한국어병학회지 제35권 제1호 (2022)
J. Fish Pathol., 35(1) : 077~092

www.ksfp.org
pISSN 1226-0819, eISSN 2233-5412
http://dx.doi.org/10.7847/jfp.2022.35.1.077

# Prevention of *Citrobacter freundii* (MW279218) infection in Nile tilapia, *Oreochromis niloticus* using zinc oxide nanoparticles

**Fatma M. M. Korni[*†], Usama K. Moawad[**], Asmaa N. Mohammed[***] and Asmaa Edrees[****]**

[*]*Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt.*
[**]*Department of Histology and Cytology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt.*
[***]*Department of Hygiene, Zoonoses and Epidemiology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt.*
[****]*Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt.*

Aquaculture development is based on the ideas of increasing production while reducing economic losses. Bacterial diseases are the leading source of fish cases. *Citrobacter freundii* has been linked to septicemia and mortality all over the world. In the current study, the cause of mortality in *O. niloticus* was *C. freundii* MW279218. External hemorrhages were seen on the affected fish, as well as paleness in the liver and kidney congestion. *C. freundii* MW279218 had a median lethal dosage of $1.5\times10^5$ CFU/mL. Zinc oxide and zinc oxide nanoparticles (ZnO-NPs) were tested for their biocidal effectiveness against *C. freundii* MW279218. The lethal effect of ZnO-NPs for *C. freundii* MW279218 was 100% when compared to zinc oxide compound, and the inhibition zone width was 2.31.1mm at the highest tested concentrations (70 mg/L) compared to the lowest (35 and 45 mg/L, respectively). Fish were fed three different diets for 28 days: diet 1 (no additives), diet 2 (100 mg of ZnO-NPs/kg of feed), and diet 3 (200 mg of ZnO-NPs/kg of feed). Organs were also collected for histopathology 96 hours after injection ($P<0.05$). In the groups given 200 mg of ZnO-NPs, there was 10% mortality and 80% RPS. The group fed 100 mg of ZnO-NPs/kg, on the other hand, had 20% mortality and 60% RPS, compared to 50% mortality in the control positive group. Histopathological examinations demonstrated significant alterations in the control positive group and mild lesions in the hepatopancreas of the groups administered 100 mg ZnO-NPs/kg of feed. The groups fed 200 mg of ZnO-NPs/kg diet, on the other hand, showed no histological alterations. ZnO-NPs were found to be effective in the up regulation of both IL-10 and complement 5 immune-related genes.

**Key words:** *O. niloticus, C. freundii*, fish immunity, gene, prevention, Zinc oxide nanoparticles.

## Introduction

*Oreochromis niloticus* (*O. niloticus*), the Nile tilapia, is one of the most widely dispersed fish species on the planet. Furthermore, there has been a significant growth in demand, resulting in intensification and high production. However, the intensification leads to a quick deterioration of water quality and an increase in the incidence of diseases caused by fish stress (Sebastião et al., 2015). Disease in aquaculture is a severe problem that aquaculturists are

[†]Corresponding author: Fatma M. M. Korni
Tel: +02-01100921799
E-mail: naglaamostafa1@yahoo.com

concerned about because of the high financial losses (Lafferty et al., 2015).

The bacterial infections that cause annual economic losses to the aquaculture industry, estimated at billions of dollars worldwide, are the most important sector for fish diseases. (Pridgeon, 2012). *Citrobacter freundii (C. freundii)* infection, which is caused by a member of the Enterobacteriaceae family, is one of these bacterial infections. It is marked by bacterial septicemia and high mortality (Jeremić et al., 2003; Abdel-Fattah et al., 2016). Furthermore, *C. freundii* is blamed for impeding the aquaculture industry's long-term development (Pan et al., 2021).

There were extensive degenerative alterations, fusion and necrosis of the catfish gill secondary lamellae, as well as inflammatory cell infiltration, congestion, and superficial epithelium detachment, all of which were caused by *C. freundii* infection (Baldissera et al., 2018). Moreover, infected Nile tilapia showed mononuclear cell infiltration and congestion of the gill main lamellae, as well as hepatic cell vacuolar degeneration (Aly et al., 2012). There was also gill lamellae destruction, hepatocyte vacuolar degeneration, and the absence of Von Kupffer cells in infected Mozambique tilapia (Thanigaivel et al., 2015).

Unfortunately, aquaculture's success is reliant on the usage of chemicals to combat a variety of diseases that plague the business. Antibiotics have been proven to be ineffective in the treatment of *C. freundii* infection in fish (Sato et al., 1982). Furthermore, due to antibiotic resistance, which poses a hazard to public health, the use of antibiotics will be prohibited. As a result, safe options to solve these issues in aquaculture are urgently needed (Lieke et al., 2020).

Nanoparticles of trace minerals have recently shown significant promise as feed additives. When compared to their bulk counterparts, nanoparticles have innovative qualities such as tiny size, improved bioavailability, higher specific surface area, increased catalytic effectiveness, stronger adsorbing ability, and use in lower doses. (Apines-Amar et al., 2004), as well as antimicrobial effects against a broad range of bacteria and fungi (Swain et al., 2016).

Zinc oxide nanoparticles showed antibacterial activity against various fish pathogens, allowing for unconventional control of disease outbreaks (Shaalan et al., 2017). They exhibited marked antimicrobial activity against *Aeromonas hydrophila*, *Aeromonas salmonicida* subsp. *salmonicida*, *Edwardsiella ictaluri, Edwardsiella tarda* and *Yersinia ruckeri* (Shaalan et al., 2017). Zinc oxide nanoparticles exhibit potent antimicrobial activities through complex mechanisms of action that include release of $Zn^{2+}$ ions, production of reactive oxygen species (ROS) and interference with bacterial replication by inhibition of cellular processes like glycolysis, acid tolerance and transmembrane proton translocation (Ramamoorthy et al 2013, Swain 2016, Gunalan et al., 2012). Previous research has found that a smaller dose of ZnO-NPs is more effective in improving fish development and immunity (Faiz et al., 2015; Tawfik et al., 2017).

The complement system and Interleukin-10 (IL-10) are key innate immune system components that are largely expressed in the head, kidneys, spleen, and gills. (Magnadóttir, 2006; Piazzon et al., 2015). In addition, the complement system is a critical component of innate immunity that plays a key role in the defense against pathogens and homeostasis (Ricklin et al., 2010). Interleukin-10 is known to suppress inflammatory responses elicited by activated macrophages (Bogdan et al., 1991). It also suppresses the generation of nitric oxide, the expression of MHC class II, and the synthesis of a variety of macrophage-derived pro-inflammatory factors (Fiorentino et al., 1991, Gazzinelli et al., 1992, Niiro et al., 1994, Aste-Amezaga et al., 1998).

There had been no previous reports of ZnO-NPs being used to inhibit *C. freundii* infection in *O. niloticus* and to measure complement and IL-10 genes. As a result, the goal of this study was to find the cause of death in *O. niloticus*. The median lethal dose (LD50) of a pathogenic isolate was identified by an

experimental infection. After in-vitro testing, the efficacy of ZnO-NPs as feed additives on the prevention of *C. freundii* infection in *O. niloticus* was also investigated. IL-10 and complement immunostimulants genes were also evaluated in the feeding groups' tissue.

## Materials and methods

### Ethical committee

All experiments were approved by Beni-Suef Institutional Animal Care and Use Committee (BSU-IACUC) of Faculty of Veterinary Medicine, Beni-Suef University, Egypt.

### Fish collection and management

**Naturally diseased *O. niloticus* for clinical examination and bacteriological isolation**

A total number of 30 diseased *O. niloticus* with an average body weight 18 ± 5 g showing extensive signs of septicemia was collected alive from Abo-Saleh fish hatchery, Beni-Suef, Egypt. Samples were transferred in plastic bags containing oxygenated water to the wet laboratory of Fish Diseases and Management Department, Faculty of Veterinary Medicine, Beni-Suef University, Egypt.

