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RESEARCH ARTICLE

Nano-Pichia guilliermondii as a Novel Dietary Supplement in Oreochromis niloticus: Impacts on Growth, Immune-Antioxidant Functions, Intestinal Morphometrics, and Resistance to Aeromonas hydrophila Challenge

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ABSTRACT

Recently, nanoparticles have been used in aquafeed, benefiting fish health and growth and promoting their sustainable use in aquaculture. The present investigation examined the potential advantages of feeding Nile tilapia (Oreochromis niloticus) with nano-Pichia guilliermondii (NPG) on their growth, antioxidant-immune metrics, digestive processes, and tolerance to the Aeromonas hydrophila challenge. For 70 days, 135 fish (body weight =33.36±0.31 g) were allocated equally into three groups of 45 fish each, with 15 fish per replicate. Basal diets fortified with 0%, 0.1 %, and 0.2% NPG /kg were given to the CONT, NPG 0.1, and NPG 0.2 groups, respectively. After the feeding experiment, each group was challenged with 0.1 mL (1.5×106) of A. hydrophila, and the fish survival was tracked for 15 days post challenge. The results demonstrated that dietary supplementation of NPG 0.2 followed by NPG 0.1 decreased serum glucose levels while increased growth metrics, growth hormone levels, and digestive enzymes' (lipase and amylase) activities as compared with the control group. Additionally, NPG-supplemented diets improved antioxidants (superoxide dismutase, catalase, and total antioxidant capacity) and immunological (lysozyme, complement 3, and phagocytic) characteristics. NPG fortification maintained the normal intestinal histology and produced no pathological abnormalities, and the NPG 0.2 diet considerably enhanced the width of the intestinal villi. The fish fed NPG were more resilient to the A. hydrophila challenge, as evidenced by the increased survival rates of the NPG 0.2 group (83.3%) and NPG 0.1 group (75%), compared to the CONT group (66.7%). Dietary NPG, particularly at the 0.2 % level, may be a viable feed additive for Nile tilapia's diets to enhance their growth, well-being, and ability to withstand bacterial pathogens.

Introduction

Aquaculture makes a substantial contribution to the world's food security. As the world's population grows and wild fish stocks become increasingly strained, aquaculture offers a viable way to meet the rising demand for seafood. Aquaculture helps boost the overall supply of seafood, which is crucial for countries that depend mainly on fish as a primary protein source [1]. Among Egypt's most important and extensively cultivated fish species is the Nile tilapia (*Oreochromis niloticus*). Its adaptability to various environments, ease of breeding, and market demand have made it a cornerstone of Egyptian aquaculture. Egypt is the biggest producer of tilapia in Africa and one of the top global producers, contributing significantly to food security and economic development [2].

Recently, a number of illness outbreaks brought on by harmful bacteria caused a high death rate of Nile tilapia and financial losses [3, 4]. *Aeromonas hydrophila* causes fatal illness epidemics in fish, specifically *Aeromonas* septicemia [5]. Infected fish become hemorrhagic and suffer serious injuries, which could result in a high death rate [6, 7]. Commercial aquaculture has traditionally employed antibiotics to lower infectious illness rates. However, overuse of antibiotics has led to resistant microbial strains and drug residues found in aquatic commodities [8-10]. The use of antibiotics is no longer advised due to their detrimental effects on the ecosystem [11]. In order to enhance the immune responses, growth, and physiological processes of species that are crucial to aquaculture, there has been a recent surge in the usage of sustainable food supplements, including yeast [12, 13].

The yeasts in the genus *Pichia*, which belong to the family *Pichiaceae*, have oblong, acuminate cells and sphere-like, oval, hat-shaped, spherical, or circular ascospores [14]. *Pichia* spp. are an effective growth promoter in fish because they enhance nutrient utilization, modulate microbiota, and support immune function [15, 16]. The mechanisms by which *Pichia guilliermondii* improves Nile tilapia growth and immune functions are not well studied in the literature. However, Sealey *et al.*[17] found that rainbow trout's growth, consumption of feed, and protein efficacy were enhanced by diets supplemented with *P. guilliermondii* at 0.3% or 0.6%. These improvements contribute to better nutrient absorption and growth performance and protect fish from infections and environmental stress. As a probiotic, *P. guilliermondii* has significant potential to improve gut health and overall productivity in aquaculture systems, making it a promising tool for sustainable fish farming [18].

By increasing the concentration and efficacy of feed ingredients at their targeted locations, nanotechnology provides a novel way to develop a safe delivery system [19, 20]. However, more research is necessary to ascertain the impacts of adding nano-*P. guilliermondii* (NPG) to fish feed. This study is the first to investigate the dietary supplementation of NPG in the Nile tilapia diet. Thus, this perspective focuses on how dietary NPG can affect growth, immune-antioxidant capacity, digestive capacity, and resistance to *A. hydrophila* of Nile tilapia.

Materials and methods

Preparation of nano-*Pichia guilliermondii (NPG)*

The commercial source of CitriStim (*Pichia guilliermondii*) was Archer Daniels Midland (ADM) Animal Nutrition in Egypt. Every chemical that was used was analytical grade. Throughout the trials, high-sensitivity deionized water (18.2 MΩ•cm) was utilized. Using normal procedures, phosphate buffer saline (PBS, pH 7.4) was made.

The sonochemical synthesis was performed utilizing the ultrasonic synthesizer (Sonics Vibra-Cell VCX 750, USA, frequency 20 kHz, power 750 W) supported with a titanium probe (13 mm diameter). A yeast suspension (5% w/v) was prepared in deionized water and subjected to ultrasonic irradiation under controlled temperature conditions (25 ± 2°C) maintained using an ice bath. The sonication was carried out using a pulse mode (5 seconds on, 2 seconds off) for a total processing time of 30 minutes with amplitude set at 40%. To remove the NPG from bigger detritus, the resultant suspension was centrifuged for 15 minutes at 10,000 rpm. After being collected, the NPG supernatant was stored at 4℃ until it could be further characterized. This technique was adapted and modified from the approach described by Suslick and Flannigan [21].

