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Expression profiling of antimicrobial peptides and immune-related genes in Nile tilapia following *Pseudomonas putida* infection and nano-titanium dioxide gel exposure

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ABSTRACT

Pseudomonas putida is a virulent bacterium that prompts major losses in fish. Recently, there has been a noticeable direction for utilizing nanomaterials in the aquaculture industry for sustaining fish health and performance. Hence, the present study is the first trial to investigate the antibacterial influence of nano titanium dioxide gel (NTG) as a watery addition for combating *P. putida* infection in Nile tilapia (*Oreochromis niloticus*). Further, antioxidant-immune capacity, and gene expression in the spleen including antimicrobial peptides and immune-related genes are assessed. Fish ($n = 200$; 47.50 ± 1.32 g of body weight) were assigned into four groups for 10 days [control, NTG (0.9 mg/L), *P. putida*, and NTG + *P. putida*]. Findings demonstrated that the infection by *P. putida* induced a decline in antioxidant immune indicators including catalase, glutathione peroxidase, and nitric oxide. Furthermore, a noteworthy rise in lipid peroxide (malondialdehyde), tumor necrosis factor-alpha (TNF-α), and stress indicator (glucose) levels was noticed. *P. putida* infection induced remarkable alterations in the expression of antimicrobial peptides genes [*tilapia piscidin* (*TP3 and TP4*), *colony-stimulating factor 1 receptor, hepcidin-2, beta-defensin1, and neutrophil cytosolic factor 4] and immune-relevant genes [transforming growth factor beta, tumor necrosis factor receptor-associated factor 6, TNF-α, interleukins* (*IL-10 and IL-11*)]. Notably, applying NTG regenerated all the negative consequences of *P. putida* infection. Inclusive, this study underscores the crucial role of NTG as a potent antibacterial and immune-antioxidant agent, highlighting its potential in protecting *O. niloticus* from *P. putida* infection and improving immune-antioxidant response.

1. Introduction

The aquaculture industry is experiencing remarkable growth and the worldwide production of aquaculture finfish touched 87.5 million tons [1]. Nile tilapia (*Oreochromis niloticus*) is the highest-ranked and most common species which is widely cultured and imported because of its higher content of protein and Omega 3 [2]. It is distinguished by rapid adaptation to temperate, subtropical, and tropical environments. In addition, it has a speedy growth rate, and resistance to various environmental conditions, and diseases [3]. Unfortunately, with the development in aquaculture production, numerous evolving diseases have ascended, specifically bacterial diseases that adversely influence fish resulting in higher mortalities [4,5].

In this regard, *Pseudomonas species* is one of the utmost pathogenic

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bacterial infections that attack human and fish species [6]. *Pseudomonas putida* is an adaptable Gram-negative bacterium belonging to the *Pseudomonaceae* family that results in higher mortalities in fish [7]. In Nile tilapia, *P. putida* is reported as a highly virulent bacterium that exhibits clinical signs including exophthalmia, ascites, and body ulcerations [8,9]. A current report elucidates the molecular mechanism of *P. putida* in inducing pathogenicity [10]. It has been detected virulence-associated genes [*Nan1*, exotoxin A (*tox A*), and exo-enzyme S (*exo S*)] which have a function of attachment, colonization, and penetrating host tissues to produce virulence impacts [10].

Reactive oxygen species (ROS) are continuously created as byproducts in regular aerobic metabolic activities along with cellular response to bacterial invasion [11]. Prior research has demonstrated that the hazardous implications of bacterial invasion might result in lipid peroxidation, oxidative stress, and DNA damage [12]. A crucial marker for lipid peroxidation and production of ROS is malondialdehyde (MDA) [13]. Fish have a system of antioxidants that can detoxify ROS and protect cells from their damaging effects. Catalase (CAT) and glutathione peroxidase (GPX) are members of this system and play a key role during oxidative stress [14,15].

In fish, innate immunity, also known as non-specific immunity, serves as the initial point of protection against invasive infections and supports the adaptive immune response [16]. A part of innate immunity is nitric oxide (NO) generated by inducible nitric oxide synthase, which is one of the main ways of killing bacteria [17]. Another component is the tumor necrosis factor-alpha (TNF-α) which is critical for the stimulation of many immune cells like macrophages and the destruction of pathogens [18]. Additionally, fish defense is correlated with cytokines, which are created during the invasion of pathogens. The anti-inflammatory cytokines include *transforming growth factor beta* (*TGF-β*) and *interleukins* (*IL-10* and *IL-11*) which regulate the immune and inflammatory response [19,20].

Furthermore, the assessment of antimicrobial peptides (AMPs) is essential to assess immune function as they have a pivotal role in the innate immune response [21]. They are naturally arising molecules that are utilized as therapeutic agents for curing bacterial diseases [22]. In fish, the AMPs are considered small peptides whose activities reflect major consequences in the innate immune response of teleost fish. In addition, they are the principal line of shield against a broad spectrum of pathogens [23]. They are characterized by exclusive properties, broad-range effectiveness, and power for overwhelming antimicrobial resistance [24]. Previous report revealed suppression of the immune-antioxidant response of fish following bacterial infection involving *P. putida* [10].

On the other side, the development of drug-resilient bacteria upsurges the request for substitute approaches to cure bacterial infections. One of the newly effective protocols is consuming nano-established materials as anti-bacterial agents [25]. The nanoparticles (NPs) are currently used as antimicrobials. NPs' surface characteristics can maximize their antimicrobial efficiency by optimizing their interactions with bacterial cells and reducing the probability of resistance [26]. Antimicrobial pathways exerted by NPs have been investigated in several studies. They included preventing cell wall production, creation of toxic ROS, impairment of energy transduction, photo-catalysis, reduced DNA synthesis, and enzyme suppression [27,28]. Above all, NPs of metal sources are drawing more consideration because of their abundant accomplishment in biological and pharmaceutical applications [29]. Metal NPs can liberate metal ions, which can trigger microbial cell death. Numerous metal oxide-NPs display strong antimicrobial activity in addition to their various features like resistance to heat and little toxicity [30]. Titanium dioxide (TiO_2) as one of the metal oxide NPs is a photo-catalytic. They generate superoxide radicals that destroy microbial cells when exposed to light, either UV or visible. In addition to, its oxidizing activity and minute nano-size [31].