**Collection of experimental fish**

A total of 336 apparently healthy *O. niloticus* with an average body weight of 15 ± 5 g were collected alive from Abo-Saleh fish hatchery, Beni-Suef, Egypt for the experimental studies. The collected fish were transferred to the wet laboratory of Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Beni-Suef University, Egypt. Fish were kept in three fiberglass tanks with 500 L capacity for each and supplied with chlorine-free tap water and continuous aeration. The fish were acclimatized for 14 days in the experimental fiberglass tanks and fed a 3% body weight pelleted commercial fish diet (Table 1).

Table 1. Composition of the commercial pelleted fish diet (Brsiek factory, Egypt)

| Composition of ingredients (25% crude protein) | The percentage (%) |
|---|---|
| Fish meal | 6 |
| Soya bean meal | 36 |
| Rice polish | 22 |
| Yellow corn | 34 |
| Mono-calcium phosphate | 1 |
| Common salt | 0.5 |
| Premix | 0.5 |

**Management of experimental fish**

After acclimatization, fish were redistributed into 90 × 25 × 40 cm glass aquaria with a water volume of 30 L. Each aquarium was supplied with continuous artificial aeration (1 air stone) through an air blower. The water exchange rate in the experimental aquaria was 10% per day. Fish were fed 3% of their body weight. Throughout the experiments, water quality parameters were measured twice a week. These parameters included water temperature (27 ± 1ºC) using a water thermometer (Yellow Spring Instrument Co., USA), dissolved oxygen (DO; 7 ± 2 mg/L) using a DO meter (Yellow Spring Instrument Co. USA) and pH (7-8) using pH indicator paper (Fisher Scientific, Denver, CO, USA).

### Clinical examination of diseased fish

External and internal examination of diseased *O. niloticus* were carried out for detection of general signs of septicemia, subcutaneous edema, hemorrhages, scale losses and other clinical abnormalities according to (Austin et al., 2012).

### Bacteriological examination

Specimens taken from liver, spleen and kidney of diseased *O. niloticus* were inoculated on TSA plates. The Plates were incubated at 25ºC for 24 h. Selected colonies were subjected to biochemical and molecular identification.

### Biochemical examination

Motility, Gram staining and morphology of the most dominant colonies in the subculture was determined as described by (Quinn et al., 2002).

### Molecular identification

It was performed according to (Lagacé et al., 2004).

**DNA extraction**

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 20 µl of proteinase K and 200 µl of lysis buffer at 56ºC for 10 minutes. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

**Oligonucleotide Primer and PCR amplification**

Primers were supplied from Metabion (Germany) and listed in (Table 2). For PCR amplification, Primers were utilized in a 25 µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

**Analysis of the PCR products**

Products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. A Gelpilot 100 bp plus ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

### Preparation and characterization of ZnO–NPs

Zinc oxide nanoparticles (Loba, Chemi, Pvt. Ltd, India) were prepared by the high-energy ball milling technique as a method described by (Salah et al., 2011). The ZnO-NPs were characterized by FT-IR spectrum (Fourier-transform infrared spectrum; VERTEX, 70) at the wave length of 500-3500 cm-1 and HR-TEM microscopy (High-resolution Transmission Electron Microscopy; a JEOL-JEM 2000EX). HR-TEM microscopy was examined in the Central lab of National Research Center, Cairo, Egypt. Whilst FT-IR spectrum of ZnO-NPs was investigated at the Faculty of Postgraduate Studies of Advanced Science, Beni-Suef University, Egypt.

### Measuring the biocidal efficacy of ZnO–NPs against the bacterial isolates

The biocidal efficacy of both ZnO and ZnO-NPs against one µL ($1 \times 10^6$ CFU/mL) of bacterial strains that isolated from kidney, liver and spleen of diseased *O. niloticus* was estimated using the broth micro-dilution, and well diffusion method according to guidelines of CLSI (Wikler, 2006 and Qi et al., 2004) at different tested concentrations (35,45,55,65,70 mg/L, respectively) .

Table 2. Primers sequences, target genes, amplicon sizes and cycling conditions

| Target gene | Primers sequences | Amplified segment (bp) | Primary Denaturation | Amplification (35 cycles) | | | Final extension | Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | Secondar denaturation | Annealing | Extension | | |
| 16S rRNA | AGAGTTTGATCMTGGCTCAG<br>TACGGYTACCTTGTTACGACTT | 1485 | 94˚C<br>5 min. | 94˚C<br>30 sec. | 56˚C<br>1 min. | 72˚C<br>1.2 min. | 72˚C<br>12 min. | Lagacé *et al.*, 2004 |

### Diet Preparation

The commercial pelleted fish diet was ground into fine powder using mortar. The ZnO-NPs were mixed directly with the prepared fine powder to obtain three fish diets. Diet 1 with no additives (control), diet 2 with 100 mg of ZnO-NPs /kg of feed and diet 3 with 200 mg of ZnO-NPs /kg of feed. Each fish diet contents were mixed with distilled water to obtain a homogenous mixture. The mixture was passed through manual hand-minced meat processing machine (Italy) for producing extruded strings which were dried at 40°C for 24 h and then broken down to about 2 mm long pellets (Rattanachaikunsopon and Phumkhachorn, 2010).

### Experimental design and feeding regime

One hundred and eighty six *O. niloticus* were separated into four groups 17 fish each except the first group had 11 fish (negative control). Fish in the first and second groups (negative and positive control, respectively) were fed diet 1, while fish in the third group were fed diet 2 and fish in the fourth group were fed diet 3. Throughout the experimental period, all groups were fed 3% of body weight of their specific diet once a day for four weeks (28 days). At the end of feeding period, three fish from each group, except the first group, with their replicates were used for collection of spleen for expression of complement 5 and IL-10 immune-related genes. Another three fish from each group with their replicates except the first group were used for histopathology. The remaining eleven fish in each group with their replicates were injected with the bacterial isolate. Ninety-six hours post injection, three fish from each group with their replicates were used for histopathology while, the remaining eight fish from each group with their replicates were observed for two weeks to examine the efficacy of ZnO-NPs for prevention of the bacterial isolate.

### Expression of complement 5 and IL-10 immune-related genes

1) Tissue collection

At the end of feeding period (28 days), three fish from each group were rapidly netted and euthanized by an overdose of tricaine methanesulfonate (MS222, Sigma-Aldrich Chemical Co. Germany). After that, pieces of spleen were collected and stored in 2 ml tubes containing RNAlater solution (Merc, Egypt). Finally, samples were kept overnight in a refrigerator at -4°C and frozen at -80°C for RNA extraction.

2) Protocol of expression of complement 5 and IL-10 immune-related genes

2.1) Extraction of RNA

RNA extraction from spleen samples was applied using QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH) when 30 mg of the tissue sample was added to 600 µl RLT buffer containing 10 µl β-mercaptoethanol per 1 ml. For homogenization of samples, tubes were placed into the adaptor sets, which were fixed into the clamps of the Qiagen tissue Lyser. Disruption was performed in high-speed (30 Hz) shaking step for two minutes. One volume of 70% ethanol was added to the cleared lysate and these steps were completed according to the purification of total RNA from animal tissues protocol of the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH).

N.B. On column DNase digestion was done to remove residual DNA.

2.2) Oligonucleotide Primers

The primers of immune-related genes were supplied from Metabion (Germany). Primers sequences, target genes, amplicon sizes and cycling conditions for SYBR green rt-PCR were listed in (Table 3) according to (Gröner et al., 2015). The thermal program for all reactions of RT-qPCR was 94°C for 15 minutes, followed by 40 cycles of 94°C for 15 s and 62°C for 30 s and an extension at 72°C for 30 s.

