2.45±0.09^{a}
Lymphocyte %	55.11±2.06^{a}	52.13±1.58^{a}	49.15±1.23^{b}	47.25±2.05^{b}	49.35±0.25^{b}	48.95±1.06^{b}
Eosinophil	2.73±0.02	2.65±1.01	2.69±0.19	3.25±0.54	2.95±0.85	3.15±1.04
Basophil	0.76±0.14	0.85±0.05	0.83±0.13	0.69±0.09	0.92±0.12	0.84±0.08
Biochemistry indices						
ALT (U/L)	36.6±1.11^{a}	24.5±1.4^{b}	25.4±1.5^{b}	22.6±1.8^{c}	20.9±0.8^{c}	21.33±1.2^{c}
AST (U/L)	113.6±3.5^{a}	78.5±2.6^{b}	76.3±2.1^{b}	68.5±3.2^{c}	63.4±6.1^{c}	65.77±2.9^{c}
Urea(mg/dl)	79.5±5.8^{a}	28.33±4.85^{b}	26.5±2.0^{b}	24.4±3.1^{bc}	20.4±0.9^{c}	20.93±1.45^{c}
Creatinine (mg/ dl)	0.36±0.042^{a}	0.25±0.026^{b}	0.24±0.03^{b}	0.22±0.05^{b}	0.18±0.03^{b}	0.19±0.06^{b}
TP (g/dl)	5.4±0.3^{c}	5.8±0.2^{b}	6.60±0.2^{a}	6.7±0.3^{a}	6.43 ±0.31^{a}	6.8±0.3^{a}
Albumin (g/dl)	3.2±0.4	2.93±0.15	2.8±0.3	3.2±0.3	3.03±0.12	3.2±0.2
Globulin (g/dl)	2.2±0.1^{c}	2.87±0.06^{b}	3.8±0.35^{a}	3.5±0^{a}	3.4±0.35^{a}	3.6±0.5^{a}
A/G Ratio	1.46±0.24^{a}	1.02±0.17^{b}	0.74±0.12^{c}	0.91±0.09^{b}	0.89±0.12^{b}	0.89±0.18^{b}

The values are the means ± SDs (n = 9/ group). There is a significant difference between letters in the same row at ($P \leq 05$). G1: Fish inoculated with *A. hydrophila* and fed a basal diet, G2: fish inoculated with *A. hydrophila* and fed a basal diet with levofloxacin for 10 days, G3: fish inoculated with *A. hydrophila* previously fed 10g Ch/kg diet for 28 days and continuously fed for 10 days post- inoculation, G4: fish inoculated with *A. hydrophila* previously fed 10g Ch/kg diet for 28 days and continuously fed plus levofloxacin for 10 days post- inoculation, G5: fish inoculated with *A. hydrophila* previously fed 100 mg ChNPs/kg diet for 28 days and continuously fed for 10 days post- inoculation, G6: fish inoculated with *A. hydrophila* previously fed 100 mg ChNPs for 28 days and continuously fed plus levofloxacin for 10 days post-inoculation. Hb: hemoglobin; RBCs: red blood cells; PCV: packed cell volume; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin, ALT: alanine aminotransferases; AST: aspartate aminotransferases; TP: total protein; A/ G Ratio: albumen/globulin ratio

Immune and oxidative stress responses post infection

Regarding (*P*-value), there was a significant effect on immune parameters due to experimental infection, supplementations, and interaction between them. The measurements of immune parameters after *A. hydrophila* infection showed a significant improvement in PA %, PI, and lysozyme in all treated groups (Table 9). Compared with those in the infected control group, the PA%, PI, and lysozyme in G6 and G5 (ChNPs with/ without levofloxacin) exhibited the most significant increases, followed by G4 and G3 (Ch with or without levofloxacin).

The post-challenge oxidative stress parameters (SOD, CAT, and MDA) are presented in (Table 9). Our results revealed that *A. hydrophila* infection caused oxidative stress, which was obvious in the untreated group G1 in which a significant decrease in CAT activity was associated with the highest level of MDA). Dietary supplementation of *O. niloticus* with Ch or ChNPs significantly ($P \leq 0.05$) decreased the MDA concentration, level maintaining CAT within the control values and thus protecting *O. niloticus* from oxidative damage. However, SOD did not significantly differ among the treatments ($P > 0.05$).