Characterization of nano-*Pichia guilliermondii (NPG)*

The NPG was characterized by the Atomic Force Microscopy (AFM) and Transmission Electron Microscopy (TEM) analysis (JEOL JEM-2100F). X-Ray Diffraction (XRD) patterns were recorded using a Rigaku Smart Lab diffractometer and the hydrodynamic diameter of the NPG particles was assessed by Dynamic Light Scattering (DLS). Using Malvern Zetasizer Nano ZS equipment, Zeta Potential Measurement tests were conducted to evaluate the NPG suspension's surface charge and colloidal stability.

Diet formulation

Three tested diets were done to satisfy the dietary demands of Nile tilapia [22]. The CONT (control diet) was a basal diet without NPG addition. The NPG 0.1 and NPG 0.2 were basal diets supplemented with 0.1% and 0.2% NPG/kg diet, respectively. A meat mincer (1.5 mm) pelletized the feed after the ingredients were mechanically combined. To ensure equal drying, the pellets were dried for 24 hours at 25 ℃, rotating frequently, and then chilled at 4 ℃ until required. The Association of Analytical Communities [23] performed the proximate chemical analysis of the basal diet (Table 1).

Table 1. Formulation and proximal chemical composition of the basal diet (% on a dry basis) for Nile tilapia.

Ingredients	(% on dry basis)
Ground yellow corn	24.30
Soybean meal 44%	25.50
Fish meal	18.00
Corn gluten 60% CP	11.00
Wheat bran	9.00
Fish oil	6.00
Wheat	5.00
Premix[#]	1.20
Calculated chemical analysis	
DE (Kcal/kg) [*]	2907.3
NFE [**]	38.56
Crude protein	33.62
Fat	9.46
Crude fiber	3.74
Lysine	1.83
Calcium	1.04
Available phosphorus	0.91
Methionine	0.71

[#] Premix: each 1kg of premix contains: vitamin A 550,000 IU, vitamin D 110,000 IU, vitamin E 11,000 mg, vitamin K 484 mg, vitamin C 50 g, vitamin B1 440 mg, vitamin B2 660 mg, vitamin B3 13,200 mg, vitamin B5 1100 mg, vitamin B6 1045 mg, vitamin B9 55 mg, Choline 110,000 mg, Biotin 6.6 mg, Iron 6.6 g, Copper 330 mg, Manganese 1320 mg, Zinc 6.6 g, Selenium 44 mg, Iodine 110 mg.

[*] Digestible energy (DE) was calculated by applying the coefficient of 0.75 to convert gross energy to digestible energy.

[**] NFE "Nitrogen free extract" = 1000 − (g/kg crude protein + fat + ash + crude fiber).

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Experimental fish and study design

The study proposal was examined and accepted by Zagazig University's Ethical Committee for Experimental Animals (ZU-IACUC/2//F/336/2023). Nile tilapia (33.36±0.31 g) was purchased from Zagazig University's Fish Research Unit in Egypt. Fish were placed in cement tanks (1 x 3 x 4 m) before the trial began, and they were kept to adjust for 14 days. According to CCAC recommendations, health tests were performed [24]. Throughout the acclimation and study periods, the water metrics were tracked [25] and stayed within the standard range: dissolved oxygen, temperature, pH, and unionized ammonia were 6.5±0.23 mg/L, 25.2± 1.5°C, 6.5±0.21, and 0.019±0.001 mg/L, respectively. The entire aquarium was drained and refilled twice a week, and the waste materials were sucked out every day. During the adaptation time, the fish received the diets three times daily at 8 am, 12 pm, and 3 pm until they were satisfied with the first week's basal food and the second week's experimental meals.

In triplicate, a total of 135 fish were divided into three groups at random (45 fish per group; 15 fish each replication). The CONT group (control) fed a basal diet, while the NPG 0.1 and NPG 0.2 groups received basal diets intervened with 0.1% and 0.2% NPG/kg diet, respectively. Throughout the 70-day experiment, the fish were received the diets three-times daily until they were satisfied. Mortalities and clinical symptoms were monitored daily.

Growth measurements

At the onset of the trial, the fish's initial weights were established. After the study, the fish's final weights and total feed intake were then noted. Following the Castell and Tiews [26] protocol, the following growth metrics were measured: feed conversion ratio, total weight gain, specific growth rate, and average daily weight gain. The Stuart and Hung [27] approach calculated the protein efficiency ratio.

Weight gain (g/fish) = Final weight- Initial weight.

Average daily gain (g/fish/day) = Weight gain /experimental days.

Feed conversion ratio = Total feed intake (g)/weight gain (g).

Protein efficiency ratio = Weight gain (g)/ protein intake (g).

Specific growth rate (%/day) = 100 (ln final weight ln initial weight)/duration/day.

Sampling procedure

A solution of 100 mg/L benzocaine [28] was utilized to sedate the selected fish (3 fish per replicate; 9 per group). Two distinct blood sets were sampled from the caudal vessels; the first was drawn using heparinized syringes to assess the phagocytic activity. The second samples were drawn without an anticoagulant, and the serum was separated for immunological and biochemical parameter measurements by centrifuging the samples for 20 minutes at 1075 ×g. Furthermore, intestinal samples (9 fish per group) were collected for histological, digestive enzyme, and intestinal bacterial load analysis. Liver samples (9/group) were collected for antioxidant indices assessment.

Digestive enzymes assay

The intestinal sample tissues were weighed and processed with plastic pistils at a ratio of 1:10 in PBS in order to assess the digestive enzyme activity [29]. Then, samples were centrifuged for 3 min at 13,000 ×g and 4 °C. To assess the lipase

and amylase enzyme activity, the supernatant was then moved to microtubes filled with ice.