2.3) SYBR green rt-PCR

Primers were utilized in 25 µl reaction containing

Table 3. Primers sequences, target genes, amplicon sizes and cycling conditions for SYBR green rt-PCR

| Target gene | Primers sequences | Reverse transcription | Reference |
|---|---|---|---|
| EF-1α | CCTTCAACGCTCAGGTCATC<br>TGTGGGCAGTGTGGCAATC | 50˚C 30 min. | Gröner *et al*., 2015 |
| IL-10 | CTGCTAGATCAGTCCGTCGAA<br>GCAGAACCGTGTCCAGGTAA | | Standen *et al*., 2016 |
| C5 | TGGCAAGGACTTTTTCTGCT<br>AGCACAGGTATCCAGGGTTG | | Syahputra *et al*., 2019 |

12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of Revert Aid Reverse Transcriptase (200 U/µL) (Thermo Fisher), 0.5 µl of each primer of 20 pmol concentration, 8.25 µl of water and 3 µl of RNA template. The reaction was performed in a Strata gene MX 3005P real time PCR machine.

2.4) Analysis of the SYBR green rt-PCR results

Amplification curves and CT values were determined by the stratagene MX3005P software. To estimate the variation of gene expression on the RNA of the different samples, the CT of each sample was compared with that of the positive control group according to the "ΔΔCt" method stated by (Yuan et al., 2006) using the ratio of ($2^{-DDct}$). Whereas ΔΔCt = ΔCt reference – ΔCt target

ΔCt target = Ct control – Ct treatment and ΔCt reference = Ct control- Ct treatment

3) Prevention of bacterial isolate in *O. niloticus*

3.1) Experimental infection and determination of $LD_{50}$ of bacterial isolate

A total of 180 apparently healthy *O. niloticus* were subdivided into six groups (10 fish/each) with three replicates. An overnight culture of the isolate was adjusted to densities $1.5 \times 10^8$, $1.5 \times 10^7$, $1.5 \times 10^6$, $1.5 \times 10^5$ and $1.5 \times 10^4$ CFU/mL. Each dilution was injected intraperitoneally into a fish group at a dose of 200 µl/fish. The fish of the $6^{th}$ group were used as control and injected with 200 µl of sterile saline. All fish groups were observed for two weeks. Mortality was recorded daily and the organs were aseptically streaked on TSA agar for re-isolation and re-identification.

3.2) Prevention scheme

At the end of dietary experimental period (28 days), the first (control negative) group with its replicates was injected intraperitoneally with 200 µl physiological saline. The second (control positive), third and fourth groups were injected intraperitoneally with 200 µl of bacterial isolate at $1.5 \times 10^5$ CFU/mL concentration. The injected fish were maintained in a separate glass aquaria for 2 weeks. The mortality was recorded and the relative percent survival (RPS) was calculated according to (Amend, 1981) using the formula of "RPS = 1 − (% of mortality in treated groups / % of mortality in control group) ×100".

## Results

### Clinical abnormalities of diseased *O. niloticus*

Diseased fish had general signs of septicemia, subcutaneous edema and hemorrhages in the eyes, on the skin, dorsal fin and base of pectoral fin (Fig. 1A). Scale loss and eye cataract were also observed (Fig. 1B). Pelvic fin disintegration (Fig. 1C). Scale loss at the two sides of the body and dorsal musculature & hemorrhages on dorsal fin were recorded (Fig. 1D). Liver paleness and edematous intestine were noticed (Fig. 1E). Also, there were congestion in gills and kidney (Fig. 1F).

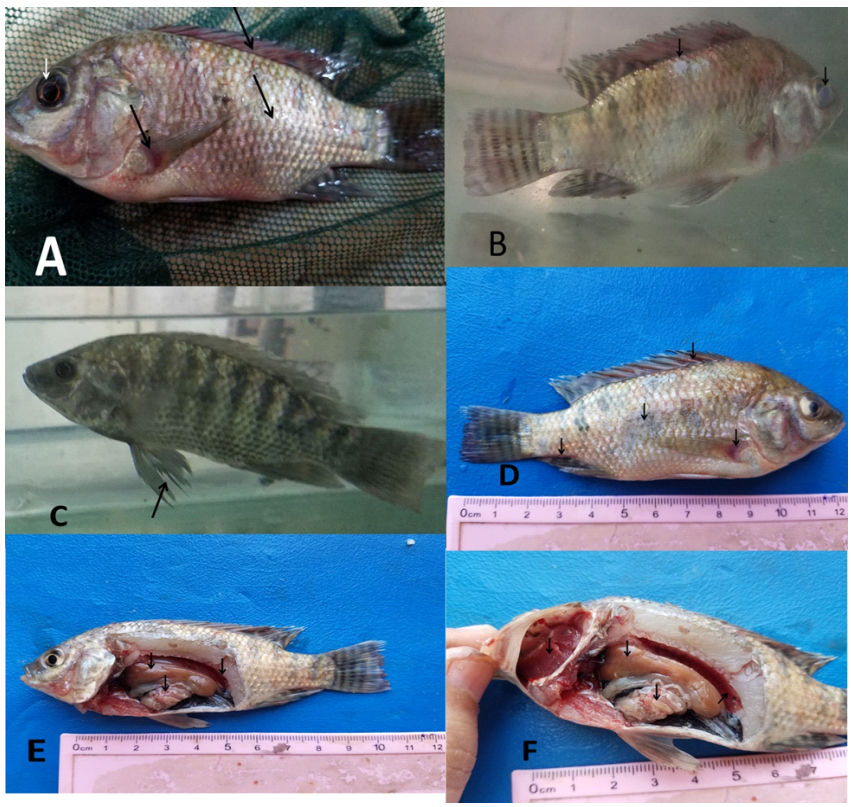


Fig. 1. Clinical abnormalities of naturally diseased *O. niloticus* with *C.freundii*. Subcutaneous edema and hemorrhages in the eyes, on the skin, dorsal fin and base of pectoral fin (A). Scale loss and eye cataract (B). Fin disintegration (C). Scale loss at the two sides of the body and dorsal musculature & hemorrhages on the dorsal fin (D). Pale liver, congested kidney and edematous intestine (E). Congested gills and kidney, pale liver and edematous intestine (F).

### Identification of bacterial isolates

Purification of isolated bacterial colonies on TSA resulted in creamy colored, round, smooth, slightly convex, entire edge with 1-2 mm diameter after 24 h incubation at 28°C. Biochemical identification showed Gram-negative, rod-shaped, oxidase negative, citrate positive, indole negative bacteria.

DNA amplification and sequencing revealed two strains of bacteria that were registered in the gene bank; *C. freundii* (MW279218_*C._freundi_Q._nilotica*) and *Klebsiella oxytoca* (MW227610_*K._oxytoca_Q._nilotica*).

