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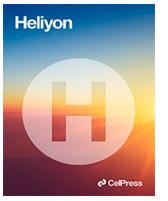

Research article

Nano-*Bacillus amyloliquefaciens* as a dietary intervention in nile tilapia (*Oreochromis niloticus*): Effects on resistance to *Aeromonas hydrophila* challenge, immune-antioxidant responses, digestive/absorptive capacity, and growth

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ABSTRACT

The present investigation proposed an innovative trial at the probable beneficial effects of nano-*Bacillus amyloliquefaciens* (NBA) dietary incorporation on the Nile tilapia (*Oreochromis niloticus*). The investigation included the impact on the growth, digestive functions, immune-antioxidant indices, and resistance to *Aeromonas hydrophila* challenge. A total of 135 fish (35.14 ± 0.12 g) were equally allocated into three groups (45 fish/group; 15 fish/replicate) in triplicates for 70 days. The control, NBA2, and NBA4 groups were fed basal diets enriched with 0, 10^2, and 10^4 CFU/kg NBA, respectively. Following the feeding experiment, all experimental groups were injected with 0.1 mL (1.5×10^6) *A. hydrophila,* and the fish mortalities were observed for 14 days. The outcomes showed that dietary NBA (NBA4 followed by the NBA2 diet) augmented the growth variables (final body weight, total weight gain, and specific growth rate) and condition factor and declined the feed conversion ratio. The intestinal digestive enzyme (amylase and lipase) and growth hormone levels were increased and the serum glucose level was decreased by dietary NBA. Furthermore, NBA diets enhanced the immune (total protein, globulin, lysozyme, complement 3, myeloperoxidase, and phagocytic activity) and antioxidant (superoxide dismutase, catalase, and total antioxidant capacity) parameters. The intestinal histology revealed no pathological lesions with a significant improvement in the intestinal histomorpho-measures (villus height and width, villus surface area, lamina propria thickness, and tunica muscularis thickness) by NBA diets in a dose-dependent manner. In the fish intestine, the *B. amyloliquefaciens* count was increased in the NBA groups (NBA4 group followed by NBA2 group) with no discernible

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difference in the total bacterial count. Fish resistance to the *A. hydrophila* challenge was increased by increasing the survival % in the NBA4 group (91.70 %) followed by the NBA2 group (83.30 %) compared to the control group (70.80 %). Overall, dietary NBA (especially 10^4 CFU/kg diet) could be a promising feed supplement in the Nile tilapia diets for improving their growth, health, and resistance to bacterial challenges.

1. Introduction

Aquaculture has risen significantly to produce fish for human consumption in recent decades. It is an important part of several sections of the world's food supply [1,2]. For many years, Nile tilapia (*Oreochromis niloticus*) aquaculture has been a profitable agricultural enterprise. Nile tilapia is the second most cultivable freshwater fish species in aquaculture [3]. Because of its fast growth, high selling price, resistance to harsh environmental conditions, revenue, and accessible genomic information, it is an economically valuable fish species and a perfect fish model for nutritional studies [4].

Unfortunately, multiple disease outbreaks caused by pathogenic bacteria have plagued the industry recently, resulting in a high rate of Nile tilapia mortality and economic losses [5,6]. One of the pathogens is *Aeromonas hydrophila*, which causes Aeromonas septicemia, devastating disease outbreaks in fish [7]. The fish exposed to the bacteria through the gills, ingestion, or wounds may become hemorrhaged and severely injured, inducing high mortalities [8]. Antibiotics have long been used in commercial aquaculture in reducing infectious diseases. However, the excessive antibiotic application has resulted in the emergence of antibiotic-resistant bacteria, mutagenic microbial strains, and residual drugs identified in aquatic products [9]. Due to their negative environmental impacts, employing antibiotics is no longer recommended [10]. Because of this, using environmentally acceptable feed additives including probiotics to enhance the physiology, growth potential, and immune responses of species relevant to aquaculture has gained appeal in recent years [11–14].

Probiotics (live bacteria) have been shown to exert various physiological impacts on their hosts [15,16]. Probiotics are identified as potential antibiotic alternative additives in aquaculture based on their effectiveness on growth performance and fish well-being versus antibiotics [17–19]. The primary beneficial impacts of probiotics include increased feed bioavailability and digestibility and strengthened immunity against pathogenic microorganisms [20–23]. They can reduce extreme inflammatory responses, maintain epithelial integrity, and inhibit the growth of pathogenic microflora in the gastrointestinal tract of fish [24–26].

Bacillus-based probiotics exhibit distinct traits such as immunomodulation, growth stimulation, and the generation of a wide range of antimicrobial peptides and extracellular compounds against a diverse variety of pathogens in fish [27,28]. *Bacillus amyloliquefaciens* is a powerful *Bacillus species* that generates several extracellular enzymes such as α-amylases, proteases, metalloproteases, and cellulose [29]. These enzymes can improve digestibility, nutrient absorption, and entire immune functioning of the gut in fish, particularly Nile tilapia [30,31]. Despite this, its application as a feed additive remains restricted since a hostile gut environment of stomach acids and bile salts can kill it and reduce its bioavailability. Also, it necessitates long-term administration and high doses to exert their therapeutic powers [32].