Recently, nano-gels (NGs), have achieved great success in diagnosing and curing a wide range of diseases [32]. Because of their nano-size, NGs can also be utilized as drug nano-transporters, via penetrating cells and tissues in a transcellular mode [33]. Recently, nano-TiO_2 gel (NTG) has been verified as an antibacterial agent because of its suppressing activity against various bacterial species [34]. In the aquaculture sector, researchers successfully tested other forms of NGs which have a major role in chelating toxicity and endorsing immune-antioxidant capacity [35,36]. It has been recently reported that NTG has a palliative role in modulating hepato-renal and histopathological/immunohistochemical disruptions induced by *P. putida* in *O. niloticus* [34].

Comprehending the antioxidant and immune systems with their defense function of the Nile tilapia against bacterial diseases is anticipated to aid in the development of tactics for controlling diseases for sustainability. Few studies articulate the effects of NTG on fish infection especially at a molecular level. Therefore, the current study is the first trial to assess the anti-bacterial activity of aqueous addition of NTG on *O. niloticus* health challenged with *P. putida.* Moreover, the assessment of antioxidant and immunomodulatory activities of NTG was evaluated via analysis of some antioxidant-immune parameters, plus gene expression including AMPs and immune-linked genes.

2. Materials and methods

2.1. Synthesis and characterization of NTG

The synthesis of TiO_2 nanoparticles (TiO_2NPs) was accomplished using a sono-chemical protocol, chosen for its efficiency in producing uniform NPs [37]. The process began with the preparation of a TiO_2 solution by dispersing 0.25 g of TiO_2 (Sigma-Aldrich Co., MO, USA) in 100 mL of 10 M sodium hydroxide (NaOH; El Naser Chemical Co., Egypt) in a 250 mL flask. This solution was then subjected to ultrasonic treatment using a Sonica 4200 EPS3 (Milano, Italy) device. The treatment conditions were carefully selected to optimize NPs formation: an amplitude of 88 % was applied for 1.5 h at room temperature (approximately 25 °C), with a cycle ratio of 0.82 (49.2 s on and 10.8 s off). These parameters ensured the efficient breakdown of TiO_2 agglomerates while preventing overheating. Following ultrasonic treatment, the solution's pH was adjusted to 7.0 using 0.1 M hydrochloric acid (HCl; El Naser Chemical Co., Egypt) to stabilize the NPs. The resulting TiO_2NPs solution was then purified through a series of three centrifugation cycles at 10,000 rpm for 15 min each, using distilled deionized water for washing. This purification step ensured the removal of any residual reactants.

The production of NTG (TiO_2NPs/carbopol hybrid nanogel) followed a multi-step process. Initially, 0.2 g of the synthesized TiO_2NPs were dispersed in 40 mL of 95 % ethanol. Concurrently, 0.4 g of carbopol was dissolved in another 40 mL of 95 % ethanol. The TiO_2NPs dispersion was then added to the carbopol solution and mechanically stirred for 65 min to ensure homogeneous mixing. Gel formation was initiated by the dropwise addition of 1.2 mL of trimethylamine to the mixture. Trimethylamine plays a crucial role as a neutralizing agent, causing the carbopol to swell and form a gel network while also facilitating the incorporation of TiO_2NPs into this network. The stirring continued for another 65 min until a white gel formed. To cater to different application needs, the NTG was prepared in both low and high-viscosity forms by adjusting the amount of trimethylamine added.

Characterization of the NTG employed a variety of techniques to assess its properties comprehensively. Morphological examination was conducted using transmission electron microscopy (TEM) to visualize particle shape and size at the nanoscale, and atomic force microscopy (AFM) to examine surface topography and 3D structure. Size distribution was determined through dynamic light scattering (DLS), which measures the hydrodynamic size of the NPs in solution. The structural properties and phase composition of the NTG were assessed using X-ray diffraction (XRD). Finally, the colloidal stability of the NTG was evaluated by measuring its zeta potential. However, a flow chart of synthesis and characterization techniques was provided as a Supplementary

Fig. 1.

2.2. Ethical approval and fish acclimation

The experimental strategy of the present report was accepted by the Institutional Animal Care and Use Committee at Zagazig University in Egypt (ZU-IACUC/2/F/333/2022). Fish (47.50 ± 1.32 g of body weight) were collected from the Al-Abbassa private fish farm at Sharkia Governorate, Egypt. Neither clinical abnormalities nor a history of disease outbreaks were found in the fish farm. Prior to the experiment, the CCAC [38] approach was used to perform a standard evaluation of the fish's health. Acclimation of fish was conducted for 14 days in 100 L of well-aerated aquaria (ten fish/aquarium). The waste materials were disposed of daily through siphoning. The fish were fed a basal diet (3 % of their body weight) twice every day during the acclimation period [39]. The physio-chemical indicators (dissolved oxygen, temperature, pH, and ammonia) of the rearing water were checked day-to-day based on APHA [40] rules, and they recorded 6.50 ± 0.11 mg/L, 23.00 ± 2.00 °C, 7.30 ± 0.12, and 0.01 ± 0.03 mg/L, respectively. The dissolved oxygen and pH were assayed using an oximeter (HI98198) and pH meter (HI2211) of HANNA Instruments, USA, while the temperature was measured by a thermometer. Total ammonia nitrogen was assayed using an ammonia medium-range photometer (HI 96715-11) of HANNA Instruments, USA. Unionized ammonia values were calculated using the previously obtained temperature, pH, and total ammonia nitrogen variables.