### Characterization of ZnO–NPs

TEM microscopy of ZnO-NPs (Fig. 2) exhibited the hexagonal and elongated morphological shape of ZnO-NPs (Fig. 2a) in addition; the NPs diameter was ranged between 31.4 to 91.2 nm as shown in (Fig. 2b). Additionally, FTIR spectra of ZnO-NPs displayed the characteristic peaks were noticed at different wave lengths of 3435.6, 1637.1, 1044.4 and 536 cm-1, which confirmed the formation of ZnO-NPs as presented in (Fig. 3)

### Efficacy of ZnO–NPs against *C. freundii* MW 279218

The efficiency of ZnO-NPs against *C. freundii* MW279218 approved that the lethal effect of ZnO-NPs on *C. freundii* MW279218 was 100% in comparison with zinc oxide compound whereas the inhibition

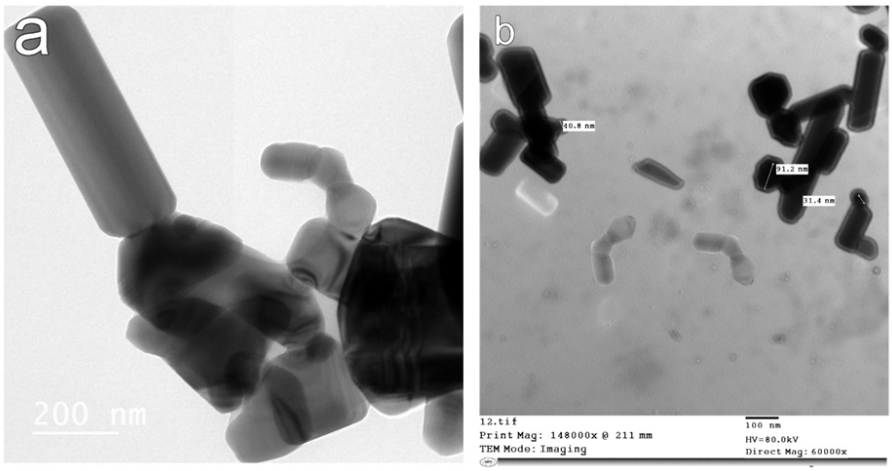


Fig. 2. TEM microscopy of Zn-OPs clarified the hexagonal morphological shape of NPs (a) in addition, the NPs diameter (b) was ranged between 31.4 to 91.2 nm.

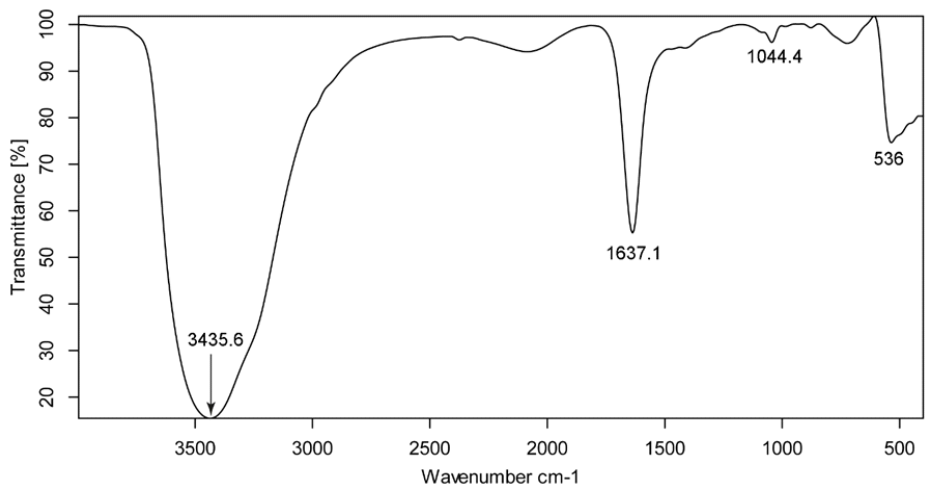


Fig. 3. FTIR spectra of ZnO-NPs showed the characteristic peaks were noted at different wavelength 3435.6, 1637.1, 1044.4 and 536 cm-1 that confirmed the creation of ZnO-NPs.

zone diameter was 2.3±1.1mm at the highest tested concentrations (70 mg/L) compared to the least one (35, and 45 mg/L, respectively) as displayed in Fig 4.

### The effect of dietary ZnO–NPs on the tissue immune–related genes of *O. niloticus*

Fish were protected significantly ($P < 0.05$) after adding ZnO-NPs to diet. Both IL-10 and complement 5 immune-related genes were up regulated in fish groups that were fed diet supplemented with ZnO-

Table 4. changes in serum level of interleukin 10 (IL-10) and complement 5 (C5) in various experimental groups

| Groups | IL 10 | C5 |
|---|---|---|
| Control | $1\pm0.00^{c}$ | $1\pm0.00^{c}$ |
| 100 mg of ZnO-NPs/kg of feed | $2.74\pm0.16^{b}$ | $3.13\pm0.14^{b}$ |
| 200 mg of ZnO-NPs/kg of feed | $3.94\pm0.14^{a}$ | $3.94\pm0.15^{a}$ |

Values are represented as mean ± SE of mean. The different superscript letters are significantly different at ($p < 0.05$) between different groups.

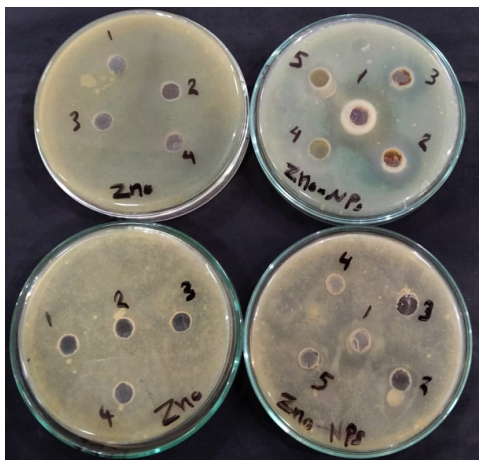


Fig. 4. The antibacterial efficacy of ZnO-NPs against bacterial isolates at different testing concentrations (1) 70, (2) 65, (3) 55, (4) 45, (5) 35 mg/L, respectively using well diffusion method. The highest lethal effect of ZnO-NPs against *C. freundii* was found at a concentration of 70 and 65 mg/L, respectively with an inhibition zone diameter (mm) 20.3±0.0 and 15.1±0.1 mm compared to other tested concentrations. Meanwhile, the isolated *C. freundii* showed their resistant to zinc oxide compound at the same tested concentrations.

NPs. In group 3, IL-10 immune-related gene was increased 2.74±0.16 times that of control group and increased 3.94±0.14 times in group 4 (Table 4) and Fig 5. On the other hand, the complement 5 immune-related gene increased in group 3 3.13±0.14 times that of control group and increased 3.94±0.15times in group 4.

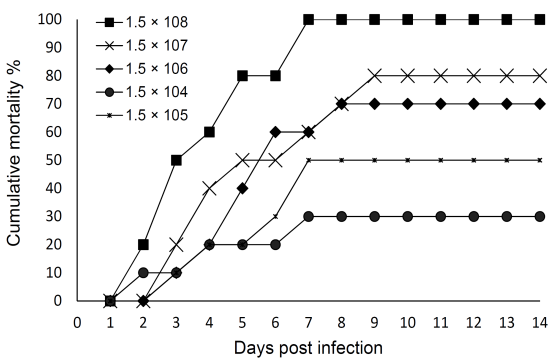


Fig. 5. Cumulative mortality and $LD_{50}$ of *C. freundii* in healthy *O. niloticus*.

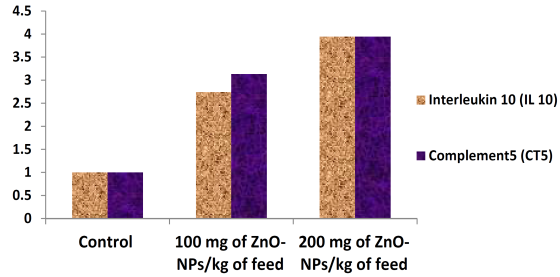


Fig. 6. Changes in serum level of IL-10 and complement 5 in control group, group fed 100mg of ZnO-NPs/kg and 200mg of ZnO-NPs/kg of feed.

## Prevention of *C. freundii* MW279218 infection in *O. niloticus* with ZnO–NPs

**Median lethal dose ($LD_{50}$) of *C. freundii***

The $LD_{50}$ of *C. freundii* MW279218 was 1.5 X $10^5$ CFU/mL (Fig. 6) and the injected fish died at the first week of injection with typical signs of septicemia.

**Prevention of *C. freundii* MW279218 infection in *O. niloticus* with ZnO-NPs**

There was no mortality and 100% RPS in the group fed normal diet then injected with physiological saline (control negative). There was 12.5% mortality and 75% RPS in the groups fed 200 mg of ZnO-NPs /kg then injected with *C. freundii*. On the other hand, there was 25% mortality and 50 RPS in the group fed 100 mg of ZnO-NPs/kg and injected by *C. freundii*. Moreover, the control positive group showed 50% mortality (Fig. 7).