To overcome these obstacles and gain their benefits, current research has investigated different formulation techniques for effectively administering probiotics to keep their beneficial effects [33]. Nanotechnology opens a new avenue for creating a safe delivery system for feed ingredients by boosting their concentration and effectiveness in their intended areas [34–38]. In contrast, the influence of utilizing nano-*B. amyloliquefaciens* (NBA) as a feed additive in fish still needs to be investigated. As a result, the current perspective is the first to look into the potential impact of dietary NBA on growth, digestive-absorptive capability, immune-antioxidant response, and *A. hydrophila* resistance in Nile tilapia.

2. Materials and methods

2.1. Preparation of NBA

Commercial *B. amyloliquefaciens* spores (10^9 CFU/g, Ecobiol Aqua, Norel Animal Production, Attaka industrial zone, Suez Gulf, Egypt) were used. NBA was synthesized utilizing a sono chemical approach. The probiotic bacteria were harvested in the stationary growth phase by centrifugation at 6000 rpm for 15 min. After removing media components from the pellet, it was suspended in sterile phosphate-buffered saline (PBS) at a concentration of 10^9 CFU/mL. This bacterial suspension was continuously sonicated using a probe sonicator with a power output of 100 W and frequency of 50 kHz for 20 min in pulse mode (on for 5 s, off for 5 s). The physical and cavitation forces induced by ultra-sonication reduce the size and formation of nanoscale cell debris and bioactive compounds. After 5 min of centrifuging the sonicated product at 10000 rpm, the supernatant containing the released nanoparticles was collected. To eliminate larger cell debris, the supernatant was filtered using a 0.22 μm syringe filter.

2.2. Characterization of NBA

The composition analysis was conducted using the Shimadzu model of UV 1800. Using transmission electron microscopy (TEM) (JEOL Co. JEM-2100), the NBA's morphology was ascertained. Dynamic light scattering (DLS) and zeta potential were used to assess the size and charge of the NBA (Malvern Co. Nano Sight NS500).

2.3. Diet preparation

To meet the nutrient needs of Nile tilapia, three experimental diets were created [39]. The first diet was a basal diet, regarded as a control diet, while NBA2 and NBA4 were basal diets supplemented with 10^2 and 10^4 CFU/kg diet NBA, respectively. The feed ingredients were mixed mechanically and then pelleted using a 1.5 mm meat mincer. The pellets were then air-dried at 25 °C for 24 h, rotating frequently to guarantee even drying, and refrigerated at 4 °C until needed. As indicated in Table 1, the proximate chemical analysis of the basal diet was performed following the Association of Analytical Communities [40].

2.4. Fish and housing condition

The Institutional Animal Care and Use Committee of Zagazig University, Egypt examined and approved the study protocol (ZU-IACUC/2//F/384/2023). The Nile tilapia was procured from the Fish Research Unit at Zagazig University in Egypt. Before the study began, fish were stocked in cement ponds (1 x 3 × 4 m) provided with hapas (15 fish/hapa; each 1.0 × 0.5 × 0.5 m) and given a 14-day acclimatization period. Health tests were conducted under CCAC guidelines [41]. The ponds were part of an outdoor system with a 12-h light cycle and a 12-h dark cycle and had a watering schedule.

During the acclimatization and experiment periods, the water parameters were measured [42] and kept within the normal levels [dissolved oxygen (6.5 ± 0.23 mg/L), temperature (25.2 ± 1.5 °C), pH (6.5 ± 0.21), and unionized ammonia (0.019 ± 0.001 mg/L)]. During the acclimatization period, the fish were fed the basal diet, three times a day (at 8 a.m., 12 p.m., and 3 p.m.) until they were satisfied. The initial body weights (IW) of the fish were determined at the start of the investigation as 35.14 ± 0.12 g.

2.5. Experimental setup

A number of 135 fish were randomly allocated into three experimental groups in triplicates (45 fish/group; 15 fish/replicate). A basal diet was given to the first group (control), while basal diets enriched with 10^2 and 10^4 CFU/kg of NBA were given to the second (NBA2) and third (NBA4) groups, respectively. The fish were fed three times (at 8 a.m., 12 p.m., and 3 p.m.) a day until they were satisfied for the duration of the 70-day experiment.

2.6. Growth metrics, condition factor, and survival rate (%)

The total feed intake (TFI) and final body weight (FW) of the fish were recorded at the end of the investigation. According to Castell

Table 1
Formulation and proximal chemical composition of the basal diet (g/kg on a dry basis).

Ingredients	g/kg
Ground yellow corn	243
Soybean meal 44 %	255
Fish meal	180
Corn gluten 60 % CP	110
Wheat bran	90
Fish oil	60
Wheat	50
Premix[a]	12
Calculated chemical analysis	
Digestible energy (Kcal/kg)[b]	2907.3
Nitrogen-free extract[c]	385.6
Crude protein	336.2
Fat	94.6
Crude fiber	37.4
Lysine	18.3
Calcium	10.4
Available phosphorus	9.1
Methionine	7.1

[a] Vitamin A (550,000 IU), vitamin E (11,000 mg), vitamin D (110,000 IU), vitamin K (484 mg), vitamin B_1 (440 mg), vitamin C (50 g), vitamin B_2 (660 mg), vitamin B_3 (13,200 mg), vitamin B_5 (1100 mg), vitamin B_6 (1045 mg), biotin (6.6 mg), choline (110,000 mg), copper (330 mg), iron (6.6 g), selenium (44 mg), zinc (6.6 g), iodine (110 mg), and manganese (1320 mg) are included in each 1 kg of premix.