The Kruger [30] approach was used to estimate each sample's total protein level to calculate the enzyme activity as per protein concentration. The amylase and lipase activities were assessed using Bernfeld's [31] and Worthington's [32] methodologies.

Bacterial community in the intestine

Wu *et al.* [33] used the approach to evaluate the intestinal bacterial count. A 0.85 % sterile normal saline was utilized to dilute the samples serially. Total bacterial counts (TBC) were measured by culturing on freshwater agar. The plates were incubated at 37°C for 28 hours. After that, 30 to 50 colonies/plate were randomly chosen from each sample and re-spread onto nutrient agar dishes to produce a pure culture. *Bacillus amyloliquefaciens* count (BAC) was recognized based on their morphology, motility, oxidation, catalytic activity, and Gram staining. The intestinal bacteria counts were determined as colony-forming units (CFU/g).

$$\text{phagocytic activity (\%)} = \frac{\text{No. of macrophages with engulfed bacteria}}{\text{No. of macrophages}} \times 100$$

Biochemical assays

The growth hormone levels were measured using a growth hormone ELISA kit (MBS266317, MyBioSource, San Diego, USA) as instructed by the manufacturer, following a previous procedure by Lugo *et al.* [34]. Serum glucose was in accordance with Trinder's method [35] utilizing the kits (Spectrum-bioscience, Egyptian Co. for Biotechnology, Cairo, Egypt). Using cellulose-acetate electrophoresis, a previous experiment [36] was used to evaluate the serum total proteins and albumin. Meanwhile, albumin values were subtracted from total protein values to determine globulin.

Immune/antioxidant indices

The serum lysozyme activity and complement 3 were assessed using the spectrophotometry method. Serum lysozyme activity was evaluated at an absorbance of 450 nm utilizing a previously reported procedure [37]. Complement 3 was assessed using a CUSABIO kit (Catalog No.: CSB-E09727s). Myeloperoxidase was measured using Palić *et al.*'s approach [38]. Using heat-inactivated *Candida albicans*, the phagocytic activity (%) was measured using the Cai *et al.* [39] methodology and computed using the following equation:

The activity of superoxide dismutase and catalase, and total antioxidant capacity level in the liver homogenates were determined by spectrophotometric analysis. The liver homogenate preparation procedure was indicated by Abdel Rahman *et al.* [40]. Utilizing commercial kits (MyBioSource, Inc., San Diego, CA 92195-3308: USA), we approximated the superoxide dismutase (catalog no. MBS2540401), catalase (catalog no. MBS038818), and total antioxidant capacity (catalog no. MBS2540515).

Histo-morphological assay

For 48 hours, 10% neutral buffered formalin was administered to the intestinal tissue specimens (anterior sections). Following fixation, the specimens were prepared for paraffin impregnation and blocking, cleaned in dimethyl benzene, and dehydrated in ethyl

alcohol. Following the instructions of Suvarna *et al.* [41], the blocks were cut off at a thickness of 5 µm and then followed by Mayer's hematoxylin solution and eosin (H&E) staining. Any changes to the histology were noted when the stained slides were seen under a light microscope.

Additionally, the following quantitative morphometric (lamina propria thickness, tunica muscularis thickness, villus height, villus width, and villus surface) analysis was carried out following Wilson *et al.* [42] methodology using the AmScope ToupView v4.8.15934 software (AmScope, Irvine, CA, USA).

Challenge test

The Department of Aquatic Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, isolated the *A. hydrophila* from sacrificed fish. The isolate was identified at the National Research Centre (NRC), Microbiology and Immunology Department, Dokki, Giza, Egypt, using the automated VITEK 2-C15 system (BioMérieux, Marcy-l'Étoile, France).

Twenty-four fish were chosen from each group at the end of the study (70 days) to assess the fish's resistance to challenge. Following our recent report, fish were intraperitoneally (IP)-challenged with 0.1 mL (1.5×10^6 CFU) of *A. hydrophila* [43]. The remaining fish in each group were given an IP injection of PBS as a control. For 15 days, the injected fish were monitored twice daily to register any unusual clinical symptoms and fatalities.

Statistical assay

The homogeneity and regularity of the results were assessed utilizing the Bartlett and Kolmogorov-Smirnov procedures. After that, a one-way ANOVA and Duncan's post hoc assay were utilized to evaluate the mean variations at the 5% possibility level. The SPSS program (version 20; Richmond, VA, USA) was utilized for all statistical evaluations. The results were presented as means ± standard error (SE). The Kaplan-Meier model was applied to assess the survival rate of fish affected by *A. hydrophila*. The log-rank (Mantel-Cox) assessment was used to see if there were any significant variations among the groups.

Results

Characterization of nano-Pichia guilliermondii (NPG)

AFM images (Figures 1A and 1B) showed uniformly distributed dome-shaped nanostructures with relatively consistent heights and diameters. The structures have 20-30 nm diameters and form a coherent film across the substrate surface. TEM imaging (Figure 1C) revealed the nanoscale morphology and aggregation behavior of the synthesized NPG. Individual particles appeared as dark spherical structures with diameters ranging from 20 to 30 nm. The XRD pattern of the NPG (Figure 1D) revealed several distinct diffraction peaks. The highest prominence peaks were observed at 2θ values of 20.86° and 21.21°, corresponding to interplanar d-spacings of 4.25 Å and 4.19 Å, respectively. Additional significant peaks were detected at 23.16° (d = 3.84 Å) and 23.56° (d = 3.77 Å). DLS analysis indicated a bimodal distribution with peaks at approximately 30 nm and 150 nm, corresponding to individual NPG particles and their assembled structures, respectively (Figure 2A). The zeta potential of the NPG suspension was measured to be approximately -30 mV at pH 7.0, indicating a moderately negative surface charge (Figure 2B).