Table 9. Immune responses and antioxidant capacity of experimentally infected *O. niloticus*

Parameter	G1	G2	G3	G4	G5	G6
PA%	41.85±4.6^{c}	40.88±1.92^{c}	51.00±0.28^{b}	50.83±3.71^{b}	56.75±5.87^{a}	56.24±2.82^{a}
PI	3.20±0.42^{b}	3.30±0.14^{b}	3.95±0.21^{a}	4.10±0.28^{a}	4.00±0.14^{a}	4.10±0.16^{a}
Lysozyme (U/ ml)	47.30±3.11^{b}	46.37 ±2.97^{b}	50.05±1.63^{b}	51.29±1.46^{b}	64.90±4.66^{a}	64.14±1.25^{a}
MDA (IU/l)	34.85±0.36^{a}	28.73±0.67^{b}	22.4±2.12^{c}	22.9±0.28^{c}	23.23±1.45^{c}	20.26±1.33^{c}
SOD (IU/l)	10.90±1.14	11.77±0.21	12.70±0.14	11.73±0.68	13.35±0.64	12.87±0.64
CAT (IU/l)	11.40±0.14^{c}	18.74±0.69^{b}	21.90±0.85^{a}	22.90±0.49^{a}	22.85±0.33^{a}	25.05±1.16^{a}

The values are the means ± SDs (n = 9/ group). There is a significant difference between letters in the same row at ($P \leq 05$). G1: Fish inoculated with *A. hydrophila* and fed a basal diet, G2: fish inoculated with *A. hydrophila* and fed a basal diet with levofloxacin for 10 days, G3: fish inoculated with *A. hydrophila* previously fed 10g Ch/kg diet for 28 days and continuously fed for 10 days post- inoculation, G4: fish inoculated with *A. hydrophila* previously fed 10g Ch/kg diet for 28 days and continuously fed plus levofloxacin for 10 days post- inoculation, G5: fish inoculated with *A. hydrophila* previously fed 100 mg ChNPs/kg diet for 28 days and continuously fed for 10 days post- inoculation, G6: fish inoculated with *A. hydrophila* previously fed 100 mg ChNPs for 28 days and continuously fed plus levofloxacin for 10 days post-inoculation. PA%: phagocytic activity percent; PI: phagocytic index; MDA: malonaldehyde; SOD: superoxide dismutase; CAT: catalase.

DISCUSSION

An aquaculture project's success is positively correlated with keeping fish in adequate health. Aquaculture biosecurity is severely impaired by infectious diseases due to mass mortalities, which can result in significant economic losses. The most common pathogen responsible for MAS in *O. niloticus* is *A. hydrophila*, which causes significant morbidity and mortality. As a result, identifying the prevalence, virulence, and antibiotic resistance profiles of this bacteria is deemed essential for developing effective control measures for farmed fish. The clinical signs detected in diseased fish match those described in earlier studies **(Aboyadak 2015; El-Bahar et al. 2019)**. *A. hydrophila* invasion and colonization and the subsequent release of extracellular toxins lead to the rupture of small blood vessels, which results in hemorrhagic sepsis, anemia, ulceration, and exhaustion **(Aoki 2011)**.

In the present study, 37 out of 100 tested samples from naturally infected fish were positive for isolation and identification of *A. hydrophila* field strain with a percentage of 37%. These results were near those obtained by **Tartor et al. (2021)**, who demonstrated that the

prevalence of *A. hydrophila* in diseased fish was 39% and lower than that obtained by **El-Ashram (2002)** who isolated *A. hydrophila* from *O. niloticus* at a percentage of 66% but higher than that reported by **El-Bahar et al. (2019)**. Differences in prevalence may result from variations in the sampling time and area covered.

In this study, 50% of the examined isolates (n=10) carried the 3 virulence genes, and 100% had 2 virulence genes (*aer* and *lip*), demonstrating the high probability of pathogenicity of *A. hydrophila* isolated from *O. niloticus* and its high ability to induce disease. The *aer* gene was the most frequently identified virulence gene in *A. hydrophila* isolates, which is consistent with findings from other studies **(El-Bahar et al. 2019; Oliveira et al. 2012)**. The pathogenesis of *A. hydrophila* may be affected by the *aer* gene which destroys membrane permeability and causes osmotic lysis and cell death **(Iacovache et al., 2016)**. *Lip*-encoding genes have frequently been detected in *A. hydrophila* isolated from fish (Oliveira et al. 2012). Which alters the structure of the cell membrane. The *hly* gene, which induces anemia by destroying RBCs, was identified in 50% of our isolates, but **Tartor et al. (2021)** and **Oliveira et al. (2012)** found this gene in only 10% of the *A. hydrophila* strains.