## Histopathological findings

**Control negative group (fed diet 1 "ordinary diet with no additives" then injected intraperitoneally with 200µl physiological saline)**

The gills showed normal histological picture of primary and secondary lamellae; (Fig. 8A). The examined liver tissues (hepatopancreas) showed normal histological picture of portal veins, polyhedral hepatic cells with spherical nuclei and hepatic cords arrangement as well as hepatic sinusoids (Fig. 8B). The spleen

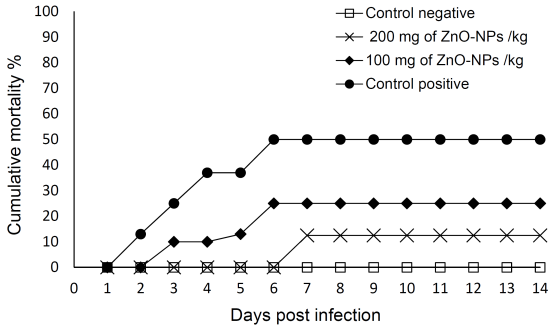


Fig. 7 Cumulative mortality of control negative, experimental feeding groups of dietary 200 mg of ZnO-NPs/kg, 100 mg of ZnO-NPs/kg of feed and the control positive

showed normal structure; red pulps that occupied most of the spleen and surrounded by areas of the white pulps and melanomacrophage centers (Fig. 8C).

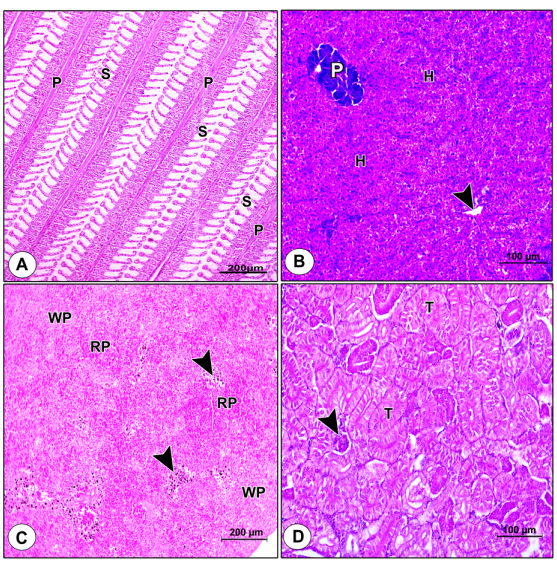


Fig. 8. Photomicrograph of control negative group of *O. niloticus* showing: A) Normal histological picture of primary (P) and secondary (S) gill lamellae; B) Normal histological structure of portal vein (arrowhead), polyhedral hepatocytes arranged in hepatic cords (H) and pancreatic acini (P); C) Normal histological architecture and arrangement of red (RP), white (W) pulps and melanomacrophage centers (arrowheads); D) normal histological picture of kidney; renal tubules (T) and glomerulus (arrowhead). H&E stain, scale bar: 200 µm, 100 µm, 200 µm and 100 µm, respectively.

The kidney showed normal histological structure including the renal glomeruli and tubules (Fig. 8D).

**Control positive group (fed diet 1 "ordinary diet with no additives" then injected intraperitoneally with 200µl of *C. freundii***

The gills showed congestions and hemorrhages of most primary lamellae. The secondary lamelle showed curling with shortening of their length (Fig. 9A). The hepatocytes showed diffuse vacuolar degeneration. The hepatic sinusoids and main hepaopancreatic vessels showed severe dilatation and congestion (Fig. 9B). In addition, some hepatic cells showed pyknosis

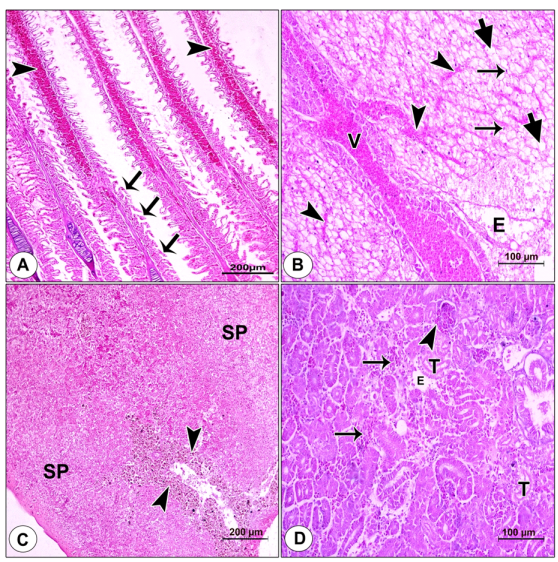


Fig. 9. Photomicrograph of control positive group of *O. niloticus* showing: A) Sever congestions of the blood vessels of the primary gill lamellae (arrowheads), and curling with shortening of secondary lamellae (arrows); B) Diffuse vacuolar degeneration (thick arrows) with nuclear loss and pyknosis (thin arrows) of some hepatic cells, dilatation and congestion of the hepatic sinusoids (arrowheads) and the hepaopancreatic vessel (V), and eduma (E); C) Numerous melanomacrophage centers formation around the splenic vessel (arrowheads) with depletion of splenic pulps (SP); D) Degeneration of renal tubules (T), interstitial infiltration of mononuclear cells (arrows), edema (E) and normal renal corpuscle (arrowhead). H&E stain, scale bar: 200 µm, 100 µm, 200 µm and 100 µm, respectively.

of their nuclei, nuclear loss of some hepatic cells and accumulation of hemosiderin around the pancreatic acini. The Spleen showed formation of numerous melanomacrophage centers around the vessels and depletion of splenic pulps (Fig. 9C). The kidney showed degeneration and necrosis of renal tubules, interstitial infiltration of mononuclear cells (Fig. 9D), interstitial edema and severe interstitial hemorrhages.

**Group of *O. niloticus* fed diet 2 "ordinary diet with 100 mg of ZnO-NPs /kg of feed"**

No histolopathological alternations were noticed in gills, liver, spleen and kidney (Figs. 10A, B, C and D).

**Group of *O. niloticus* fed diet 2 "ordinary diet with 100 mg of ZnO-NPs /kg of feed" then injected intraperitoneally with 200µl of *C. freundii***

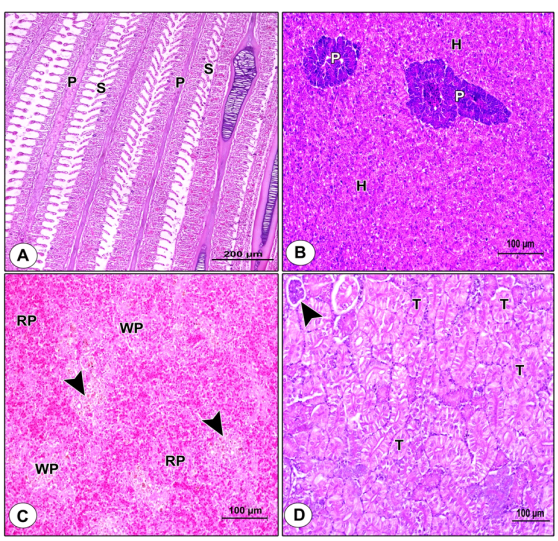


Fig. 10. Photomicrograph of third group of *O. niloticus* fed diet 2 showing: A) Normal histological structure and arrangement of gill lamellae (primary "P" and secondary "S"); B) Normal histological architecture and arrangement of of hepatocytes in cords (H) and normal pancreatic tissues (P); C) Normal histological picture and arrangement of splenic pulps (red "RP" and white "WP") and melanomacrophage centers (arrowheads); D) Normal histological picture of renal tubules (T) and renal corpuscle (arrowhead). H&E stain, scale bar: 200 µm, 100 µm, 100 µm and 100 µm, respectively.