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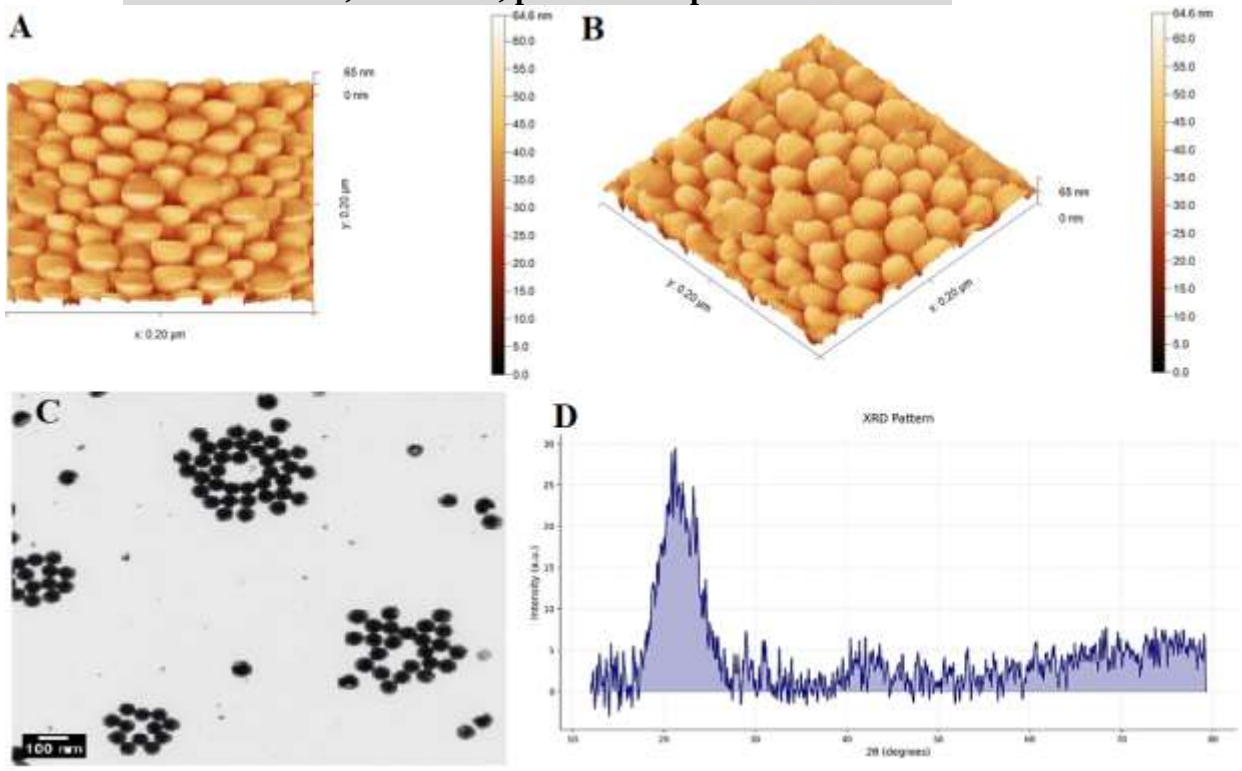

Figure 1. Characterization of the nano-*Pichia guilliermondii* (NPG). (A) Top-view Atomic Force Microscopy (AFM) image. (B) Three-dimensional AFM representation structures. (C) Transmission Electron Microscopy (TEM). (D) X-Ray Diffraction.

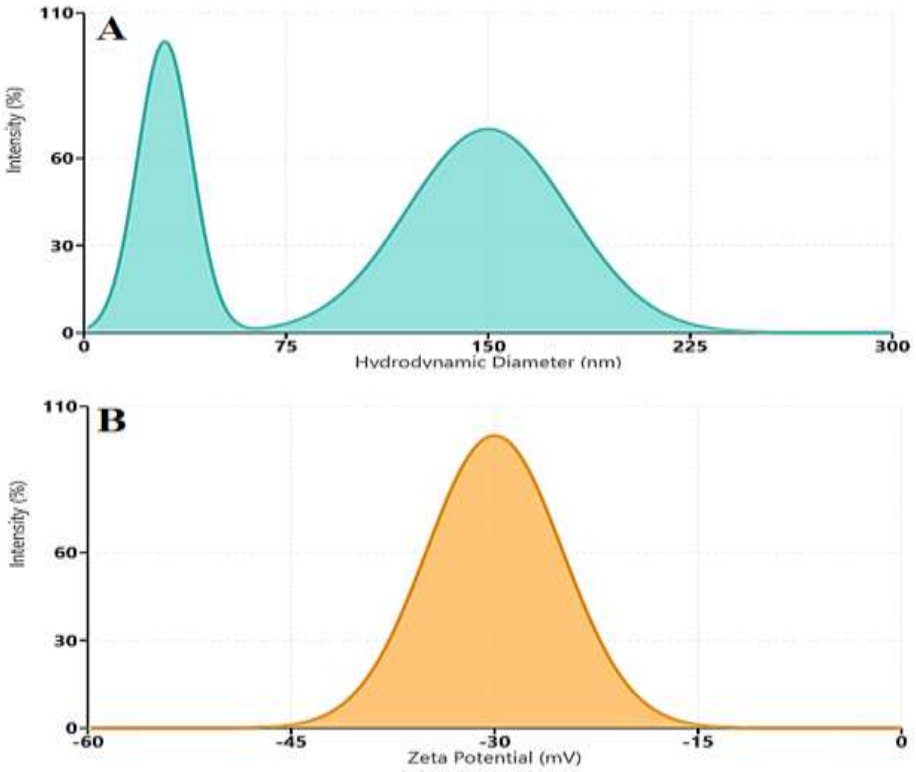

Figure 2. Dynamic Light Scattering (DLS) size distribution profile of NPG (A). Zeta potential distribution of nano-*Pichia guilliermondii* (NPG) particles (B).