Development of antibiotic-resistant *A. hydrophila* in aquaculture constitutes a threat to public health because these bacteria can potentially spread throughout the ecosystem by horizontally transmitting mobile genetic elements to other bacteria or humans through contaminated water **(Jacobs and Chenia, 2007)**. Moderate resistance rates (51.35%, 54.05%, and 59.46%) were recorded for ampicillin, amoxicillin, and doxycycline respectively, possibly resulting from the production of chromosomal beta-lactamases **(Tayler et al., 2010)** however, these findings were inconsistent with those of **El-Bahar et al. (2019)** and **Tartor et al. (2021)**, who reported that all the tested *A. hydrophila* isolates had high resistance to Ampicillin and Amoxicillin, and that approximately 90% and 96.7% respectively, were susceptible to Doxycycline. Higher resistance rates (91.89, 83.78, and 78.38%) were reported for Erythromycin, Tylosin, and Sulfadimethexine respectively. This finding agrees with that of **Vivekanandhan et al. (2002)**, who reported a higher resistance (97.3%) to Erythromycin among *A. hydrophila* isolates. Most of our isolates (94.59%) were sensitive to Levofloxacin, so it might be the drug of choice for treating this infection and a recent study **(El-Sherbeny et al. 2022)** supports this up.

The in vitro analysis of the antibacterial activity of ChNPs revealed changes in the ultrastructure of the bacterial cells, such as disruption of the cell membrane, which caused atypical changes in cell morphology and size; cytoplasmic lysis; and damage to the cells; these results are consistent with those of **El-Sherbeny et al. (2022)**.

Our study established that the dietary addition of the Ch or ChNPs had positive impacts on the growth performance, immune responses, antioxidant capacity, and general health parameters of *O. niloticus*. Our results established that the incorporation of 10 g Ch or 100 mg ChNPs/kg diet significantly improved the weight gain and feed intake of *O. niloticus* and increased the feed efficiency in comparison with those of the CTR and fish-fed ChNPs demonstrated the greatest increase in growth performance. The reasons for these results might be that ChNPs eliminate potential microorganisms, increase the number of beneficial bacteria, increase the activity of microbial enzymes (amylase and lipase), and increase the height of intestinal villi **(Abd El-Naby et al. 2019)**, which could all improve feed digestion and nutrient absorption. Comparable results were attained by **Zaki et al. (2015)**, who reported that providing Ch diets to *Dicentrarchus labrax* at doses of 1 and 2 g/kg diet greatly increased feed efficiency and growth performance. However, **Shiau and Yu (1999)** reported that the addition of chitosan to tilapia diets at 2%, 5%, and 10% suppressed growth. This was due to the high fiber content of the diet, which reduces the digestibility of dry matter and lipids and inhibits nutrient absorption via the intestinal epithelium.

According to our hematological findings, the uppermost erythrocytic count, Hb level,

and Ht were recorded in the Ch or ChNP-treated groups. The ChNPs-treated group exhibited a nonsignificant increase in hematological parameters compared to those of the Ch group. These outcomes are comparable to those of **Ranjan et al. (2014)**, who reported that feeding *Lates calcarifer* a diet supplemented with Ch significantly increased the number of RBCs compared to that in the CTR. **Aly et al. (2023)** reported that compared with control diet-fed fish, fish-fed ChNPs exhibited a substantial increase in WBC count from (34.23 × 103/ml) to (53.27 × 10^3/ml). This finding is in agreement with our results, which showed a significant increase in WBC count in the groups fed Ch (34.19 × 10^3/ml) or ChNPs (34.38 × 10^3/ml) compared with the control group (32.103 × 10^3/ml). Heterophils are key elements of the cellular defense mechanism against a wide range of infections. The assessment of their function is valuable in evaluating fish health status. In this study, the most significant percentage of heterophils ($P \leq 0.05$) was detected in the Ch (55.99%) and ChNP (56.19%) treated groups, which was comparable to that in the CTR (48.60%).