[b] The conversion of gross energy to digestible energy was computed using the coefficient of 0.75.

[c] Nitrogen-free extract = 1000 − (crude protein + fat + ash + crude fiber).

and Tiews [43], the sequential growth measures [total weight gain (TWG), average daily weight gain (ADWG), feed conversion ratio (FCR), and specific growth rate (SGR)] were established. The protein efficiency ratio (PER) was computed using the method described by Stuart and Hung's [44] technique. Also, equation (1) was used to compute the condition factor (CF) according to Mozsár et al. [45]. The survival rate % (SR) was estimated according to equation (2).

$$CF = \frac{W}{L^3} \times 100 \tag{1}$$

Where, W stands for the body weight (g), and L is the total fish length.

$$\frac{\text{SR (\%)} = \text{No. of survived fish at the end of the feeding trial (70 days)}}{\text{Total No. of fish at the beginning of the feeding trial}} \times 100 \tag{2}$$

2.7. Sampling

Blood samples (3 fish/replicate; 9/group) were chosen, and they were anesthetized with 100 mg/L of benzocaine solution [46]. The caudal vessels were then used to drain two separate blood samples. Using sterile, heparinized syringes, blood samples were obtained to evaluate the phagocytic activity (PA %). Other blood samples were taken without anticoagulant, and the samples were centrifuged at 1075 $\times g$ for 20 min to separate the serum for measurements of the biochemical and immunological parameters. Additionally, hepatic and intestinal samples (9 fish/group) were taken for performing antioxidant, digestive enzymes, and histological investigation. Another intestinal sample (9 fish/group) was used for intestinal bacterial load assay.

2.8. Digestive enzymes analysis (amylase and lipase)

To measure the activity of digestive enzymes, the intestinal samples were weighted and homogenized using plastic pistils at a ratio of 1:10 in PBS [47]. Every sample was centrifuged for 3 min at 4 °C and 13000 $\times g$. After that, the supernatant was transferred to ice-filled micro-tubes to evaluate the amylase and lipase enzyme activities.

Using the Kruger [48] methodology, the total protein (TP) content of each sample was calculated to ascertain the expression of enzyme activity per g of protein. Twenty microliters of homogenate diluted in PBS (proportion 1:500) were added to micro-tubes holding 980 μL of Bradford reagent diluted in distilled water (proportion of 1:5). A microplate reader (model EON, Biotek company, USA) was used to measure the absorbance of each solution at 595 nm after it had been incubated for 10 min. The methodology of Bernfeld [49] and Worthington [50] was utilized to determine the amylase and lipase activity, respectively.

2.9. Intestinal bacterial load assay

The intestinal bacterial load was assessed following the methodology of Wu et al. [51]. Whole intestinal samples (9 samples/group) were serially diluted using a sterilized normal saline solution (0.85 % w/v NaCl). Total bacterial counts (TBC) were obtained by plating on freshwater agar (Oxoid, England). For 28 h, the plates were incubated at 37 °C. Subsequently, a pure culture was obtained by randomly selecting 30–50 colonies per plate from each sample and re-spreading them onto nutrient agar plates (Oxoid, England). Based on their motility, morphology, Gram staining, oxidation, and catalytic activities, *B. amyloliquefaciens* count (BAC) was observed and identified. The colony-forming units (CFU/g) of bacteria in the intestine were measured.

2.10. Biochemical analysis

A growth hormone (GH) ELISA kit (MBS266317, MyBioSource, San Diego, USA) was used following the manufacturer's instructions to measure GH level in accordance with a prior method by Lugo et al. [52]. Using colorimetric diagnostic kits (Spectrum-Bioscience, Egyptian Co. for Biotechnology, Cairo, Egypt) and Trinder's methods [53], serum glucose was estimated. An earlier assay [54] was followed to assess the qualitative fractionation of serum TP and albumin (ALB) using cellulose-acetate electrophoresis. In the meantime, globulin (GLB) was calculated by deducting ALB values from TP values.

2.11. Immune/antioxidant analysis

The spectrophotometry approach was used to evaluate the serum lysozyme (LYZ) activity and complement 3 (C3). Serum LYZ activity was assessed as previously described protocol [55] at an absorbance of 450 nm. A CUSABIO kit (Catalog No.: CSB-E09727s) was used to evaluate C3. Myeloperoxidase (MPO) was assessed according to the methodology of Palić et al. [56]. The protocol of Cai et al. [57] was utilized to assess the PA % and phagocytic index (PI) of the phagocytes using heat-inactivated *Candida albicans* calculated according to the subsequent equation (3):

$$\frac{\text{PA (\%)} = \text{No. of macrophages with engulfed bacteria} \times 100}{\text{No. of macrophages}} \tag{3}$$

Spectrophotometric analysis was used to measure the levels of superoxide dismutase (SOD), catalase (CAT), and total antioxidant capacity (TAC) in the hepatic tissue homogenates. The process of preparing the hepatic homogenate was described by Abdel Rahman

et al. [58]. We estimated the SOD (catalog no. MBS2540401), CAT (catalog no. MBS038818), and TAC (catalog no. MBS2540515) using commercial kits (MyBioSource, Inc., San Diego, CA 92195-3308: USA).