2.3. Bacterial challenge using P. putida strain

2.3.1. Bacterial isolation and identification

The present work was conducted on *P. putida*. Initially, *P. putida* was isolated from unhealthy Nile tilapia at the Department of Aquatic Animal Medicine, Faculty of Veterinary Medicine, Zagazig University). The fish demonstrated different external body lesions involving fin rot, body darkness, and hemorrhages during an outbreak on a local farm. It was identified at the National Research Centre (NRC), Dokki, Giza, Egypt by the VITEK 2-C15 automated system to identify bacterium (BioMérieux, Craponne France). Additionally, it was also recognized with the aid of conventional biochemical analyses depending on the manufacturer's guidelines as mentioned by Scheidegger et al. [41]. The isolate was maintained at −80 °C in glycerol stocks.

2.3.2. Culture preparation

The bacterium was lined onto pseudomonas agar base (Oxoid, England) for a day at 37 °C. One colony was picked to be incubated in brain heart infusion broth (Sigma-Aldrich) for a day at 37 °C with 120 rpm shaking [42]. Following centrifuging at 3000×*g* for 10 min of the cultured broth at 4 °C, the pellet was resuspended in sterile phosphate-buffered saline (PBS).To quantify the bacterial solution employed to infect fish, spectrophotometric absorbance (600 nm optical density) and standard plate-count procedure (colony-forming unit; CFU) were applied.

The lethal dose (LD_{50}) of *P. putida*, which causes 50 % mortality of fish, was assessed to determine a sub-lethal dose [43]. Fish ($n = 80$) were dispersed into four groups in replicates (10 fish/replicate; 20 fish/-group). Fish were challenged via intraperitoneal route (IP) with various doses of 24-h-old alive *P. putida* culture suspension (10^6–10^9 CFU/fish). For the control group, fish were IP injected with 0.1 mL sterile saline. Mortalities were then monitored four days after the inoculation. Based on the Probit Analysis Program, version 1.5 (US Environmental Protection Agency), the LD_{50} recorded 3.9×10^8 CFU/mL. A sub-lethal dose of 1.5×10^8 CFU/mL (equal to 38.46 % from the LD_{50}) was utilized in the trial [10].

2.3.3. Challenge protocol

A total of two hundred fish were randomly divided into four groups ($n = 50$ fish/group) with five replicates ($n = 10$ fish/replicate) for 10 days. The first (control) group was not exposed to nano-titanium dioxide gel (NTG) or challenged with *P. putida*. The second group (NTG) was exposed to NTG (0.9 mg/L), meanwhile, the third group was IP challenged with *P. putida* (0.1 mL; 1.5×10^8 CFU/mL). The fourth group was exposed to NTG (0.9 mg/L) and challenged with *P. putida* (1.5×10^8 CFU/mL. The desired amount of NTG (0.9 mg/l) was first dissolved in 5 mL of distilled water and then added to the aquarium water to achieve the final concentration. The NTG was added to the aquarium water of all groups only after symptoms appeared in the infected groups according to the previous experimental protocol [9].

Siphoning of waste materials was performed daily and the entire water was changed three times per week using dechlorinated water at the same temperature as the old water. The addition of new water should be done slowly without removal of the fish from the aquarium. Following the water change, the desired NTG concentration was prepared and added.

2.4. Sampling

By the end of the experiment (after 10 days), fish (15/group) were randomly selected to collect samples. Depending on Neiffer and Stamper [44] protocol, fish were subjected to anesthetizing using a benzocaine solution (100 mg/L), and then blood was collected, using tubes devoid of anticoagulant, from the caudal blood vessels (0.1 mL blood/fish). Samples were centrifuged at 1750 × g for 10 min at room temperature (22.00 ± 2.00 °C). Clear serum was then kept at −20 °C to investigate antioxidant-immune and stress biomarkers. For gene expression assay, splenic samples (15 fish/group) were maintained in 1 mL TRIazole (Thermo Fisher Scientific, USA) and kept at −80 °C.

2.5. Antioxidant/oxidant biomarkers assay

The activity of CAT (Cat. No. CA 25 17) and GPX (Cat. No. GP 25 24) enzymes as antioxidant biomarkers were assessed in the serum samples. Moreover, serum oxidant biomarker [MDA; Cat. No. MD 25 29] was estimated. The diagnostic kits (Bio-diagnostics CO., Cairo, Egypt) were applied. CAT activity was investigated by spectrophotometrically assessing the rate of hydrogen peroxide disappearance at 510 nm [45]. The GPX activity was measured calorimetrically at an absorbance rate of 340 nm [46]. Using the thiobarbituric acid approach, MDA was determined spectroscopically at 532 nm [47].

2.6. Immune and stress biomarkers assay

Using Cusabio kits (Catalogue No.: CSB-E13254Fh) and the protocol included in the kit packages, serum TNF-α was measured by ELISA technique. Additionally, the Bryan and Grisham [48] method was applied to assess the amount of NO. The glucose level (GLU) as a stress biomarker was established [49] using colorimetric diagnostic kits (Ref: 1001192; Spinreact Co., Santa Coloma, Spain).

2.7. RNA extraction and expression profiling of AMPs and immune-relevant genes assays

Using TRIazole (1 mL/50 mg sample), total RNA was recovered from spleen samples in accordance with the manufacturer's (Thermo Fisher Scientific, USA) instructions. In brief, 50 mg from the tissue samples was collected on 1 mL TRIzol reagent and then homogenized with Eppendorf homogenizer on ice. After that, 200 μL of chloroform HLPC grade (Sigma Aldrich, USA) was added to the prepared homogenate and centrifuged at 14,000 × g for 15 min. Then, the interface was collected on 1.5 mL Eppendorf with the addition of 400 μL propanol HPLC grade (Sigma Aldrich, USA) and centrifuged at 14,000 × g for 10 min. Finally, the RNA pellet was washed twice with 75 % ethanol (Sigma Aldrich, USA) and centrifuged at 14,000 × g for 5 min. The RNA pellet was air-dried and

suspended in 100 μL nuclease-free water. The amount of RNA was determined using a 260/280 nm (absorbance ratio of 1.80:2.00), and purity was confirmed using a NanoDrop® ND-1000 UV–Vis spectrophotometer (thermos scientific, Waltham, MA, USA). Using a high-capacity cDNA reverse transcription kit (Applied Biosystem, USA), reverse transcription was carried out from 500 ng of the total RNA as directed in the supplier instructions.