The liver tissues showed mild vacuolar degeneration of the hepatocytes with mild dilatation and congestion of hepatic sinusoids (Fig. 11A). The gills, kidney and spleen showed no histopathological alternations (Figs. 11B, C and D).

**Group of *O. niloticus* fed diet 3 ordinary with 200mg of ZnO-NPs /kg of feed**

All examined organs of this group showed normal histological pictures including; gills, liver, spleen and kidney (Fig. 12).

**Group of *O. niloticus* fed diet 3 ordinary with 200mg of ZnO-NPs /kg of feed then injected intraperitoneally with 200µl of *C. freundii***

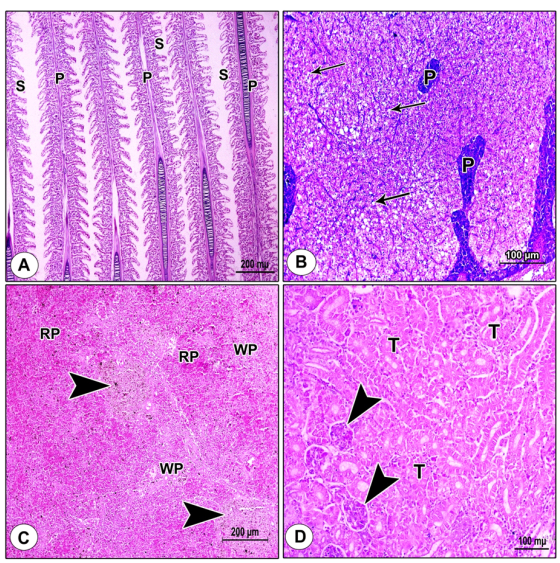


Fig. 11. Photomicrograph of fourth group of *O. niloticus* fed diet 2 then injected intraperitoneally with 200µl of pathogenic *C. freundii*at $1.5 \times 10^5$ concentration showing: A) Normal histological picture of primary (P) and secondary (S) gill filaments; B) Mild vacuolar degeneration of hepatocytes (arrows) and normal pancreatic tissues (P); C) Normal arrangement of splenic pulps (red "RP" and white "WP") and melanomacrophage centers (arrowheads); D) Normal histological structure of renal corpuscles (arrowheads) and tubules (T). H&E stain, scale bar: 200 µm, 100 µm, 200 µm and 100 µm, respectively.

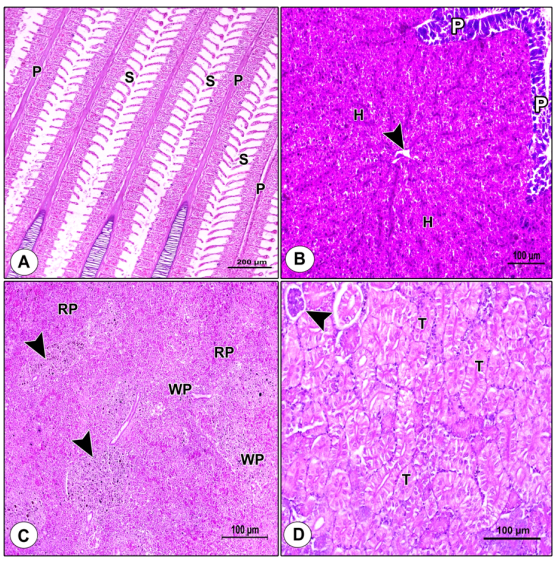


Fig. 12. Photomicrograph of fifth group of *O. niloticus* fed diet 3 showing: A) Normal histological picture of gills (primary "P" and secondary "S" filaments); B) Normal histological architecture of hepatocytes (H), central vein (arrow) and pancreatic tissues (P); C) Normal histological architecture of red (RP), white (WP) pulps and melanomacrophage centers (arrowheads); D) Normal histological picture of renal corpuscle (arrowhead) and tubules (T). H&E stain, scale bar: 200 µm, 100 µm, 100 µm and 100 µm, respectively.

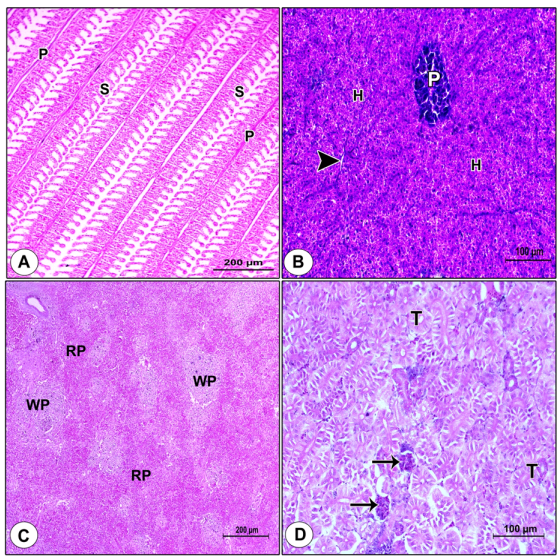


Fig. 13. Photomicrograph of six group *O. niloticus* fed diet 3 then injected intraperitoneally with 200µl of pathogenic *C. freundii* at 1.5 × 105 concentration showing: A) Normal histological picture of primary (P) and secondary (S) gill filaments; B) Normal histological structure of hepatic cells and cords (H) as well as central vein (arrow) and pancreatic tissues (P); C) Normal histological architecture of splenic red (RP) and white (WP) pulps; D) Normal histological architecture of renal glomerulus (arrowheads) and tubules (T). H&E stain, scale bar: 200 µm, 100 µm, 200 µm and 100 µm, respectively.

The gills, liver, kidney, spleen and kidney showed normal histological architecture (Fig. 13).

## Discussion

Infection with *C. freundii* is one of the most common bacterial infections, affecting both wild and farmed fish globally and resulting in significant economic losses. (Yang et al., 2021). Antibiotics and antimicrobial compounds cause not just pollution in aquaculture but also consumer aversion. As a result, looking for other options has been highlighted. ZnO-NPs have been shown to be effective against a variety of bacteria and fungi (Liu et al., 2009; Raghupathi et al., 2011; Xie et al., 2011). Recently, there has been a lot of work done in the subject of employing nanomaterials in aquaculture (nano-aquaculture) to increase fish output, health, and minimize disease cases (Dawood et al., 2020).

ZnO-NPs have a high growth inhibitory effect on all gram-negative and positive bacteria, even at low doses. As a result, it can be employed in food systems to prevent pathogenic germs from growing. (Nazoori and Kariminik, 2018). Previous literatures found that ZnO-NPs could completely lyse cell walls of Gram-negative pathogenic bacteria causing food poisoning such as *Salmonella typhimurium, E. coli*, and *S. aureus* and Gram-positive bacteria as well such as *B. subtilis* which failed to grow at a 200 mg/L concentration of ZnO-NPs (Liu et al., 2009; Jiang et al., 2009; Santimano and Kowshik, 2013).

In the current investigation, *O. niloticus* samples taken from Abo-Saleh fish hatchery, Beni-Suef Uni-

versity, Egypt, showed mortality and symptoms of septicemia. *C. freundii* (MW279218 *C. freundii Q. nilotica*) and *Klebsiella oxytoca* (MW227610 *K. oxytoca Q. nilotica*) were the two bacteria strains that were registered in the gene bank after isolation and molecular identification. To the best of our knowledge, *C. freundii*, rather than *Klebsiella oxytoca*, is the cause of mortality and economic losses in fish farms, particularly in *O. niloticus* (Abdel-Latif and Sedeek, 2017). As a result, after in-vitro testing, ZnO-NPs were used to prevent C. freundii infection. A histological analysis was also performed to determine the product's effect on fish organs.