ALT and AST might serve as indicators of liver damage because they are free of blood when hepatocytes are destroyed. The current trial revealed that the mean levels of ALT and AST were insignificantly ($P > 0.05$) lower in the treated groups than in the CTR; this difference might be related to the safety of the tested doses of Ch and ChNPs. These findings agreed with the results of an earlier trial by **Cha et al. (2008)** which established a significant decrease in ALT levels in fish-fed Ch in comparison with those in CTR. Urea and creatinine are indicators of the general health of the kidneys and gills. In our study, urea and creatinine were insignificantly changed in both the Ch and ChNPs groups versus the CTR. Fish fed either Ch or ChNPs displayed higher levels of total serum protein and globulin, which indicates an improvement in innate immunity, and these results are inconsistent with those of **El-Naggar et al. (2021)**.

Similar outcomes have been observed in olive flounder **(Cha et al., 2008)** and *Epinephelus bruneus* **(Harikrishnan et al. 2012)**, following feeding on Ch-supplemented diets. In our study, the decrease in the A/G ratio in the Ch-fed groups in comparison with that in CTR may be due to the increase in the globulin level compared with the albumin level, which did not significantly change among the fish groups. The present study revealed a significant increase in PA % in groups supplemented with Ch/ ChNPs. Our findings are consistent with those of **Shanthi Mari et al. (2014)**, who reported a significant increase in the percentage of PAs in the fish groups fed 1% chitin and chitosan in the 2nd and 4th weeks. The PI was noticeably greater in all the groups receiving Ch/ ChNPs than in the control group, and the ChNP group exhibited the highest PI. Additionally, lysozyme activity improved significantly after feeding Ch or ChNP supplemented diets and peaked in ChNPs-fed fish. Similar findings have been reported by **Shanthi Mari et al. (2014)** and **Ahmed et al. (2021)**. These reports support our results showing that the stimulatory effects of Ch. Moreover, the concentration of MDA, which is a crucial analytical indicator of oxidative cell damage, significantly decreased in the Ch or ChNP groups, and the levels of antioxidant enzymes (SOD and CAT) significantly ($P \leq 0.05$) increased in the same groups. Previous studies demonstrated the antioxidant activities of Ch, which may be related to its ability to chelate substances and scavenge reactive oxygen species (ROS). These findings matched those of **Aly et al. (2023)**, **El-Naggar et al. (2021)**, and **Abdel-Tawwab et al. (2019)**, who reported that SOD activity was considerably greater in fish-fed diets supplemented with ChNPs.

Chemical analysis of the fish body revealed that the addition of 10 g Ch or 100 mg ChNPs/ kg diet significantly increased the crude protein content. This change was accompanied by increased serum protein levels and high growth performance. Conversely, moisture, crude lipids, CHO, and total energy were significantly lower in the same groups. These results may be attributed to ability of Ch to improve nutrient absorption, leading to better utilization of dietary proteins. Also, Ch promotes gut health by enhancing the gut microbiota and increasing

the length of intestinal villi **(Abd El-Naby et al. 2019)**. A healthier gut can improve nutrient absorption and protein deposition in the body. Chitosan can boost the immune system of fish as reported in our study, leading to better overall health and potentially affecting protein composition. **Yan et al. (2017)** reported that the gut lipid content and mRNA expression levels of fatty acid binding protein 2 and intestinal lipoprotein lipase decreased with increasing chitosan concentration, which suggested that the variations in crude lipids could also be related to the dose of chitosan. Moreover, **Shiau & Yu (1999)** attributed the decrease in crude lipids to the impact of chitosan adsorbing on the reducing absorption of dietary lipids.

In the present study, feeding fish Ch or ChNPs caused a significant decrease in the total aerobic bacterial count in the fish meat before the bacterial challenge. Studies have demonstrated that Ch coatings or films can effectively decrease the microbial spoilage of fish and extend its shelf life. The antimicrobial properties of chitosan disrupt the bacterial cell membrane, leading to bacterial death and significant reduction in the total bacterial count in fish muscle. It has been proposed that its capacity to increase the permeability of the outer membrane of gram-negative bacteria contributes for its antibacterial activity **(Edo et al. 2024)**. Ch's positive charges disrupt negatively charged macromolecular residues at the surface of bacteria, likely by competing with calcium for electronegative positions on the membrane, making the membrane unstable and then binding with DNA preventing bacterial replication **(Rajeshkumar et al. 2009).**

The lowest cumulative mortality was detected in G6 (ChNPs+levofloxacin; 20%), followed by G5 (ChNPs; 26.67%), compared to 73.33% in infected control G1. Several earlier studies revealed decreased mortality in fish-fed Ch-supplemented feeds after they were experimentally infected **(Gopalakannan and Arul, 2006)**. As reported by **Abdel-Razek (2019)**, fish fed a diet supplemented with ChNPs showed low mortalities (6.7–20%) from all the studied bacterial strains, including *A. hydrophila* which was one of the most susceptible strains to ChNPs, while the control group had a low mortality rate (66.7-100%).