Applying the quantitative real-time PCR (RT-qPCR) required utilizing the Rotor-Gene Q2 plex real-time thermal cycler (Qiagen, Germany). The expression of AMPs genes [*tilapia piscidin* (*TP3 and TP4*), *colony-stimulating factor 1 receptor* (*CSF-1*), *hepcidin-2, beta-defensin1* (*β-Defen 1*), *and neutrophil cytosolic factor 4* (*NCF-4)*] and immune-relevant genes [*TGF-β, tumor necrosis factor receptor-associated factor 6* (*TRAF-6*), *TNF-α*, *IL-10* and *IL-11*] were assessed using specified primers (Stratagene, Metabion, Germany) (Table 1). The RT-qPCR conditions comprised a dissociation analysis phase in between 40 cycles of 95 °C for 10 s and 60 °C for 15 s, with an initial denaturation temperature of 95 °C for 10 min. To ensure that every amplifier was at its peak, the melting curve analysis was carried out after the amplification. Once the efficiency of the primers was nearly 100 %, the $2^{-\Delta\Delta CT}$ procedure was used to evaluate the results of gene expression [50]. Expression of these genes was measured concerning *elongation factor 1-alpha* (*Ef-1α*) which remained constant in all exposure groups.

2.8. Data analysis

Using Shapiro–Wilk normality, all obtained data were inspected for norm homogeneity. Subsequently, the data were statistically scrutinized using an analysis of variance test (one-way-ANOVA) using SPSS version 22 (SPSS, Richmond, VA, USA). Tukey's range assessment was directed to appraise the variances between means at a 95 % confidence level. The means standard error (*SE*) was utilized to highlight the data.

Table 1
Primers for RT-qPCR amplification of Nile tilapia genes.

Target gene	Primers sequences	Accession no.
TP3	GCTGTCGATGGTTGTCCTCA AGCGTTGGTTTAGCTGCTCT	JX006072.1
TP4	TCCTTGTGCTGTCGATGGTC CCAGTTGTTGCTGCAGTTCC	XM_003456613.3
CSF-1	AAAGCCAGTGCCCATTCAGA TGCAGGCTCCAAGTAGGTTG	XM_003455186.5
Hepcidin-2	GCATTGCAGTTGCAGTGACA GGAAAGATGGCTCTGACGCT	XM_003450530.5
β-Defen 1	TTGTGTCCTCTGCTCCGTTC TCGCGTCTCTCAGTTTTGCT	KJ577575.1
NCF-4	TCGGCACGAGGCAATATACA AATGGCCTGGCTCATACCTC	FF280256.1
TGF-β	GAGCAGGAGGGGAGACACTA CTGTCCGTTGTGTCAGTGGA	NM_001311325.1
TRAF-6	CAGGCCAAACCAATGGAAGC GCCAGGGTTAGTTCACTGCT	MK227433.1
TNF-α	CTGCTCCCTTCCACTCCTTG CCGCTATCTGTGAGAGGCTG	XM_013266975.3
IL-10	ATGAGCAGAAGGCCTGTCAC ACCCAGTGAGGTGATGGGTA	XM_013269188.3
IL-11	AAAATGCTGGCGAACAGACG ATGGACAAACAGCTCAGCCA	XM_025902512.1
Ef-1α	ACGTCAAGAACGTCTCCGTC CATAGCCTGCAGCGATCTGA	NM_001279647.1

TP3: tilapia piscidin 3; TP4: tilapia piscidin 4; CSF-1: colony-stimulating factor 1 receptor; β-Defen 1: beta-defensin1; NCF-4:neutrophil cytosolic factor 4; TGF-β, transforming growth factor beta; TRAF-6: tumor necrosis factor receptor-associated factor 6; TNF-α, tumor necrosis factor alpha; IL-10, interleukin 10; IL-11, interleukin 11; Ef-1α: elongation factor 1-alpha.

3. Results

3.1. Characterization of NTG

Morphological analysis through TEM (Fig. 1A) and AFM (Fig. 1B and C) revealed that the NTG particles possess a spherical shape. The AFM images offered both 2D and 3D visualizations of the surface topography. The XRD pattern (Fig. 2A) showed no distinctive peaks, confirming the amorphous nature of the formed gel. DLS analysis (Fig. 2B) indicated a uniform particle size distribution with an average diameter of approximately 47.5 nm. Furthermore, zeta potential measurement (Fig. 2C) yielded a value of −35 mV.

3.2. Antioxidant/oxidant biomarkers

Table 2 presents that the activity of CAT and GPX enzymes in the NTG group markedly enhanced ($P < 0.0001$) relative to the control one with no significant alterations in the level of MDA. *P. putida* infection caused a significant decline in the activity of CAT and GPX enzymes and elevation of the MDA level ($P < 0.0001$) compared to the control. However, the treated group (NTG + *P. putida*) exhibited elevation ($P < 0.0001$) of these enzymes and a decline in the MDA level relative to the infected group (*P. putida*).

3.3. Immune and stress (glucose) response

Table 3 reveals no observed significant differences in the level of TNF-α and GLU between the NTG and control groups with a substantial increase ($P < 0.0001$) in the NO level. *P. putida* infection induced marked elevation in the TNF-α and GLU levels and a decline in the NO value ($P < 0.0001$) compared to the control. On the contrary, TNF-α and GLU levels decreased significantly with an increase of NO ($P < 0.0001$) in the NTG + *P. putida* group relative to the *P. putida* group.