2.12. Intestinal histo-morphological analysis

The intestinal tissue specimens (anterior parts) were infused with 10 % neutral buffered formalin for 48 h. Following fixation, the specimens underwent the appropriate steps of being cleared in dimethyl benzene, dehydrated in ethyl alcohol, and prepped for paraffin impregnation and blocking. Under the recommendations of Suvarna et al. [59], the tissue blocks were sectioned at a thickness of 5 μm and stained using Mayer's hematoxylin solution and eosin stains (H&E). After that, a light microscope was used to review the stained slides, and any changes to the histology were noted.

Furthermore, using Wilson et al.'s guidelines [60], a quantitative morphometric analysis was performed as follows: villus height (VH), villus width (VW), villus surface area (VSA), lamina propria thickness (LP), and tunica muscularis thickness (TM). The AmScope ToupView v4.8.15934 software (AmScope, Irvine, CA, USA) was used for all microscopic morphometric measurements.

2.13. Bacterial challenge assay

The *A. hydrophila* isolate was previously identified as pathogenic for Nile tilapia after being isolated from sacrificed fish at the Department of Aquatic Animal Medicine, Faculty of Veterinary Medicine, Zagazig University. The isolate was identified at the Microbiology and Immunology Department, National Research Centre (NRC), Dokki, Giza, Egypt, using the automated VITEK 2-C15 system for bacterial identification (BioMérieux, Marcy-l'Étoile, France). To calculate the bacterium's lethal dose 50 (LD_{50}), 100 fish were divided into five equal groups and kept in duplicates. The first group received an intraperitoneal (IP) injection of 0.1 mL of PBS, while the bacterial suspensions (10^4–10^7 CFU/mL) were injected in the remaining four groups. The mortality rates were noted for four days, and the LD_{50} was calculated using the Finney Probit analysis [61], determining that it was 3.00×10^6 CFU/mL. For the challenge test, a sub-lethal dose of 1.50×10^6 CFU/mL was employed.

At the end of the feeding investigation (70 days), twenty-four fish were randomly chosen from each group and fastened for 24 h to evaluate fish resistance to infection. About 0.1 mL (1.50×10^6 CFU/mL) of *A. hydrophila* suspension was IP injected into the fish. As a control, the remaining fish in each group received an IP injection of PBS. After the infection, fish were fasted for 12 h and were given the proper diets. The inoculated fish were observed twice daily for 14 days to document any abnormal clinical signs and mortalities.

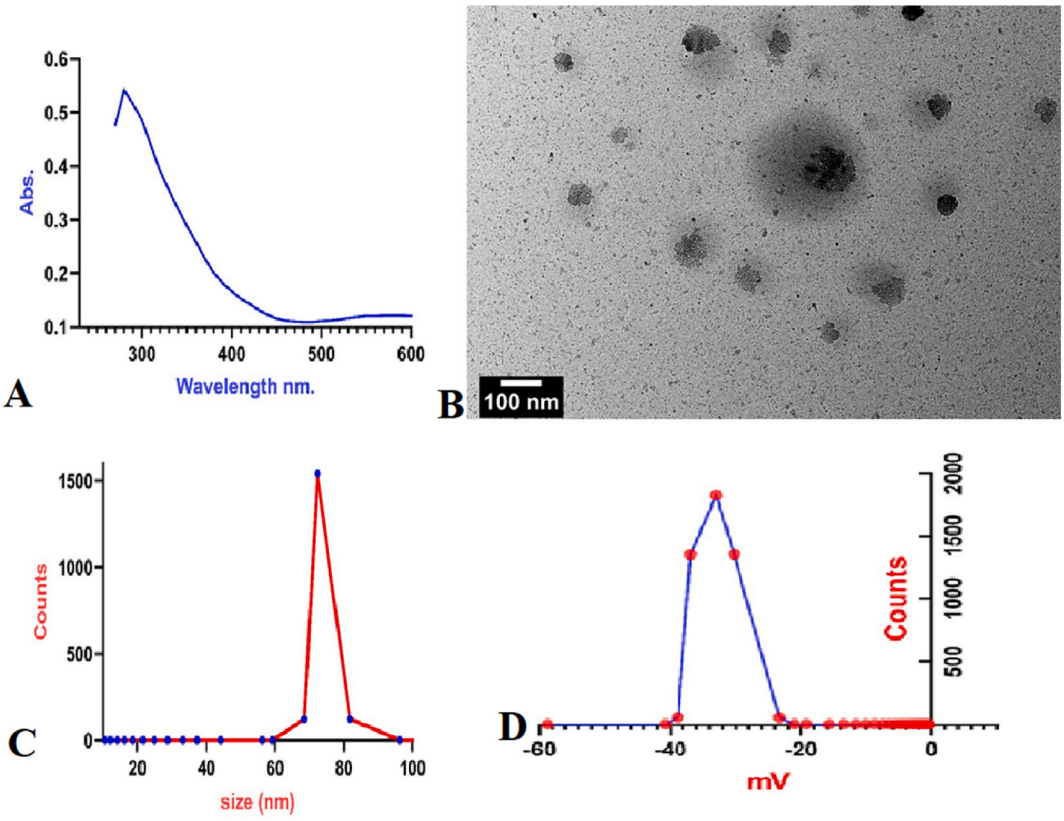

Fig. 1. Characterization of the nano-*Bacillus amyloliquefaciens* (NBA). (A) UV pattern. (B) TEM. (C) DLS pattern. (D) Zeta potential.