Ranjan et al. (2014) reported that chitosan could be employed as a preventive agent against experimental infection in cultured Asian seabass. Chitosan had an immunostimulant effect, as evidenced by the increase in total serum protein, globulin, PA, PI, and lysozyme activity before or after the experimental infection. The experimental infection with *A. hydrophila* confirmed the protective effect of chitosan against fish mortality. Compared with those in the infected control group, the percentages of PA%, PI, and lysozyme in G6 and G5 (ChNPs with/without levofloxacin) significantly increased, followed by those in G4 and G3 (Ch with or without levofloxacin). Several fish species showed enhanced PA% and lysozyme activities after feeding diets enriched with chitin and Ch **(Cha et al. 2008, Shanthi Mari et al. 2014, and Rajeshkumar et al. 2009)**. Ch was found to be an efficient immunomodulator that prevents bacterial organization in *Cyprinus caprio* **(Mustafa et al. 2014)**.

Zhou et al. (2015) established that Ch increased SOD activity and nitrogen monoxide levels, which are essential for antibacterial and antiviral defense, and enhanced gibel carp immunity. In this study, the immune response of the CTR group increased significantly after experimental infection, which may be attributed to bacterial challenge, as shown by **Gray et al. (2016)**. The experimental infection triggered a significant increase in liver enzymes, urea, and creatinine and reduced hematological parameters (Hg, RBCs, and PCV) in the infected control (G1). While supplementation with Ch/ChNP showed significant improvement toward CTR values before the infection. All the infected-treated groups displayed a significant reduction in liver enzymes and an increase in total protein and globulin levels compared to those in the G1 group, demonstrating the efficacy of these treatments by preventing the bacteria's progressive degeneration of important organs, including the liver. *Epinephelus bruneus* fed a diet containing 2% chitin and chitosan showed a significant increase in RBCs from the 1st to 4th week against *V. alginolyticus* **(Harikrishnan et al. 2012)**.

Compared to those in the infected control group, all the infected-treated groups displayed a noticeable reduction in MDA levels and an increase in CAT levels to varying degrees. The vital effects of these treatments on enhancing fish health status could be evidenced by the antioxidant defense (SOD and CAT) response, which are sensitive indicators of oxidative stress, and ensure that ROS generation and removal occur in dynamic equilibrium to avoid oxidative damage **(Tang et al. 2018)**, in addition to the inhibition of lipid peroxidation caused by these ROS.

CONCLUSION

Our findings indicated that supposedly virulent identified field strain of *A. hydrophila* isolates exist in *O. niloticus* and emphasize the importance of appropriate antibiotic usage in the aquaculture industry. The use of alternative antimicrobial agents with stronger immune-stimulatory activities, such as chitosan, has now gained significance due to resistance that has evolved against synthetic antibiotics employed for treatment. All the therapies used in current trial overwhelmed the adverse effects of *A. hydrophila* infection, demonstrating their efficacy. LEV and ChNPs act effectively and synergistically as antibacterial agents and increase fish survival, which is a valued marker for fish health. Finally, the addition of 10 g Ch or 100 mg ChNPs/kg diet may be a suitable feed additive and growth promoter for *O. niloticus* culture. Both Ch and ChNPs enhance the nonspecific immune response of *O. niloticus* and increase the chance of fish survival.

REFERENCES

Abd El-Naby FS, Naiel MAE, Al-Sagheer AA & Negm SS. 2019. Dietary chitosan nanoparticles enhance the growth, production performance, and immunity in *Oreochromis niloticus*. Aquaculture (501): 82–89.

Abdel-Latif HMR, Abdel-Tawwab M, Khafaga AF, Dawood MAO. 2020. Dietary origanum essential oil improved antioxidative status, immune-related genes, and resistance of common carp (*Cyprinus carpio L.*) to *Aeromonas hydrophila* infection. Fish & Shellfish Immunology (104): 1–7.

Abdel-Tawwab M, Razek NA, Abdel-Rahman AM. 2019. Immunostimulatory effect of dietary chitosan nanoparticles on the performance of Nile tilapia, *Oreochromis niloticus* (L.). Fish & Shellfish Immunology (88): 254–258.