3.4. Expression profiling of AMP genes in the spleen

Figs. 3 and 4 show the expression profile of *TP3, TP4, CSF-1, hepcidin-2*, *β-Defen 1*, and *NCF-4* genes as AMPs-relevant genes in the spleen of *O. niloticus*. The expression of these genes did not differ significantly between the NTG and control fish except for *β-Defen 1* and *NCF-4* which showed marked up-regulation (1.35- and 1.89-fold), respectively. A noteworthy up-regulation ($P < 0.0001$) of the *TP3* (2.89-fold), *TP4* (3.86-fold), *CSF-1* (4.06-fold), hepcidin-2 (3.23-fold), *β-Defen 1*(5.05-fold), and *NCF-4* (4.37-fold) genes was obvious in the NTG + *P. putida* group followed by the *P. putida* group (1.25-, 1.66-, 2.03-, 1.60-, 1.87-, and 2.84-fold), respectively, relative to the control.

3.5. Expression profiling of immune-relevant genes in the spleen

Figs. 5 and 6 demonstrate the expression profile of *TGF-β, TRAF-6, TNF-α, IL-10,* and *IL-11* genes as immune-relevant genes in the spleen of *O. niloticus*. No discernible differences in the expression of these genes were observed between the NTG and control fish except for *TRAF-6* and *IL-11* which showed notable up-regulation (1.15-and 1.86-fold), respectively. The treated group (NTG + *P. putida*) revealed a marked up-regulation ($P < 0.0001$) in the expression of *TGF-β* (3.07-fold), *TRAF-6* (4.19-fold), *TNF-α* (3.59-fold), and *IL-11* (3.38-fold) genes followed by the *P. putida* group (1.86-, 2.27-, 2.07-, and 2.40-fold), respectively, relative to the control. In contrast, *P. putida* infection caused significant down-regulation of the *IL-10* (0.19-fold) compared with the control. This gene was up-regulated in the treated group (NTG + *P. putida*) compared with the *P. putida* group with a 0.67-fold.

4. Discussion

Handling bacterial diseases has been ominously convoluted by the

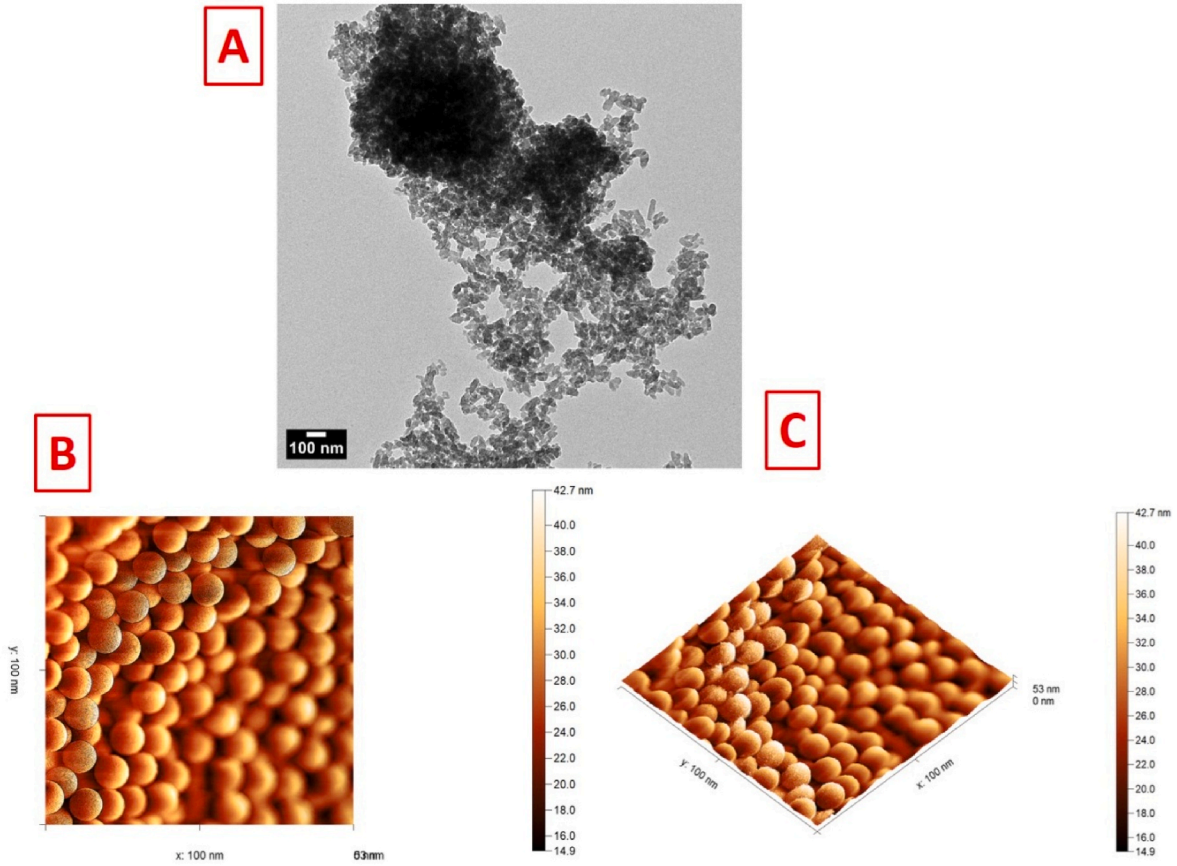

Fig. 1. The characterization patterns of NTG. [A]TEM image (100 nm). [B and C] AFM images (2D and 3D).