The biocidal efficacy of nano-zinc oxide against isolated strains of *C. freundii* demonstrated that ZnO-NPs were 100 percent effective at the maximum concentration of 70 mg/L as compared to the lowest concentrations (35 and 45 mg/l). The average size of ZnO-NPs ranged from 31.4 to 91.2 nm, according to the TEM micrograph. Furthermore, both (Shi et al., 2014) and (Dutta et al., 2013) revealed that the biocidal efficacy of ZnO-NPs is due to a buildup of ZnO-NPs in the cytoplasm of bacterial cells, which causes Zn2+ to be produced, which is then directed to damage the protein in the bacterial membrane, resulting in bacterial death. Furthermore, ZnO-NPs demonstrated a possible biocidal effect at a diameter of 30 nm, causing bacterial mortality by disrupting the cell membrane integrity (Jiang et al., 2016).

After injection with *C. freundii* MW279218, the group fed 200 mg ZnO-NPs / kg of fish feed had a high survival rate. The current findings were supported by Das et al., (2020) who approved that using Zn-ONPs had bactericidal activity against *Aeromonas veronii* infection in *Xiphophorus hellerii.* Moreover, Shaalan et al., (2017) recorded that ZnO-NPs inhibited the growth of *A. salmonicida*, *Y. ruckeri* and *A. invadans.* This could be due to ZnO-NPs producing hydrogen peroxide (H2O2) and reactive oxygen species (ROS). When ZnO-NPs came into contact with microbial cells, they induced membrane malfunction, cell membrane breakdown, and cell damage. In addition to interrupting transmembrane electron transport, which is one of its antibacterial actions against harmful microbes, ZnO-NPs have other antimicrobial properties (Sirelkhatim et al., 2015). ZnO-NPs also boosted fish growth and metabolic activities, which improved fish immunity. (Thangapandiyan and Monika, 2020). These is supported by the elevation of both IL-10 and complement 5 immune-related genes in fish fed a diet enriched with ZnO-NPs in the current study. Previous research has looked into the impact of ZnO-NPs in improving the immunological response of Oreochromis mossambicus in the form of Portunus pelagicus-1, 3 glucan binding protein based ZnO-NPs (Pp-GBP-ZnO NPs) supplemented diets (Anjugam et al., 2018). Cellular immune responses (myeloperoxidase activity, lysozyme activity, and reactive oxygen species activity) were also improved, as were humoral immunological responses (complement activity, antiprotease activity, and alkaline phosphatase activity) (Anjugam et al., 2018). Moreover, Ghazi et al., (2021) reported that fish received Se/Zn-NP had the highest phagocytic activity, phagocytic index, lysozyme activity, and immunoglobulin M, followed by Se-NP, Zn-NP. Histopathological changes were also demonstrated in all fish groups in this study before and after injection of *C. freundii* to understand the effect of ZnO-NPs on preventing the devasting effect of *C. freundii* in fish tissue as up till now there are few data on the pathogenesis of this disease in fish (Baldissera et al., 2018). In the present study, infected fish presented congestions of gill tissue, diffuse vacuolar degeneration in the hepatocytes together with dilatation and congestion of hepatic sinusoids and main hepaopancreatic vessels. Spleen also showed formation of numerous MMCs around the vessels and depletion of splenic pulps. The kidney showed degeneration and necrosis of renal tubules, interstitial infiltration of mononuclear cells. These results were supported by (Junior et al., 2018) who mentioned that *C. freundii* infection was characterized by liver degeneration, de-

crease in the amount of renal hematopoietic tissue, and the presence of melanomacrophage centers (MMCs) in the spleen and cephalic kidney of infected fish. These close results illustrate that mortality occurs in *C. freundii* infected fish may be contributed to histopathological lesions in fish vital organs. Amazingly, fish fed diet containing 100 mg of ZnO-NPs /kg of feed had mild vacuolar degeneration of the hepatocytes with mild dilatation and congestion of hepatic sinusoids which may explain the mortality in this group. Moreover, fish fed on diet containing 200 mg of ZnO-NPs /kg of feed had normal histological architecture of gills, liver, kidney, spleen and kidney.

## Conclusion

There were two strains of bacteria that were registered in the gene bank; *C. freundii* (MW279218_ *C._freundii_Q._nilotica*) and *Klebsiella oxytoca* (MW 227610_*K._oxytoca_Q._nilotica*). These strains may be the cause of mortality in *O. niloticus* samples. ZnO-NPs have higher antibacterial activity against *C. freundii* MW279218 than traditional ZN-O. Adding 200 mg ZnO-NPs per kg feed succeeded in preventing *C. freundii* MW279218 in *O. niloticus* with lower mortality and without histopathological alterations compared to a 25% mortality rate and mild histopathological alterations in the group fed 100 mg ZnO-NPs /kg feed group and 50% mortality rate with severe histopathological changes in the positive control group.

## Acknowledgement

We would like to thank Biotechnology unit Reference lab of veterinary quality control on poultry production Animal health research institute, Dokki Giza Egypt. Agricultural Research Centre for determination of virulence genes and transcription levels of the immune-related genes.

Declarations:-

Funding

No funding was received.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval

All experiments were approved by the Beni-Suef Institutional Animal Care and Use Committee (BSU-IACUC) of the Faculty of Veterinary Medicine, Beni-Suef University, Egypt.

Competing interests

Not applicable

Availability of data and material:- n/a

Code availability (software application or custom code)

Not applicable

Author contributions

Asmaa M.K. Edrees:

Applied experimental scheme, management of fish throughout the experimental period, prevention experiments, writing - review & editing.

Usama K. Moawad:

Applied histopathological study, writing, reviewing

Asma N. Mohamed:

Applied the In vitro study and nano-particles preparation, writing, reviewing.

Fatma M. M. korni:

Applied experimental scheme, management of fish throughout the experimental period, prevention experiments, writing - review & editing.

## References

Abdel-Fattah, A. A., et al. (2016). "Some Studies on *Citrobacter Freundii* in Experimentally Infected Nile Tilapia" Journal of the Arabian Aquaculture Society 374(6077): 1-10.

Abdel-Latif, H. M. and E. K. Sedeek (2017). "Diversity of Enterobacteriaceae retrieved from diseased cultured *Oreochromis niloticus*." International Journal of Fisheries and Aquatic Studies 5(1): 29-34.

Aly, S. M., et al. (2012). "Bacteriological and histopatho-

logical studies on Enterobacteriaceae in Nile Tilapia, *Oreochromis niloticus*." J Pharm Biomed Sci 2: 94-104.

Amend, D. F. (1981). "Potency testing of fish vaccines." Fish biologics: serodiagnostics and vaccines: 447-454.

Anjugam, M., et al. (2018). "Effect of β-1, 3 glucan binding protein based zinc oxide nanoparticles supplemented diet on immune response and disease resistance in *Oreochromis mossambicus* against *Aeromonas hydrophila*." Fish & shellfish immunology 76: 247-259.

Apines-Amar, M. J. S., et al. (2004). "Amino acid-chelate: a better source of Zn, Mn and Cu for rainbow trout, *Oncorhynchus mykiss*." Aquaculture 240(1-4): 345-358.

Aste-Amezaga, M., et al. (1998). "Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10." The Journal of Immunology 160(12): 5936-5944.

Austin, B. A., D.A.; Austin, B. and Austin, D.A. (2012). Bacterial fish pathogens.

Baldissera, M. D., et al. (2018). "*Citrobacter freundii i*mpairs the phosphoryl transfer network in the gills of Rhamdia quelen: Impairment of bioenergetics homeostasis." Microbial pathogenesis 117: 157-161.

Bogdan, C., et al. (1991). "Macrophage deactivation by interleukin 10." Journal of Experimental Medicine 174(6): 1549-1555.

Dawood, M. A. O., et al. (2020). "The evaluation of growth performance, blood health, oxidative status and immune-related gene expression in Nile tilapia (*Oreochromis niloticus*) fed dietary nanoselenium spheres produced by lactic acid bacteria." Aquaculture 515: 734571.