Growth metrics

The NPG 0.2 group, followed by the NPG 0.1 group, displayed significant enhancement in the growth measures ($P < 0.001$) (final weight, weight gain, and average daily gain) compared to the CONT group (Table 2). The protein efficiency ratio ($P = 0.001$) and specific growth rate ($P = 0.002$) were significantly improved with a low feed conversion ratio ($P < 0.001$) value in the NPG 0.1 and NPG 0.2 groups comparable to the CONT. The feed intake ($P = 0.74$) did not change significantly among the experimental groups.

Table 2. Growth performance of Nile tilapia fed nano-*Pichia guilliermondii* (NPG) for 70 days.

Parameter	CONT	NPG 0.1	NPG 0.2	*P*-value
Initial weight (g/fish)	33.23±0.86	33.13±0.24	33.73±0.52	0.76
Final weight (g/fish)	81.59±0.22^{c}	93.25±0.58^{b}	96.92±0.37^{a}	<0.001
Weight gain (g/fish)	48.36±0.71^{c}	60.12±0.36^{b}	63.19±0.88^{a}	<0.001
Average daily gain (g/fish/day)	0.69±0.01^{c}	0.85±0.05^{b}	0.90±0.01^{a}	<0.001
Feed intake (g/fish)	91.80±1.29	93.08±0.92	92.91±1.44	0.74
Feed conversion ratio	1.89±0.04^{a}	1.54±0.02^{b}	1.47±0.03^{b}	<0.001
Protein efficacy ratio	1.63±0.04^{b}	1.98±0.02^{a}	2.09±0.04^{a}	0.001
Specific growth rate	1.28±0.03^{b}	1.47±0.02^{a}	1.50±0.02^{a}	0.002

Variation in the data was expressed as means ± *SE*. $^{a, b, \text{and } c}$ Mean values in the same row with different superscripts differ significantly ($P < 0.05$). CONT, NPG 0.1, and NPG 0.2 groups were fish fed basal diet supplemented with 0 %, 0.1 %, and 0.2 % nano-*Pichia guilliermondii* (NPG), respectively.

Activity of digestive enzymes, intestinal bacterial load, and biochemical indices

The activity of digestive enzymes, intestinal bacterial load, and biochemical variables of Nile tilapia are listed in Table 3. The amylase (P<0.001) and lipase (P=0.006) activities were increased significantly by dietary NPG supplementation (NPG 0.2 followed by NPG 0.1 diet) compared to the CONT group. Dietary NPG produced no significant change in the TBC (P = 0.07), while BAC count was substantially risen (P<0.001) in the NPG 0.2 group than in the NPG 0.1 group as compared to the CONT group.

Growth hormone level was substantially risen (P<0.001) by dietary NPG 0.2 followed by NPG 0.1 comparable to the CONT group. Blood glucose level was notably lower in the NPG 0.1 and NPG 0.2 groups relative to the CONT group. The total proteins, albumin, and globulin did not substantially differ (P >0.05) by dietary NPG.

Table 3. Biochemical parameters of Nile tilapia fed nano-*Pichia guilliermondii* (NPG) for 70 days.

Parameter	CONT	NPG 0.1	NPG 0.2	*P*-value
Amylase (U/g)	324.45±2^{c}	410.24±0.58^{b}	435.47±2.47^{a}	<0.001
Lipase (U/g)	22.52±0.83^{c}	26.29±1.51^{b}	30.02±0.22^{a}	0.006
TBC/g (x10^{7})	6.09±0.05	6.12±0.01	6.33±0.11	0.07
BAC/g (x 10^{3})	2.28±0.10^{c}	3.13±0.012^{b}	3.64±0.21^{a}	<0.001
Growth hormone (ng/mL)	2.22±0.03^{c}	2.52±0.02^{b}	2.91±0.01^{a}	<0.001
Glucose (mg/dL)	70.20±0.57^{a}	67.36±0.48^{b}	67.01±0.77^{b}	0.02
Total proteins (g/dL)	2.20±0.05	2.65±0.02	2.96±0.42	0.17
Albumin (g/dL)	1.32±0.02	1.60±0.04	1.56±0.13	0.11
Globulin (g/dL)	0.88±0.03	1.05±0.02	1.40±0.25	0.08

Variation in the data was expressed as means ± *SE*. $^{a, b, \text{and } c}$ Mean values in the same row with different superscripts differ significantly ($P < 0.05$). TBC is the total bacterial count; BAC is the *Bacillus amyloliquefaciens* count. CONT, NPG 0.1, and NPG 0.2 groups were fish fed basal diet supplemented with 0 %, 0.1 %, and 0.2 % nano-*Pichia guilliermondii* (NPG), respectively.

Immune-antioxidant indices

The immune variables, including lysozyme (*P*= 0.04), complement 3 (*P*=0.002), and phagocytic activity % (*P*= 0.001), increased significantly in the NPG 0.2 group followed by the NPG 0.1 group as compared to the CONT group (Table 4). Dietary intervention with NPG 0.2 followed by NPG 0.1 significantly improved (*P* < 0.001) the antioxidant variables (catalase, superoxide dismutase, and total antioxidant capacity) relative to the CONT. The myeloperoxidase (*P*= 0.78) and phagocytic index (*P*= 0.08) did not substantially change among the experimental groups.

Table 4. Immune-antioxidant parameters of Nile tilapia fed nano-*Pichia guilliermondii* (NPG) for 70 days.