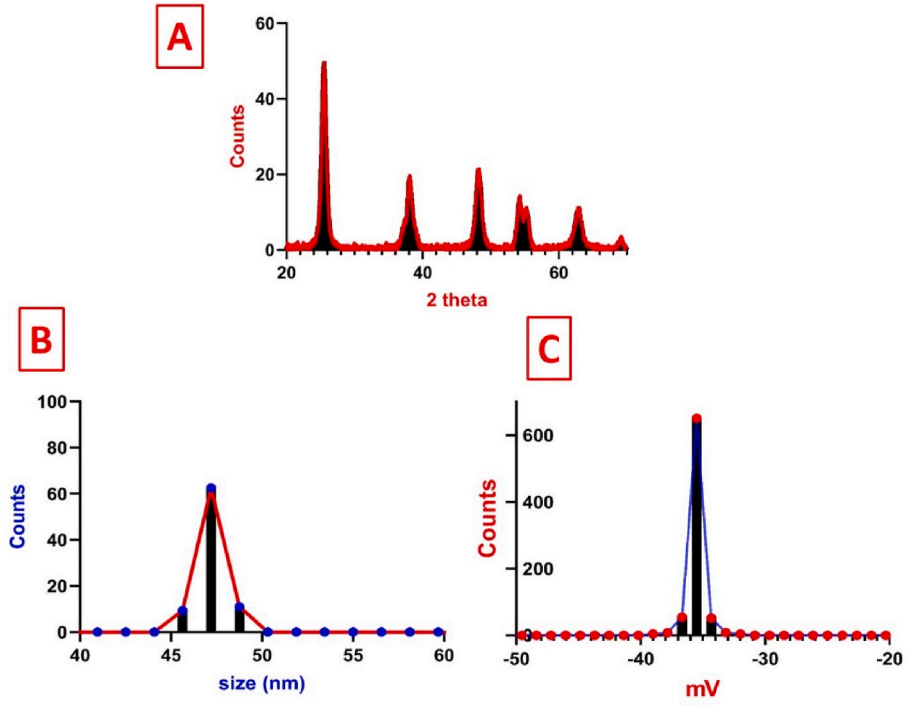

Fig. 2. The characterization patterns of NTG. [A] XRD image. [B] DLS image. [C] Zeta potential image.

Table 2
Antioxidant/oxidant biomarkers of *O. niloticus* experimentally infected with *P. putida* and exposed to nano-titanium dioxide gel (NTG) for 10 days.

Parameters	CAT (ng/mL)	GPX (U/mL)	MDA (nmol/mL)
Control	1.43 ± 0.07^{b}	124.45 ± 1.32^{b}	1.19 ± 0.17^{c}
NTG	2.35 ± 0.20^{a}	159.50 ± 3.86^{a}	1.83 ± 0.59^{c}
P. putida	0.37 ± 0.04^{d}	52.70 ± 1.33^{d}	12.92 ± 0.63^{a}
NTG + *P. putida*	1.02 ± 0.05^{c}	110.50 ± 1.86^{c}	8.50 ± 0.29^{b}
P-value	<0.0001	<0.0001	<0.0001

CAT: catalase; GPX: glutathione peroxidase; MDA: malondialdehyde. Values (means± *SE*) in the same column that do not share the same superscripts differ substantially ($P < 0.05$; one-way ANOVA).

Table 3
Immune and stress indicators of *O. niloticus* experimentally infected with *P. putida* and exposed to nano-titanium dioxide gel (NTG) for 10 days.

Parameters	TNF-α (Pg/mL)	NO (μmol/L)	GLU (mg/dL)
Control	118.56 ± 0.90^{c}	37.12 ± 1.22^{b}	106.00 ± 1.38^{c}
NTG	121.06 ± 1.19^{c}	44.88 ± 1.66^{a}	107.70 ± 0.17^{c}
P. putida	633.20 ± 1.85^{a}	11.68 ± 0.39^{d}	152.35 ± 0.37^{a}
NTG + *P. putida*	368.42 ± 1.97^{b}	18.55 ± 0.26^{c}	117.50 ± 1.44^{b}
P-value	<0.0001	<0.0001	<0.0001

TNF-α: tumor necrosis factor-alpha; NO: nitric oxide; GLU: glucose. Values (means ± *SE*) in the same column that do not share the same superscripts differ substantially ($P < 0.05$; one-way ANOVA).

development of biofilms to which antibiotics are ineffectual. Hence, prior studies on nanomaterials have been established to combat biofilm-associated infections. Among them, NGs have presented aptitude as a therapeutic scheme for many bacteria because of their nano size which allows their cell penetration, biocompatibility, biodegradability, and firmness [51]. From this fact on, this report continues by first conferring the efficacy of NTG as an antibacterial agent in *O. niloticus* experimentally challenged by *P. putida*.

The significance of titanium nanomaterial is attributed to some of its characteristics, like thermally stable, and it is not considered a harmful substance (based on the UN GHS chemical labeling and the classification system) [52,53]. In the current study, the characterization of NTG provided comprehensive insights into its properties. TEM and AFM patterns revealed a spherical shape which enhanced our understanding of the nanostructure. In addition, the amorphous nature of the produced gel is proven by the XRD pattern that revealed no distinctive peaks. This amorphous structure is particularly beneficial for the intended application as it allows for better incorporation of the TiO_2 NPs into the gel network. Outcomes of DLS analysis demonstrated a uniform particle size distribution with 47.5 nm diameter. This nanoscale size is crucial for the gel's properties and potential applications, as it influences factors such as surface area and reactivity [54]. Zeta potential as a marker for NTG stability which yielded a value of −35 mV. This value indicates good colloidal stability, as generally, zeta potential values greater than ±30 mV are associated with stable colloidal systems [55]. The combination of nanoscale size and high colloidal stability suggests that the synthesized NTG has properties suitable for a wide range of potential applications in various fields involving drug delivery [37,56]. A recent study by Karg et al. [57] supported these findings. However, a previous report by Okuda-Shimazaki et al. [58] was partially inconsistent with the study findings which revealed cytotoxic impacts of titanium nanocomposite at small size (166 nm).