Dutta, R., et al. (2013). "Antibacterial effect of chronic exposure of low concentration ZnO nanoparticles on *E. coli*." Journal of Environmental Science and Health, Part A 48(8): 871-878.

Faiz, H., et al. (2015). "Zinc oxide, zinc sulfate and zinc oxide nanoparticles as source of dietary zinc: comparative effects on growth and hematological indices of juvenile grass carp (Ctenopharyngodon idella)." International Journal of Agriculture and Biology 17(3).

Fiorentino, D. F., et al. (1991). "IL-10 inhibits cytokine production by activated macrophages." The Journal of Immunology 147(11): 3815-3822.

Gazzinelli, R. T., et al. (1992). "IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma-activated macrophages." The Journal of Immunology 148(6): 1792-1796.

Ghazi, S., et al. (2021). "Synergistic Effects of Selenium and Zinc Oxide Nanoparticles on Growth Performance, Hemato-biochemical Profile, Immune and Oxidative Stress Responses, and Intestinal Morphometry of Nile Tilapia (*Oreochromis niloticus*)." Biological trace element research: 1-11.

Gröner, F., et al. (2015). "Effects of the pharmaceuticals diclofenac and metoprolol on gene expression levels of enzymes of biotransformation, excretion pathways and estrogenicity in primary hepatocytes of Nile tilapia (*Oreochromis niloticus*)." Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 167: 51-57.

Gunalan S, Sivaraj R, Rajendran V. (2012). Green synthesized ZnO nanoparticles against bacterial and fungal pathogens. Prog Nat Sci Mater Int. 22:693-700.

Jeremić, S., et al. (2003). "*Citrobacter freundii* as a cause of disease in fish." Acta veterinaria 53.

Jiang, Y., et al. (2016). "Role of physical and chemical interactions in the antibacterial behavior of ZnO nanoparticles against *E. coli*." Materials Science and Engineering: C 69: 1361-1366.

Junior, G. B., et al. (2018). "*Citrobacter freundii* infection in silver catfish (Rhamdia quelen): hematological and histological alterations." Microbial pathogenesis 125: 276-280.

Lafferty, K. D., et al. (2015). "Infectious diseases affect marine fisheries and aquaculture economics."

Lagacé, L., et al. (2004). "Identification of the bacterial community of maple sap by using amplified ribosomal DNA (rDNA) restriction analysis and rDNA sequencing." Applied and environmental microbiology 70(4): 2052-2060.

Lieke, T., et al. (2020). "Sustainable aquaculture requires environmental-friendly treatment strategies for fish diseases." Reviews in Aquaculture 12(2): 943-965.

Liu, Y.-j., et al. (2009). "Antibacterial activities of zinc oxide nanoparticles against Escherichia coli O157: H7." Journal of applied microbiology 107(4): 1193-1201.

Magnadóttir, B. (2006). "Innate immunity of fish (overview)." Fish & shellfish immunology 20(2): 137-151.

Nazoori, E. S. and A. Kariminik (2018). "In vitro evaluation of antibacterial properties of zinc oxide nanoparticles on pathogenic prokaryotes." Journal of Applied Biotechnology Reports 5(4): 162-165.

Niiro, H., et al. (1994). "IL-10 inhibits prostaglandin E2 production by lipopolysaccharide-stimulated monocytes." International immunology 6(4): 661-664.

Padmavathy, N. and R. Vijayaraghavan (2008). "Enhanced bioactivity of ZnO nanoparticles－an antimicrobial study." Science and technology of advanced materials 9(3): 035004.

Pan, L., et al. (2021). "The novel pathogenic *Citrobacter freundii* (CFC202) isolated from diseased crucian carp (*Carassius auratus*) and its ghost vaccine as a new prophylactic strategy against infection." Aquaculture 533: 736190.

Piazzon, M. C., et al. (2015). "Carp IL-10 has anti-inflammatory activities on phagocytes, promotes proliferation of memory T cells, and regulates B cell differentiation and antibody secretion." The Journal of Immunology 194(1): 187-199.

Pridgeon, J. (2012). "Major bacterial diseases in aquaculture and their vaccine development." CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 7.

Qi, L., et al. (2004). "Preparation and antibacterial activity of chitosan nanoparticles." Carbohydrate research 339(16): 2693-2700.

Quinn, P., et al. (2002). Veterinary microbiology and microbial disease, Blackwell science.

Raghupathi, K., et al. (2011). "Size-Dependent Bacterial Growth Inhibition and Mechanism of Antibacterial Activity of Zinc Oxide Nanoparticles." Langmuir : the ACS journal of surfaces and colloids 27: 4020-4028.

Ramamoorthy S, Kannaiyan P, Moturi M, Devadas T, Muthuramalingam J, Natarajan L, Arunachalam N, Ponniah AG (2013). Antibacterial activity of zinc oxide nanoparticles against *Vibrio harveyi*. Indian J Fish. 60: 107-12.

Rattanachaikunsopon, P. and P. Phumkhachorn (2010). "Assessment of factors influencing antimicrobial activity of carvacrol and cymene against Vibrio cholerae in food." Journal of bioscience and bioengineering 110(5): 614-619.

Ricklin, D., et al. (2010). "Complement: a key system for immune surveillance and homeostasis." Nature immunology 11(9): 785-797.

Salah, N., et al. (2011). "High-energy ball milling technique for ZnO nanoparticles as antibacterial material." International journal of nanomedicine 6: 863.

Sato, N., et al. (1982). "Systemic *Citrobacter freundii i*nfection among sunfish Mola mola in Matsushima aquarium [Japan]." Bulletin of the Japanese Society of Scientific Fisheries (Japan).

Sebastião, F., et al. (2015). "Identification of bacterial fish pathogens in Brazil by direct colony PCR and 16S rRNA gene sequencing." Advances in Microbiology 5(06): 409.

Shi, L.-E., et al. (2014). "Synthesis, antibacterial activity, antibacterial mechanism and food applications of ZnO nanoparticles: a review." Food Additives & Contaminants: Part A 31(2): 173-186.

Sirelkhatim, A., et al. (2015). "Review on zinc oxide nanoparticles: antibacterial activity and toxicity mechanism." Nano-micro letters 7(3): 219-242.

Swain, P. S., et al. (2016). "Nano zinc, an alternative to conventional zinc as animal feed supplement: A review." Animal Nutrition 2(3): 134-141.

Tawfik, M., et al. (2017). Evaluation of Nano Zinc Oxide feed additive on tilapia Growth and Immunity. 15th International Conference on Environmental Science and Technology, Rhodes, Greece.

Thangapandiyan, S. and S. Monika (2020). "Green synthesized zinc oxide nanoparticles as feed additives to improve growth, biochemical, and hematological parameters in freshwater fish Labeo rohita." Biological trace element research 195(2): 636-647.

Thanigaivel, S., et al. (2015). "In vivo and in vitro antimicrobial activity of Azadirachta indica (Lin) against *Citrobacter freundii* isolated from naturally infected Tilapia (*Oreochromis mossambicus*)." Aquaculture 437: 252-255.

Wikler, M. A. (2006). "Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard." CLSI (NCCLS) 26: M7-A7.

Xie, J., et al. (2011). "Synthesis and photocatalysis properties of ZnO structures with different morphologies via hydrothermal method." Applied surface science 257(15): 6358-6363.

Yang, J., et al. (2021). "Pathogenic infection and immune-related gene expression of Chinese sturgeon (Acipenser sinensis) challenged by *Citrobacter freundii."* Developmental & Comparative Immunology 114: 103872.

Yuan, J. S., et al. (2006). "Statistical analysis of real-time PCR data." BMC bioinformatics 7(1): 1-12.

Manuscript Received : May 05, 2022
Revised : May 29, 2022
Accepted : Jun 11, 2022