2.14. Statistical analysis

The normality and homogeneity tests (Bartlett and Kolmogorov-Smirnov tests, respectively) were applied to the data. After that, to assess mean differences at the 5 % probability level, a one-way ANOVA and Duncan's post hoc test were used. For all statistical analyses, the SPSS software (version 20; Richmond, VA, USA) was utilized. The means ± standard error (*SE*) was used to express the data. The survival probability of fish in each treatment group impacted by *A. hydrophila* was calculated using the Kaplan-Meier model. To determine whether there were any pairwise differences between the groups, the log-rank (Mantel-Cox) test was employed.

3. Results

3.1. Characterization of NBA

The UV–vis spectrum of the NBA showed a peak at 280 nm (Fig. 1A). No aggregation of the NBA was evidenced by the absence of shifts or broadening of the UV peak over time. TEM analysis revealed near spherical nanoparticles with a size distribution concentrated in the range of 70–75 nm (Fig. 1B). The DLS analysis showed the NBA has an average hydrodynamic diameter of 72 nm with a polydispersity index of 0.18, indicating a narrow size distribution (Fig. 1C). The zeta potential of the nanoparticles was determined to be −33.23 mV (Fig. 1D).

3.2. Growth metrics, condition factor, and SR (%)

The growth measures (FW, TWG, ADWG, FCR, and SGR) were improved ($P<0.0001$) in the NBA4 group, followed by the NBA2 group compared to the control group (Table 2). FW, TWG, and FCR were improved by 16.3 %, 26.97 %, and 19.88 % in the NBA2 group, while they were improved by 40.36 %, 66.65 %, and 39.18 % in the NBA4 group, respectively. TFI was not affected ($P = 0.80$) by NBA diets. PER value was improved ($P<0.0001$) in both NBA2 and NBA4 groups compared to the control group. The CF was significantly increased ($P = 0.001$) in the NBA4 group followed by the NBA2 group compared to the control. All groups exhibited 100 SR (%) at the end of the feeding trial (70 days) (Table 2).

3.3. Digestive enzymes, intestinal microflora, and biochemical indices

In comparison to the control group, the NBA4 group, followed by the NBA2 group, showed a significant improvement in the activity of the digestive enzymes [amylase ($P<0.0001$) and lipase ($P = 0.001$)] as well as GH values ($P<0.0001$) (Tables 3 and 4). There was a significant increase in the BAC ($P = 0.001$) in the NBA groups (NBA4 group followed by NBA2 group) over the control. Still, there was no discernible difference in the TBC ($P = 0.06$) between the experimental groups (Table 3). In comparison to the control group, the glucose level was significantly lower ($P = 0.002$) in the NBA2 and NBA4 groups (Table 4).

3.4. Immune-antioxidant indices

The immune indices in terms of TP ($P<0.0001$), GLB ($P<0.0001$), LYZ ($P<0.0001$), C3 ($P = 0.002$), PA% ($P<0.0001$), and PI ($P<0.0001$) were significantly enhanced in the NBA groups (NBA4 group followed by NBA2 group) compared with the control. No substantial variance in the ALB ($P = 0.24$) was between the experimental groups. Meanwhile, the activity of MPO ($P = 0.01$) increased markedly in the NBA4-fed group (Table 5). Dietary inclusion of NBA induced a substantial increase in the SOD ($P = 0.001$), CAT ($P<0.0001$), and TAC ($P = 0.002$) as antioxidant indices over the control, where the NBA4 diet recorded the highest level (Table 6).

Table 2
Effect of dietary nano-*Bacillus amyloliquefaciens* (NBA) on the growth metrics, condition factor, and survival rate (%) of Nile tilapia for 70 days.

Parameters	Control	NBA2	NBA4	*P*-value
Initial body weight (g/fish)	35.23 ± 0.28	35.13 ± 0.24	35.06 ± 0.20	0.89
Final body weight (g/fish)	90.00 ± 0.57^{c}	104.67 ± 3.84^{b}	126.33 ± 1.85^{a}	<0.0001
Total body weight gain (g/fish)	54.77 ± 0.33^{c}	69.54 ± 3.60^{b}	91.27 ± 2.03^{a}	<0.0001
Average daily weight gain (g/fish/day)	0.78 ± 0.004^{c}	0.99 ± 0.05^{b}	1.30 ± 0.028^{a}	<0.0001
Total feed intake (g/fish)	93.80 ± 1.29	95.05 ± 0.86	94.96 ± 1.96	0.80
Feed conversion ratio	1.71 ± 0.02^{a}	1.37 ± 0.08^{b}	1.04 ± 0.04^{c}	<0.0001
Protein efficiency ratio	1.80 ± 0.03^{b}	2.24 ± 0.13^{a}	2.95 ± 0.11^{a}	<0.0001
Specific growth rate	1.33 ± 0.004^{c}	1.55 ± 0.04^{b}	1.83 ± 0.02^{a}	<0.0001
Condition factor	2.16 ± 0.01^{c}	2.63 ± 0.06^{b}	3.16 ± 0.08^{a}	0.001
Survival rate (%)	100	100	100	–

Significant differences ($P < 0.05$; one-way ANOVA; Duncan's post hoc test) are seen between values (means ± *SE*) in the same row containing various superscripts. Control, NBA2, and NBA4 groups: supplementation of the diets with 0, 10^2, and 10^4 CFU/kg NBA, respectively.