Parameter	CONT	NPG 0.1	NPG 0.2	*P*-value
Lysozyme (U/mL)	42.79±1.81^{c}	74.87±1.21^{b}	92.32±1.15^{a}	0.04
Complement 3 (g/L)	1.12±0.01^{c}	1.41±0.09^{b}	1.64±0.02^{a}	0.002
Myeloperoxidase (OD value)	0.72±0.09	0.74±0.08	0.77±0.08	0.78
Phagocytic activity (%)	13.48±0.31^{c}	15.93±0.63^{b}	18.38±0.30^{a}	0.001
Phagocytic index	1.03±0.02	1.06±0.02	1.12±0.01	0.08
Superoxide dismutase (U/g)	40.50±0.86^{c}	44.14±0.81^{b}	52.71±0.79^{a}	<0.001
Catalase (U/g)	110.34±1.56^{c}	117.24±0.88^{b}	130.75±0.75^{a}	<0.001
Total antioxidant capacity (μmol/g)	181.91±1.19^{c}	205.13±3.62^{b}	216.72±2.21^{a}	<0.001

Variation in the data was expressed as means ± *SE*. $^{a, b, \text{and } c}$ Mean values in the same row with different superscripts differ significantly (*P* < 0.05). CONT, NPG 0.1, and NPG 0.2 groups were fish fed basal diets supplemented with 0 %, 0.1 %, and 0.2 % nano-*Pichia guilliermondii* (NPG), respectively.

Histological results

The intestine of the CONT fish showed regular histological features (Figure 3A). Supplementation with NPG 0.1% had a non-significant effect on the villus morphology and the thickness of lamina propria, and tunica muscularis (Figure 3B), while supplementation with NPG 0.2% resulted in a significant increase in the villus width with non-significant effects on the other intestinal morphometric indices (Figure 3C). Table 5 provided a summary of the intestinal morphometric measures.

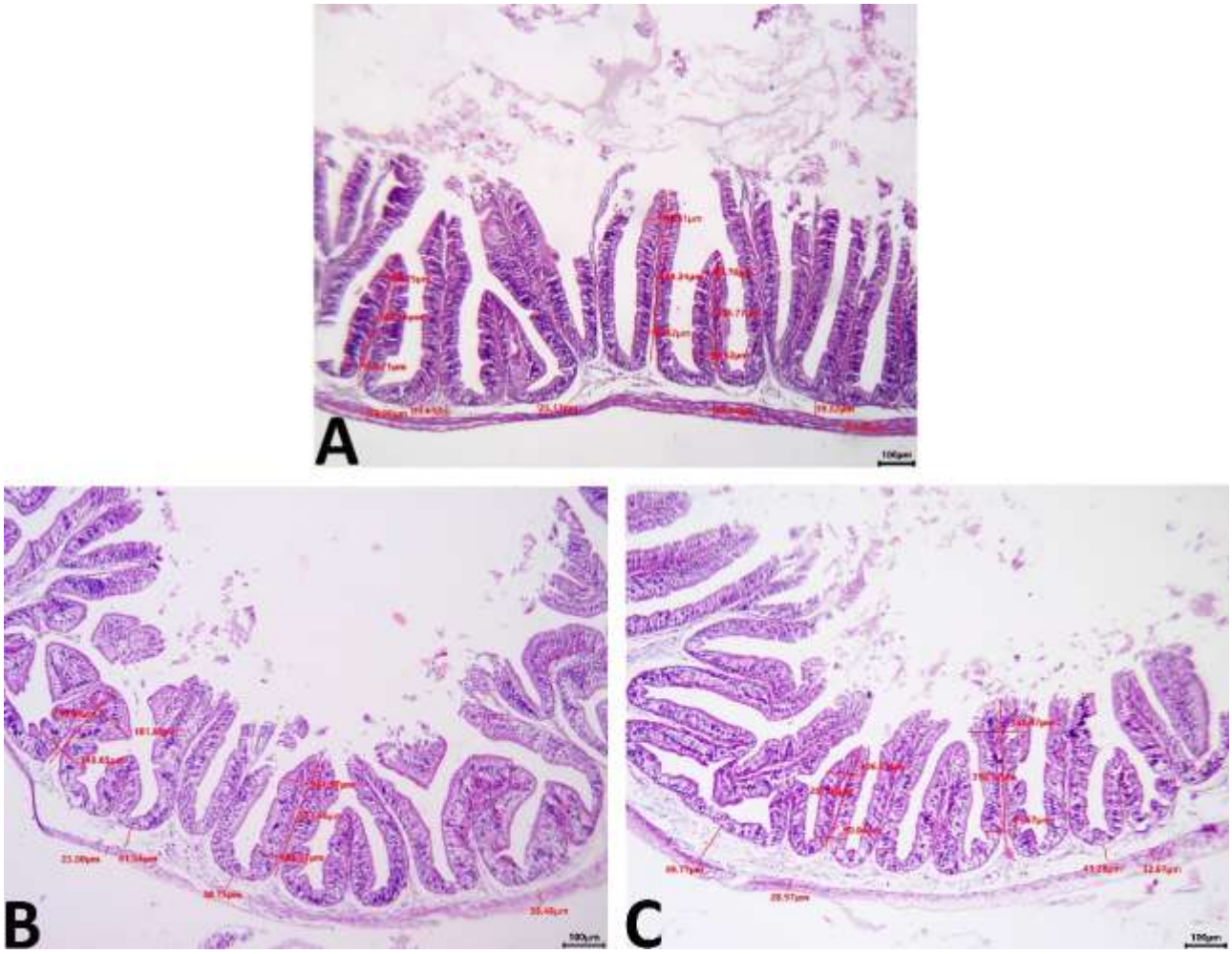

Figure 3. Representative light micrographs of the H&E-stained intestinal tissue sections (anterior part) show the normal histological picture in the CONT group (A); no significant histological changes are seen in the NPG 0.1 (**D**) or NPG 0.2 (**E**) groups except the increase in the villus width in the latter group. CONT, NPG 0.1, and NPG 0.2 groups: supplementation of the diets with 0, 0.1%, and 0.2% NPG/kg diet, respectively. Scale bar: 100 µm. CONT, NPG 0.1, and NPG 0.2 groups were fish fed basal diets supplemented with 0 %, 0.1 %, and 0.2 % nano-*Pichia guilliermondii* (NPG), respectively.

Table 5. Intestinal histomorphometric measures of Nile tilapia fed nano-*Pichia guilliermondii* (NPG) for 70 days.