Abo Elsoud MM, El Kady EM. 2019. Current trends in fungal biosynthesis of chitin and chitosan. Bulletin of the National Research Centre 43(59): 1-12.

Aboyadak IM. 2015. Molecular Detection of *Aeromonas hydrophila* as the Main Cause of Outbreak in Tilapia Farms in Egypt. Journal of Aquaculture & Marine Biology 2.

Abu-Elala NM, Mohamed SH, Zaki MM, Eissa AE. 2015. Assessment of the immune-modulatory and antimicrobial effects of dietary chitosan on Nile tilapia (*Oreochrmis niloticus*) with special emphasis to its bio-remediating impacts. Fish & Shellfish Immunology (46): 678–685.

Abu-Zahra NIS, ElShenawy AM, Ali GIE, Alsokary ET, Mousa MA, El-Hady HAMA. 2024. Mentha piperita powder enhances the biological response, growth performance, disease resistance, and survival of *Oreochromis niloticus* infected with *Vibrio alginolyticus*. Aquaculture International (32): 6353–6379.

Aebi H. 1984. [13] Catalase in vitro. Methods in Enzymology. Elsevier 121–126.

Ahmed SAA, Ibrahim RE, Farroh KY, Moustafa AA, Al-Gabri NA, Alkafafy M, Amer SA. 2021. Chitosan vitamin E nanocomposite ameliorates the growth, redox, and immune status of Nile tilapia (*Oreochromis niloticus*) reared under different stocking densities. Aquaculture (541): 736804.

Ajith MP, Aswathi M, Priyadarshini E, Rajamani P. 2021. Recent innovations of nanotechnology in water treatment: A comprehensive review. Bioresource Technology (342): 126000.

Aly SM, Eissa AE, Abdel-Razek N, El-Ramlawy AO. 2023. The antibacterial activity and immunomodulatory effect of naturally synthesized chitosan and silver nanoparticles against Pseudomonas fluorescence infection in Nile tilapia (*Oreochromis niloticus*): An in vivo study. Fish & Shellfish Immunology (135): 108628.

AOAC International. 2019. AOAC Official Method 925.55 Loss on Drying (Moisture) in Salt." Edited by George W. LatimerJr. Official Methods of Analysis of AOAC International (Volume I).

Aoki T. 2011. Fish diseases and disorders. Volume 3: Viral, bacterial and fungal infections. infections - CABI.org CABI Publishing, Wallingford, UK.

APHA (American Public Health Association). 2001. Compendium of methods for the microbiological examination of food. 4th Ed. APHA, Washington, D.C.

Bergmeyer HU, Horder M, Rej R. 1986. International Federation of Clinical Chemistry (Ifcc)1),2). Clinical Chemistry and Laboratory Medicine (24): 481–510.

Blaxhall PC, Daisley KW. 1973. Routine hematological methods for use with fish blood. Journal of Fish Biology (5): 771–781.

Cha S-H, Lee JS, Song CB, Lee KJ, Jeon YJ. 2008. Effects of chitosan-coated diet on improving water quality and innate immunity in the olive flounder, *Paralichthys olivaceus*. Aquaculture (278): 110–118.

Chikwendu CI, Obi RK, Ibe KC, Orji JC. 2020. The Isolation of Antimicrobial Resistant *Aeromonas* spp. from Aquaculture Samples and their Susceptibility to Medicinal Plant Extracts. Microbiology Research Journal International 56–67.

CLSI. 2020. Performance Standards for Antimicrobial Susceptibility Testing of Bacteria Isolated from Aquatic Animals. 3rd Ed. CLSI Supplement VET04. Wayne, PA: Clinical and Laboratory Standards Compendium of Methods for the Microbiological Examination of Foods. American Public Health Association.

Edo GI, Yousif E, Al-Mashhadani MH. 2024. Chitosan: An overview of biological activities, derivatives, properties, and current advancements in biomedical applications. Carbohydrate Research (542): 109199.

El-Ashram A. 2002. On *Aeromonas hydrophila* Infection Among Cultured Tilapias: A Biological, Histopathological and Management Study. Egyptian Journal of Aquatic Biology and Fisheries (6): 181–202.

El-Bahar HM, Ali NG, Aboyadak IM, Khalil SAES, Ibrahim MS. 2019. Virulence genes contributing to *Aeromonas hydrophila* pathogenicity in *Oreochromis niloticus*. International Microbiology (22): 479–490.

Ellis AE, lysozyme assays: In stolen aal;sos. Tech. Fish immunology, 101-103. 1990. (n.d.).