Table 3
Effect of dietary nano-*Bacillus amyloliquefaciens* (NBA) on the intestinal digestive enzymes and microflora of Nile tilapia for 70 days.

Parameters	Control	NBA2	NBA4	*P*-value
Amylase (U/g)	328 ± 10.96^{c}	447 ± 6.63^{b}	506 ± 19.05^{a}	<0.0001
Lipase (U/g)	24.60 ± 3.34^{c}	30.80 ± 1.96^{b}	41.80 ± 3.11^{a}	0.001
Total bacterial count/g (× 10^{7})	6.60 ± 0.17	6.10 ± 0.11	6.20 ± 0.05	0.06
B. amyloliquefaciens count/g (× 10^{3})	2.35 ± 0.14^{c}	3.40 ± 0.17^{b}	4.10 ± 0.46^{a}	0.001

Significant differences ($P < 0.05$; one-way ANOVA; Duncan's post hoc test) are seen between values (means ± *SE*) in the same row containing various superscripts. Control, NBA2, and NBA4 groups: supplementation of the diets with 0, 10^{2}, and 10^{4} CFU/kg NBA, respectively.

Table 4
Effect of dietary nano-*Bacillus amyloliquefaciens* (NBA) on the biochemical parameters of Nile tilapia for 70 days.

Parameters	Control	NBA2	NBA4	*P*-value
Growth hormone (ng/mL)	2.60 ± 0.11^{c}	3.25 ± 0.08^{b}	5.30 ± 0.34^{a}	<0.0001
Glucose (mg/dL)	70.00 ± 4.04^{a}	42.50 ± 4.3^{b}	39.50 ± 1.44^{b}	0.002

Significant differences ($P < 0.05$; one-way ANOVA; Duncan's post hoc test) are seen between values (means ± *SE*) in the same row containing various superscripts. Control, NBA2, and NBA4 groups: supplementation of the diets with 0, 10^{2}, and 10^{4} CFU/kg NBA, respectively.

3.5. Histological findings

The intestine of the control fish exhibited normal histology (Fig. 2A). Typically, the tilapia intestine showed villous mucosa lined with columnar epithelium with goblet cells over a connective tissue core with the absence of the crypts of Lieberkühn, followed by lamina propria, and submucosa composed of loose connective tissue. The tunica muscularis comprises inner circular and outer longitudinal smooth muscle layers, and the serosa comprises squamous epithelium. The values of the intestinal morphometric indices in terms of VH (P<0.0001), VW (P<0.0001), VSA (P<0.0001), LP (P<0.01), and TM (P<0.02) were substantially increased in the NBA4 followed by the NBA2 group compared to the control group (Fig. 2B and C and Table 7).

3.6. Challenge test

Kaplan-Meier curves in Fig. 3 demonstrate that the NBA4 group (91.70 %) recorded the highest SR (%), followed by the NBA2 (83.30 %) compared to the control group (70.80 %). Furthermore, statistical significance was established for the variances across the groups ($P < 0.0001$).

4. Discussion

The current aquaculture industry is concentrated on upholding exacting conditions for fish growth. It is important to produce healthy aquafeeds with substantial food content and useful additions [62]. Aquafeed additives can improve survivability and hasten the absorption rate in the digestive tract, especially when they include live bacteria in the form of nanoparticles [63]. Since there is currently no information on the use of NBA in fish diets, the purpose of this study was to examine the growth, digestive-absorptive capacity, and antioxidant-immune response, as well as the total bacterial load of Nile tilapia in response to various NBA diets.

An effective method of determining the health of fish is to measure the growth rate and intestinal morphometric traits of fish in

Table 5
Effect of dietary nano-*Bacillus amyloliquefaciens* (NBA) on the immune parameters of Nile tilapia for 70 days.

Parameters	Control	NBA2
Total protein (g/dL)	2.62 ± 0.05^{c}	3.50 ± 0.42 ^{b}
Albumin (g/dL)	1.52 ± 0.04	1.56 ± 0.13
Globulin (g/dL)	1.10 ± 0.01^{c}	1.94 ± 0.25 ^{b}
Lysozyme (U/mL)	64.00 ± 8.08^{c}	103.00 ± 10.96^{b}
Complement 3 (μg/mL)	1.17 ± 0.18^{c}	1.85 ± 0.08^{b}
Myeloperoxidase (OD value)	0.71 ± 0.017^{b}	0.74 ± 0.02^{b}
Phagocytic activity (%)	14.00 ± 1.73^{c}	18.00 ± 0.57^{b}
Phagocytic index	1.01 ± 0.003^{c}	1.21 ± 0.008^{b}
Parameters	Control	NBA2
Superoxide dismutase (U/g)	43.00 ± 0.72^{c}	59.00 ± 1.27^{b}
Catalase (U/g)	113.00 ± 2.30^{c}	132.50 ± 2.02^{b}
Total antioxidant capacity (μmol/g)	183.50 ± 10.68^{c}	210 ± 19.91^{b}

Significant differences ($P < 0.05$; one-way ANOVA; Duncan's post hoc test) are seen between values (means ± *SE*) in the same row containing various superscripts. Control, NBA2, and NBA4 groups: supplementation of the diets with 0, 10^{2}, and 10^{4} CFU/kg NBA, respectively.