Parameter	CONT	NPG 0.1	NPG 0.2	*P*-value
Villus height (µm)	310.30±12.04	312±14.79	312.30±16.12	0.99
Villus width (µm)	106.30±3.12^{c}	111.10±7.08^{b}	124.90±4.66^{a}	0.04
Villus surface area (µm^2)	329.18±1.40	345.46±2.49	388.28±2.09	0.12
Lamina propria (µm)	39±1.79	42±2.68	44±6.30	0.65
Tunica muscularis (µm)	45.10±4.12	50.10±5.30	54.80±6.23	0.44

Variation in the data was expressed as means ± *SE*. $^{a, b, and c}$ Mean values in the same row with different superscripts differ significantly ($P < 0.05$). CONT, NPG 0.1, and NPG 0.2 groups were fish fed basal diets supplemented with 0 %, 0.1 %, and 0.2 % nano-*Pichia guilliermondii* (NPG), respectively.

Challenge test

The Kaplan Meier curves (Fig.4) show the survivability of the Nile tilapia during *A. hydrophila* challenge. The NPG 0.2 group showed the highest survivability (83.3 %), followed by the NPG 0.1 group (75%) compared to the CONT group (66.7 %).

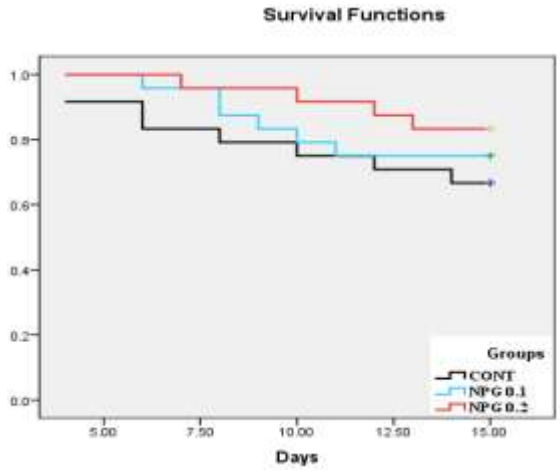

Figure 4. Effect of dietary supplementation of nano-*Pichia guilliermondii* (NPG) on the survival rate % (Kaplan-Meier curves) of Nile tilapia for 15 days. CONT, NPG 0.1, and NPG 0.2 groups were fish fed basal diets supplemented with 0 %, 0.1 %, and 0.2 % nano-*Pichia guilliermondii* (NPG), respectively.

Discussion

When aquatic feed supplements are used in the form of nanoparticles (NPs), they can speed up the rate of uptake in the intestines [44, 45]. There is little knowledge on the usage of NPG in Nile tilapia diets; the goal of this study was to look into the growth, digestive enzyme activity, immune-antioxidant status, and intestinal bacterial community of Nile tilapia concerning different NPG diets.

The results of the current investigation displayed that the intervention of NPG in Nile tilapia diets with the tested concentrations, especially the highest one (0.2 % NPG/kg diet), boosted the fish growth metrics and improved the feed conversion ratio. Fish growth depends on

various factors, including digestive enzyme activity, hormonal control, and intestinal tissue health [46]. Our investigation supported this by improving the digestive enzymes (amylase and lipase) activity and increasing growth hormone concentrations in the NPG-fed fish. In addition, NPG diets maintained the normal histological picture of the intestine with increased villous width at 0.2 % NPG level. Yeast strains may stimulate the secretion of lipases, amylases, and proteases, which would positively affect fish growth and digestion [47, 48]. Growth hormone is secreted from the pituitary gland's somatotropic cells and contributes to fish growth. This trial's rise in growth hormone levels confirms this concept [49]. Furthermore, the increased villous width in the NPG 0.2-fed fish indicated an enlarged surface area for absorption.

Pichia spp. enhances intestinal structure, supports a healthy and diverse gut microbiota, and strengthens immune defenses. These improvements contribute to better nutrient absorption and growth performance and protect fish from infections and environmental stress [18]. Applying yeasts to fish as a supplement to their food may have improved their growth by colonizing the fish's intestines, changing the microbial content of the fish's guts [50, 51]. Non-detectable changes in the TBC and increased BAC in the trial due to feeding on NPG diets supported this concept.

Along with the previously discussed benefits, the bioavailability of *P. guilliermondii* nanoform allowed it to stay in the bloodstream for a long duration, promoting excellent uptake and dissemination during the investigation (70 days). Combining feed digestion, consumption, and absorption led to a notable rise in growth. Furthermore, the characteristics of the NPs may improve the uptake of *P. guilliermondii* by forming tight junctions at cell membranes [43, 52].

Sealey *et al.* [17] previously observed similar results in diets supplemented with *P. guilliermondii* (0.3 and 0.6 %) in rainbow trout (*Oncorhynchus mykiss*). These studies confirmed the beneficial impact of probiotic yeast nanoforms in lowering the dosage and boosting efficacy by using larger concentrations than we did. Furthermore, a previous report on another nano-probiotic bacterium (*B. amyloliquefaciens*) reported improved growth of Nile tilapia [43].

A fish's stress status and general health can be assessed using glucose levels in the blood [53]. The composition of the diet is an essential element affecting the level of glucose in the blood [54]. Since fish use blood glucose as their primary energy supply to survive stressful situations, it is a reliable sign of stress in fish [55]. In our investigation, the glucose level was noticeably decreased in the blood of the NPG-fed fish relative to the CONT-fish. The effect of NPG on the level of blood glucose needs further investigation to explore the mechanism of reducing blood glucose levels by such an additive. At the same time, Al-Refaiee *et al.* [56] established that using yeast (50% *Saccharomyces cerevisiae*) reduced the blood glucose concentration in common carp (*Cyprinus carpio*).