El-Naggar M, Salaah S, El-Shabaka H, El-Rahman FA, Khalil M, Suloma, A. 2021. Efficacy of dietary chitosan and chitosan nanoparticles supplementation on health status of Nile tilapia, *Oreochromis niloticus* (L.). Aquacult. Rep., (19):100628.

El-Sherbeny EMEl, Khoris EA, Kassem S. 2022. Assessment the efficacy of some various treatment methods, in vitro and in vivo, against *Aeromonas hydrophila* infection in fish with regard to side effects and residues. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology (253): 109246.

Gray MA, Choy CH, Dayam RM, Ospina-Escobar E, Somerville A, Xiao X, Ferguson SM, Botelho RJ. 2016. Phagocytosis enhances lysosomal and bactericidal properties by activating the transcription factor TFEB. Current Biology (26): 1955–1964.

Harikrishnan R, Kim JS, Balasundaram C, Heo MS. 2012. Immunomodulatory effects of chitin and chitosan enriched diets in *Epinephelus bruneus* against *Vibrio alginolyticus* infection. Aquaculture (326–329): 46–52.

Harugade A, Sherje AP, Pethe A. 2023. Chitosan: A review on properties, biological activities and recent progress in biomedical applications. Reactive and Functional Polymers (191): 105634.

Iacovache I, De Carlo S, Cirauqui N, Dal Peraro M, van der Goot FG, Zuber B. 2016. Cryo-EM structure of aerolysin variants reveals a novel protein fold and the pore-formation process. Nature Communications 7.

Ibrahim RE, Amer SA, Farroh KY, Al-Gabri NA, Ahmed AI, El-Araby DA, Ahmed SAA. 2021. The effects of chitosan-vitamin C nanocomposite supplementation on the growth performance, antioxidant status, immune response, and disease resistance of Nile tilapia (*Oreochromis niloticus*) fingerlings. Aquaculture (534): 736269.

Ishikawa ANM, Ranzani-Paiva MJT, Lombardi JV. 2008. Total Leukocyte Counts Methods in Fish, *Oreochromis niloticus*. Archives of Veterinary Science (13): 54–63.

Jacobs L, Chenia HY. 2007. Characterization of integrons and tetracycline resistance determinants in Aeromonas spp. isolated from South African aquaculture systems. International Journal of Food Microbiology (114): 295–306.

Maturin L, Peeler JT. 2001. BAM: Aerobic plate count. US Food and Drug Administration: Silver Spring, MD, USA.

Meng X, Wang J, Wan W, Xu M, Wang T. 2017. Influence of low molecular weight chitooligosaccharides on growth performance and non-specific immune response in Nile tilapia *Oreochromis niloticus*. Aquaculture International (25): 1265–1277.

Mustafa S.A, Alfaragi JK, Aref Z. 2014. The influence of chitosan on immune status and survival rate of *Cyprinus carpio* L. challenged with *Aeromonas hydrophila*. Kufa Journal of Veterinary Medical Science (5): 93–104.

Nagarajan Y, Ramaiyan B, Chanthuru A, Nila KM (2008, November 30): Detection of Virulence Gene in *Aeromonas hydrophila* Isolated from Fish Samples Using PCR Technique. Global J Biotech Biochem 4

Nishikimi M, Appaji Rao N, Yagi K. 1972. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochemical and Biophysical Research Communications (46): 849–854.

Oliveira STL, Veneroni-Gouveia G, Costa MM. 2012. Molecular characterization of virulence factors in *Aeromonas hydrophila* obtained from fish. Pesquisa Veterinária Brasileira (32): 701–706.

Patton CJ, Crouch SR. 1977. Spectrophotometric and kinetics investigation of the Berthelot reaction for the determination of ammonia. Analytical Chemistry (49): 464–469.

Pauzi NA, Mohamad N, Azzam-Sayuti M, Yasin ISMd, Saad MZ, Nasruddin NS, Azmai MNA. 2020. Antibiotic susceptibility and pathogenicity of *Aeromonas hydrophila* isolated from red hybrid tilapia (*Oreochromis niloticus*× *Oreochromis mossambicus*) in Malaysia. October-2020 (13): 2166–2171.

Platt N, Fineran P. 2015. Measuring the phagocytic activity of cells. Methods in Cell Biology. Elsevier 287–304.