Table 6
Effect of dietary nano-*Bacillus amyloliquefaciens* (NBA) on the antioxidant parameters of Nile tilapia for 70 days.

Parameters	Control	NBA2	NBA4	*P*-value
Superoxide dismutase (U/g)	43.00 ± 0.72^{c}	59.00 ± 1.27^{b}	68.20 ± 1.65^{a}	0.001
Catalase (U/g)	113.00 ± 2.30^{c}	132.50 ± 2.02^{b}	141.50 ± 1.44^{a}	<0.0001
Total antioxidant capacity (μmol/g)	183.50 ± 10.68^{c}	210 ± 19.91^{b}	229.50 ± 4.33^{a}	0.002

Significant differences ($P < 0.05$; one-way ANOVA; Duncan's post hoc test) are seen between values (means ± *SE*) in the same row containing various superscripts. Control, NBA2, and NBA4 groups: supplementation of the diets with 0, 10^2, and 10^4 CFU/kg NBA, respectively.

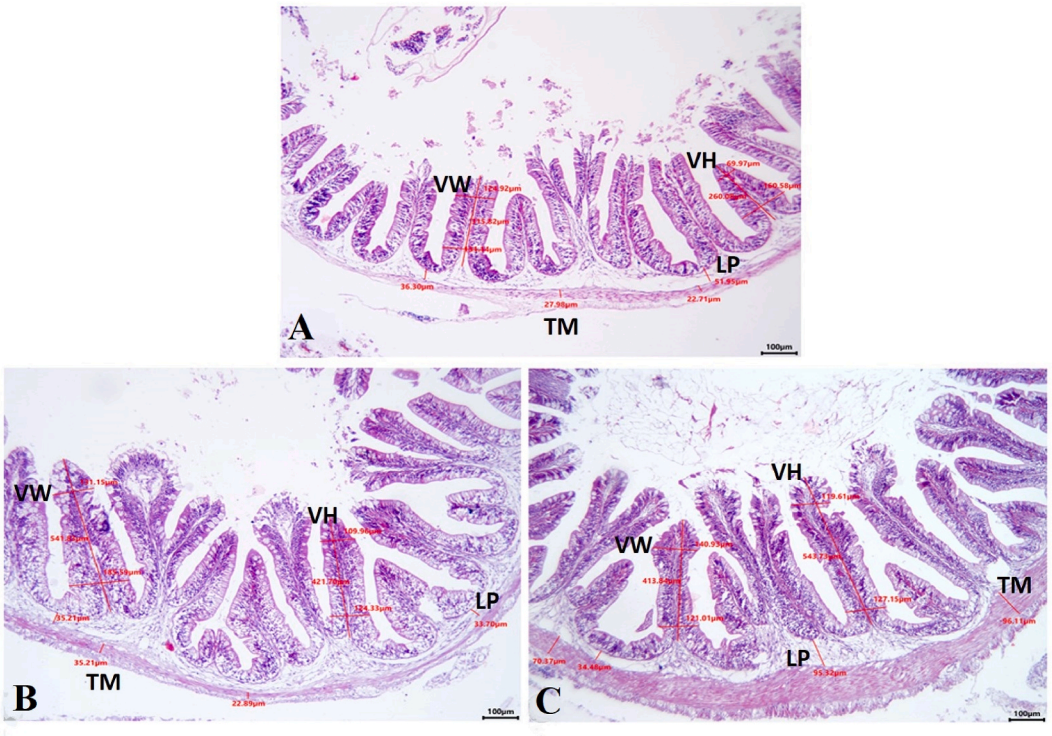

Fig. 2. Representative light micrographs of intestinal sections (anterior part; H&E-stained) show a typical histological picture in the control group (A), with significant increases in the villus height (VH) and width (VW), and the thicknesses of lamina propria (LP) and tunica muscularis (TM) in the NBA2 (B) and NBA4 (C) groups. Control, NBA2, and NBA4 groups: supplementation of the diets with 0, 10^2, and 10^4 CFU/kg NBA, respectively. Scale bar: 100 μm.

Table 7
Effect of dietary nano-*Bacillus amyloliquefaciens* (NBA) on the intestinal histomorphometric measures of Nile tilapia for 70 days.