The present report supported the occurrence of oxidative stress verified by a noticeable imbalance in the antioxidant system upon

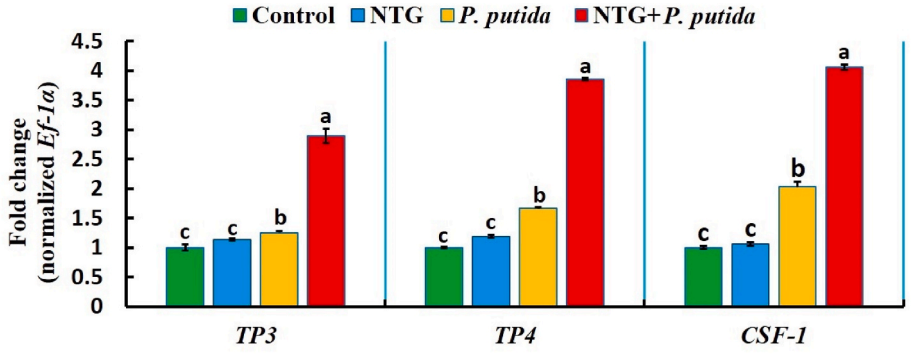

Fig. 3. Expression of *tilapia piscidin* (*TP3* and *TP4*) and *colony-stimulating factor 1 receptor* (*CSF-1*) genes ($P < 0.0001$) of *O. niloticus* experimentally infected with *P. putida* and exposed to nano-titanium dioxide gel (NTG) for 10 days. Bars (means ± *SE*) that do not share the same superscripts differ substantially ($P < 0.05$; one-way ANOVA).

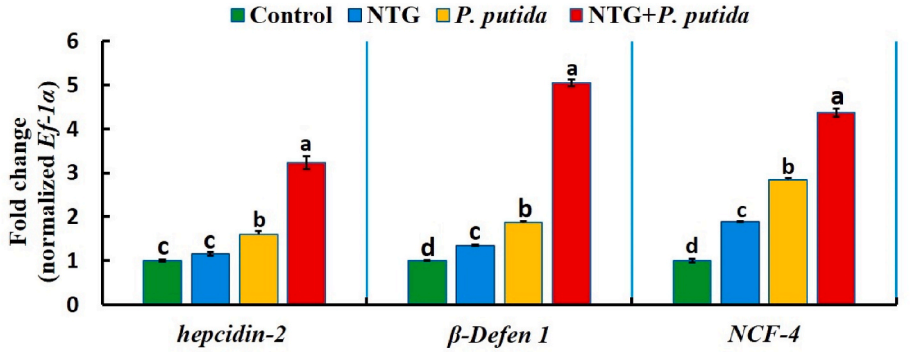

Fig. 4. Expression of *hepcidin-2*, *beta-defensin1* (*β-Defen 1*), and *neutrophil cytosolic factor 4* (*NCF-4*) genes ($P < 0.0001$) of *O. niloticus* experimentally infected with *P. putida* and exposed to nano-titanium dioxide gel (NTG) for 10 days. Bars (means ± *SE*) that do not share the same superscripts differ substantially ($P < 0.05$; one-way ANOVA).

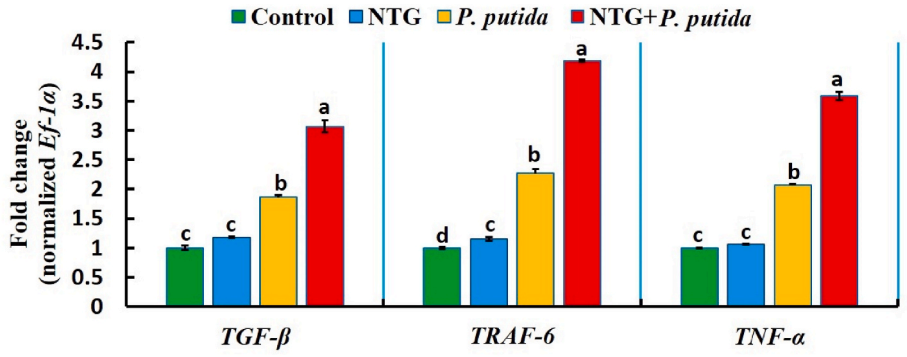

Fig. 5. Expression of *transforming growth factor beta* (*TGF-β*), *tumor necrosis factor receptor-associated factor 6* (*TRAF-6*), and *tumor necrosis factor-alpha* (*TNF-α*) genes ($P < 0.0001$) of *O. niloticus* experimentally infected with *P. putida* and exposed to nano-titanium dioxide gel (NTG) for 10 days. Bars (means ± *SE*) that do not share the same superscripts differ substantially ($P < 0.05$; one-way ANOVA).

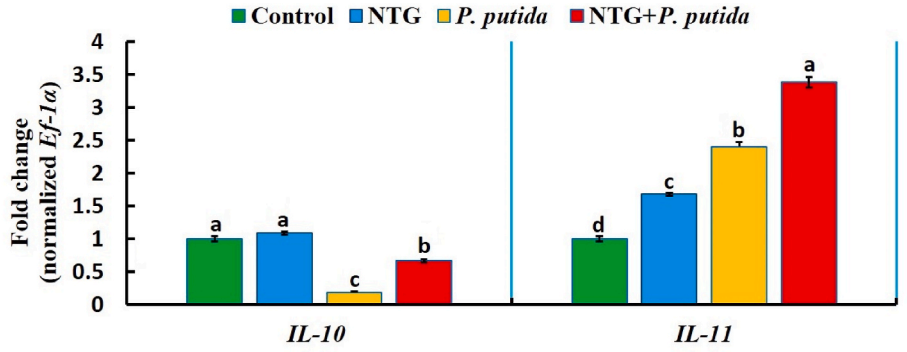

Fig. 6. Expression of *interleukins* (*IL-10* and *IL-11*) genes ($P < 0.0001$) of *O. niloticus* experimentally infected with *P. putida* and exposed to nano-titanium dioxide gel (NTG) for 10 days. Bars (means ± *SE*) that do not share the same superscripts differ substantially ($P < 0.05$; one-way ANOVA).