Nonspecific immunity is a crucial component of fish resistance against any disease and contributes to the creation of the immune system's adaptive response [57, 58]. Pathogens are phagocytosed by phagocytic cells, critical components of nonspecific immunity. Without the action of another element, lysozyme, which is generated by leucocytes and lyses the

bacterial cell wall, phagocytosis cannot start [59]. Complement proteins are crucial in lysozyme activity by rupturing the bacterium's surface layer, permitting lysozyme access to the peptidoglycan layer [60]. The antioxidant defenses (superoxide dismutase and catalase) are vital to fish well-being because they remove free radicals and protect cellular components. Probiotics and other dietary supplements strengthen these defenses [61].

Surprisingly, when fish were fed NPG-fortified diets, there was a discernible increase in the immunological (lysozyme, complement-3, and phagocytic activity) and antioxidant (superoxide dismutase, catalase, and total antioxidant capacity) variables. We assert that both dietary concentrations (0.1 and 0.2 %) successfully elicit immunological and antioxidant responses in Nile tilapia. *Pichia* spp. can stimulate components of the innate immune responses involved in pathogen recognition and elimination. Additionally, *Pichia* spp. Stimulates phagocytosis, lysozyme, and respiratory burst activity. These immune responses are essential for rapidly responding to infections before they cause widespread damage [18].

A challenge test is used to assess the fish's immunological capacity. This study showed that feeding fish with NPG increased their survival rate and protected them from infection *by A. hydrophila*. According to our research, the improved immunological and antioxidant responses are responsible for the beneficial effects of NPG on fish survival. Additionally, the primary mechanisms of probiotics' antibacterial activity include the production of antibacterial molecules, competition with pathogens for attachment sites and nutrition, and prevention of gut microbial colonization [62, 63]. Previous research [17] reported that *P. guilliermondii* increased the resistance of *O. mykiss* against *Flavobacterium pyschrophilum* infection.

Conclusion

As we are aware, this trial was the first to look into NPG as a dietary additive for Nile tilapia. Dietary NPG boosted digestive enzyme activity, beneficial microbiota in the intestine, and growth in Nile tilapia. Furthermore, the immune-antioxidant responses of fish were improved with decreased glucose levels as a result of the NPG dietary supplement. Notably, NPG diets increased the fish's resistance to *A. hydrophila* by improving their survival rate. As a probiotic, NPG significantly enhances gut health and productivity in Nile tilapia, making it a promising tool for sustainable fish farming. Future research is required to study how NPG improves the health of other fish species. Moreover, it can be used as an ameliorative feed additive against different environmental stressors.

Author contribution statement

Mohammed E. Hassanin, Abdelhakeem El-Murr, Amr R. EL-Khattib, Mohamed M. M. Metwally, Sameh H. Ismail, Rowida E. Ibrahim: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – review & editing. Rowida E. Ibrahim: Writing – original draft. All authors reviewed and approved the final version of the manuscript before submission.

Data availability statement

Data will be available on request.

Declaration of Competing Interest

The authors have no competing of interests.

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الملخص العربي

خميرة البيكيا جوليرموندي النانومتريه كأضافة جديدة لعليقة البلطي النيلي: التأثيرات علي النمو، الحالة المناعية ومضادات الأكسدة، القياسات المورفومتريه للأمعاء و مقاومة بكتريا الإيروموناس هيدروفيلا

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تم إستخدام الجسيمات النانوية مؤخرًا في أعلاف الأسماك، مما يُحسّن صحة الأسماك ونموها، ويعزز إستخدامها المستدام في تربية الأحياء المائية. تناولت هذه الدراسة المزايا المحتملة لتغذية أسماك البلطي النيلي بجسيمات النانو لخميرة Pichia guilliermondi (NPG) ضمن نظامها الغذائي لمدة 70 يومًا. ودُرست آثارها على النمو، ومقاييس المناعة ، مضادة للأكسدة، وعمليات الهضم، ومقاومه العدوي ببكتيريا الإيروموناس هيدروفيلا ، وُزّعت 135 سمكة (33.36 ± 0.31 غرام) بالتساوي على ثلاث مجموعات، كل مجموعة تحتوي على 45 سمكة، بواقع 15 سمكة لكل تكرار. أُعطيت وجبات أساسية مُدعّمة بـ 0، و0.1%، و0.2% منNPG لكل كيلو جرام علف. بعد تجربة التغذية، تم عدوي المجموعات ببكتريا الايروموناس هيدروفيلا بجرعة0.1 مل (1.5 × 106) ، وتم تتبع بقاء الأسماك لمدة 15 يومًا. أظهرت النتائج أن NPG بجرعة.1 % متبوعًا بـ 0.2 % أدى إلى انخفاض مستويات الجلوكوز في الدم مع زيادة مقاييس النمو، ومستويات هرمون النمو، ونشاط الإنزيمات الهضمية (الليبيز والأميليز). بالإضافة إلى ذلك، حسّنت إضافات NPG من مضادات الأكسدة (سوبر أكسيد ديسميوتاز، كاتالاز، والقدرة الكلية لمضادات الأكسدة) والخصائص المناعية (ليزوزيم، المتمم 3، والقدره علي البلعمة). حافظت عملية تدعيم NPG على النسيج المعوي الطبيعي ولم تُسبب أي تغيرات مرضية، كما عززت الاضافة NPG بجرعه 0.2 % من عرض الزغابات المعوية بشكل ملحوظ. كانت الأسماك أكثر قدرة على تحمل العدوي ببكتريا الايروموناس هيدروفيلا ، كما يتضح من زيادة معدلات البقاء على قيد الحياة في مجموعة 0.2% NPG (83.3%) ومجموعة 0.1 % NPG (75%)، مقارنةً بالمجموعة الضابطة (66.7%). قد يكون NPG وخاصةً مستوى 0.2%، مُضافًا غذائيًا فعالًا لعلف أسماك البلطي النيلي لتعزيز نموها وسلامتها وقدرتها على مقاومة مسببات الأمراض البكتيرية.