Rajeshkumar S, Venkatesan C, Sarathi M, Sarathbabu V, Thomas J, Anver Basha K, Sahul Hameed AS. 2009. Oral delivery of DNA construct using chitosan nanoparticles to protect the shrimp from white spot syndrome virus (WSSV). Fish & Shellfish Immunology (26): 429–437.

Ranjan R, Prasad KP, Vani T, Kumar R. 2014. Effect of dietary chitosan on hematology, innate immunity and disease resistance of Asian seabass, *Lates calcarifer* (Bloch). Aquaculture Research (45): 983–993.

Rozi, Rahayu K, Daruti DN. 2018. Detection and analysis of hemolysin genes in *Aeromonas hydrophila* isolated from Gouramy (*Osphronemus gouramy*) by polymerase chain reaction (PCR). IOP Conference Series: Earth and Environmental Science

(137): 012001.

Sax SM. 1975. Clinical Chemistry—Principles and Technics, 2nd ed. R. J. Henry, J. W. Winkelman, and D. C. Cannon, Eds. Harper & Row, Publishers, New York, N. Y., 1974, xii + 1629 pp. 267 illustrations. $37.50. Clinical Chemistry (21): 273–274.

Sen K, Rodgers M. 2004. Distribution of six virulence factors in Aeromonas species isolated from US drinking water utilities: A PCR identification. Journal of Applied Microbiology (97): 1077–1086.

Shah SL, Altindag A. 2004. Hematological Parameters of Tench (*Tinca tinca L.*) after Acute and Chronic Exposure to Lethal and Sublethal Mercury Treatments. Bulletin of Environmental Contamination and Toxicology (73): 911–918.

Shanthi Mari LS, Jagruthi C, Anbazahan SM. 2014. Protective effect of chitin and chitosan enriched diets on immunity and disease resistance in *Cirrhina mrigala* against *Aphanomyces invadans*. Fish & Shellfish Immunology (39): 378–385.

Shiau SY, Yu YP. 1999. Dietary supplementation of chitin and chitosan depresses growth in tilapia, *Oreochromis niloticus*× *O. aureus*. Aquaculture (179): 439–446.

Singh V, Rathore G, Kapoor D, Mishra BN, Lakra WS. 2008. Detection of aerolysin gene in *Aeromonas hydrophila* isolated from fish and pond water. Indian Journal of Microbiology (48): 453–458.

Tahmasebi P, Javadpour F, Sahimi M. 2015. Three-Dimensional Stochastic Characterization of Shale SEM Images. Transport in Porous Media (110): 521–531.

Tang Y, Han L, Chen X, Xie M, Kong W, Wu Z. 2018. Dietary Supplementation of Probiotic *Bacillus subtilis* Affects Antioxidant Defenses and Immune Response in Grass Carp Under *Aeromonas hydrophila* Challenge. Probiotics and Antimicrobial Proteins (11): 545–558.

Tartor YH, ELNaenaeey ESY, Abdallah HM, Samir M, Yassen MM, Abdelwahab AM. 2021. Virulotyping and genetic diversity of *Aeromonas hydrophila* isolated from Nile tilapia

Tayler AE, Ayala JA, Niumsup P. 2010. Induction of B-lactamase production in *Aeromonas hydrophila* is responsive to B-lactam-mediated changes in peptidoglycan composition. Microbiology (156): 2327–2335.

Tietz NW. 1995. Clinical Guide to Laboratory tests. 3rd ed. Philadelphia. WB. Saunders, 268-273.

Vivekanandhan G, Savithamani K, Hatha AAM, Lakshmanaperumalsamy P. 2002. Antibiotic resistance of *Aeromonas hydrophila* isolated from marketed fish and prawn of South India. International Journal of Food Microbiology (76): 165–168.

Yan J, Guo C, Dawood MAO, Gao J. 2017. Effects of dietary chitosan on growth, lipid metabolism, immune response and antioxidant-related gene expression in *Misgurnus anguillicaudatus*. Beneficial Microbes (8): 439–449.

Zaki MA, Salem ME-S, Gaber MM, Nour AM. 2015. Effect of Chitosan Supplemented Diet on Survival, Growth, Feed Utilization, Body Composition & Histology of Sea Bass (*Dicentrarchus labrax*). World Journal of Engineering and Technology 03: 38–47.

Zhou Q, Xie J, Ge X, Habte-Tsion HM, Liu B , Ren M. 2015. Growth performance and immune responses of gibel carp, *Carassius auratus gibelio*, fed with graded level of rare earth-chitosan chelate. Aquaculture International 24: 453–463.