exposure to *P. putida.* This finding was indicated by an elevation in the oxidant parameter (MDA) and a decline in the CAT and GPX. It is suggested that *P. putida* toxins are responsible for impairing the oxidant \antioxidant mechanism via elevating the production of ROS as documented by Liu et al. [59]. Concurrent with a current report [60], clarified that *P. putida* can depress the antioxidant defense mechanism. Additionally, Sazykin et al. [61] noticed that *P. putida* has the ability to alter the antioxidant biomarkers including CAT, superoxide dismutase, lipid peroxidation, and glutathione reductase. Furthermore, a state of stress was noticed in *P. putida*-challenged fish indicated by elevating level of GLU. *P. putida* induces catabolism of GLU which commences with as a substrate (6-phosphogluconate), shaped via detaching and joining routes for oxidation in the periplasm or hexose phosphorylation in the cytoplasm [62–64]. During stress (infection), the liver is trigged by cortisol to GLU for quick energy [65], which confirms our findings.

Interestingly, the exposure to NTG regenerated the antioxidant defense system as well as modulating the elevated level of stress indicator (GLU) reflecting the antioxidant activity of NTG. This was concluded by lessening the oxidant indicator (MDA) and elevating the antioxidant biomarkers (CAT and GPX). A previous study sustained these outcomes and elucidated that TiO_2 has an antioxidant-antibacterial effect by prompting an abrupt decline in the bacterial cell membrane integrity and a release of ROS where superoxide species are created to destroy the biomolecules found in the bacterial cell [66]. Recently, it has been reported that the surface of titanium, along with its photocatalytic characteristics, releases oxygen free radicals and induces oxygen poisoning for the bacterium [67]. Furthermore, Abdel Rahman [9] found that NTG has the power to boost antioxidant activity via enhancing levels of total antioxidant capacity and reducing glutathione content post-exposure of Nile tilapia to bacterial infection.

Assessment of immune function is fundamental to emphasize the role of nano-based materials in aquaculture [68–70]. Herein, we demonstrate the immunosuppressing activity elicited by *P. putida* which was confirmed by a clear decline in the immune parameter (NO) with an increase of pro-inflammatory biomarker (TNF-α), and an alteration in immune-related genes (*TGF-β*, *TRAF-6, TNF-α*, *IL-10*, and *IL-11*). In line with Alzahrani [10] who reported that the immune-inhibitory activity of *P. putida* was indicated by disrupting the immune indicators and attributed the virulence of this bacterium because of the existence of the virulence genes including *nan1, tox A,* and *exo S*.

The elementary consideration is dedicated to the immune-stimulating action of NTG after the fish is challenged by *P. putida* which is verified by elevating values of NO and a decline in the TNF-α level plus improvement in the expression of the immune-related genes. It is suggested that TiO_2 has a potent antibacterial activity that can inhibit bacterial activity because of its nano-size as reported by Hajipour et al. [71], resulting in a notable enhancement in the immune indices and turn, reinforces the immune function. A previous study verified the study outcomes and detected that titanium resulted in a decline in the secretion of pro-inflammatory cytokines secretion including *IL-1α, TNF-α,* and *IL-1β* producing a controlled immuno-inflammatory response [72]. Nevertheless, Taira et al. [73] dispute the study findings who found that the titanium particles enhance inflammation-related genes, involving the *IL-6* gene by using DNA microarray technology. Additionally, it has been reported that TiO_2 NPs induce genotoxicity via the occurrence of DNA strand breaks and chromosomal damages, depending on the particles' size and the duration of exposure [74].

Antimicrobial peptides (AMPs) are strong antimicrobial agents which are used for curing bacterial diseases [22]. Also, they are reported to have a vital role in the innate immune defense mechanism [75]. The present study revealed that the highest expression level of the AMP

genes (*TP3, TP4, CSF-1, hepcidin-2*, *β-Defen 1*, and *NCF-4*) was obvious in the treated group (NTG + *P. putida*) followed by the infected group (*P. putida*). In line with a recent study, Masso-Silva and Diamond [76] and Chen et al. [77] verified our findings and found that fish AMPs and their associated genes are stimulated by infection to kill fish pathogens as well as the immuno-stimulatory molecules. Our finding reflected an efficient immune modulatory role of NTG. A current study elucidates its mechanism of action against the bacterium which is represented in the permeability of the anions found in the outer membrane, inducing lysis of cells [78]. AMPs can smoothly integrate into the cell membrane or cross via the cytosol [79], then intermingle with the bacterial cell membrane and impair the structure of the inner bacterial membrane, producing cell death [80]. Weakened membrane integrity is produced because of the interaction of AMPs with a negative-charged cell membrane, and suppression of DNA, protein, and RNA synthesis [81,82].

5. Conclusion

Overall, the aqueous application of NTG at 0.9 mg/L demonstrated significant antibacterial activity against *P. putida*, effectively modulating oxidative stress, and immune dysfunction, and enhancing the expression of AMPs and immune-related genes in the spleen. The absence of growth performance data is a limitation that needs to be addressed in future studies.

CRediT authorship contribution statement

Heba H. Mahboub: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – review & editing, Writing – original draft. **Morteza Yousefi:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – review & editing. **Hosny Ahmed Abdelgawad:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – review & editing. **Abdelwahab A. Abdelwarith:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – review & editing. **Elsayed M. Younis:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – review & editing. **Emad Sakr:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – review & editing. **Tarek Khamis:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – review & editing. **Sameh H. Ismail:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – review & editing. **Afaf N. Abdel Rahman:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – review & editing, Writing – original draft. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2024.110037.

Data availability

Data will be made available on request.

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