Parameters	Control	NBA2	NBA4	*P*-value
Villus height (μm)	308.50 ± 15.17^{c}	438.70 ± 25.20^{b}	499.40 ± 24.36^{a}	<0.0001
Villus width (μm)	106.60 ± 3.93^{c}	133.20 ± 6.56^{b}	145.40 ± 4.08^{a}	<0.0001
Villus surface area (μm^2)	328.11 ± 1.77^{c}	594.75 ± 5.55^{b}	727.74 ± 4.41^{a}	<0.0001
Lamina propria (μm)	39.50 ± 2.20^{c}	52.00 ± 5.55^{b}	70.90 ± 5.60^{a}	0.01
Tunica muscularis (μm)	46.25 ± 5.12^{c}	58.40 ± 6.81^{b}	71.60 ± 4.88^{a}	0.02

Significant differences ($P < 0.05$; one-way ANOVA; Duncan's post hoc test) are seen between values (means ± *SE*) in the same row containing various superscripts. Control, NBA2, and NBA4 groups: supplementation of the diets with 0, 10^2, and 10^4 CFU/kg NBA, respectively.

response to a dietary natural supplement [64]. This study demonstrated that adding NBA to Nile tilapia diets for 70 days improved growth and feed utilization metrics (FW, TWG, SGR, FCR, and PER), and CF with a significant improvement observed in the highest dose (10^4 CFU/kg diet). In line with this, probiotics can alter the intestinal ecology by stimulating the digestive enzyme activity in fish [27,65]. It is well known that bacillus probiotics, including *B. amyloliquefaciens*, produce short-chain fatty acids and organic acids, which can improve the digestive system's performance [29]. Our study confirmed this by the augmentation that occurred in the activity of digestive enzymes (amylase and lipase). In fish fed a dietary NBA, there was also a rise in GH level and intestinal

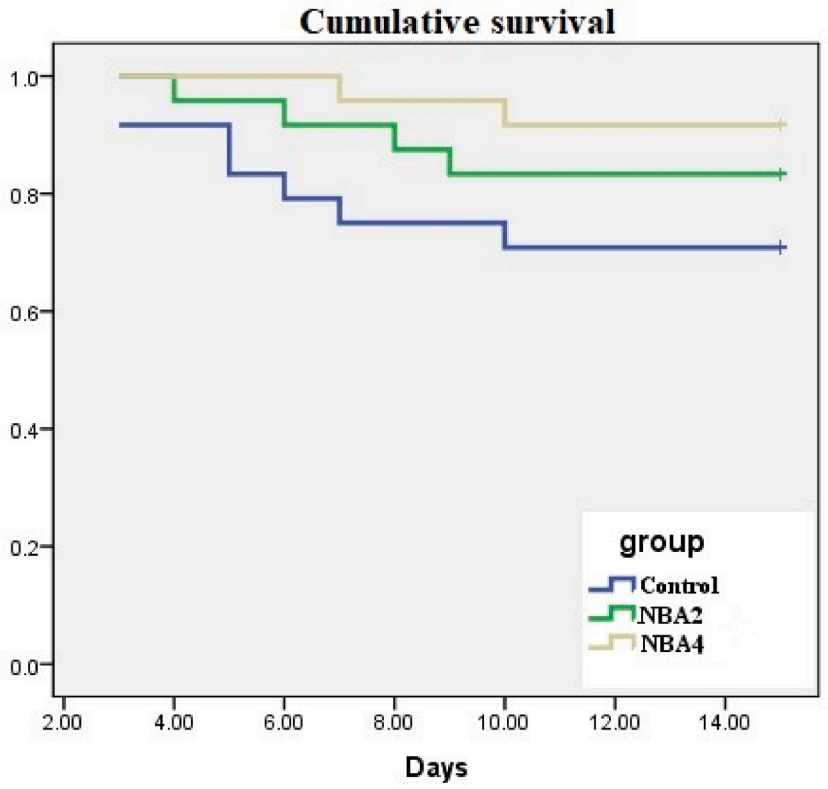

Fig. 3. Effect of dietary nano-*Bacillus amyloliquefaciens* (NBA) on the survival rate % (Kaplan-Meier curves) of Nile tilapia for 70 days. Control, NBA2, and NBA4 groups: supplementation of the diets with 0, 10^2, and 10^4 CFU/kg NBA, respectively.

morphometries (VW, VH, VSA, LP, and TM) without any pathological alterations. Since GH is produced by the pituitary gland (its somatotropic cells) and plays a crucial role in animal growth, the elevated level of GH supports this notion [66].

Additionally, the modified intestinal architecture suggested improved intestinal brush border integrity and enlarged surface area for absorption. Also, *B. amyloliquefaciens* contributes to elevating the diversity of beneficial gastrointestinal tract bacteria in fish [67]. According to Van Hai [68], the effect of intestinal microbiota is to enhance nutrient absorption and digestion through epithelial cells. Furthermore, pathogenic bacteria's negative effects on intestinal immunity are mitigated by the beneficial bacteria found in the gastrointestinal tract. Thus, the local intestine immunity and the body's overall immune system are connected [28,69]. This was confirmed by non-observed alteration in the TBC and enhanced BAC in the findings of this study.

In addition to the previously mentioned aspects, *B. amyloliquefaciens* nanoform's bioavailability allowed it to remain in the bloodstream for longer, facilitating optimal absorption and dispersion during the experiment (70 days). This resulted in a significant increase in growth rate through feed digestibility, utilization, and absorption. Furthermore, by creating tight junctions at cell membranes, the properties of the NPs may enhance *B. amyloliquefaciens* absorption [63].

Comparable outcomes were previously reported by Al-Deriny et al. [30] and Ghalwash et al. [31] in Nile tilapia-provided *B. amyloliquefaciens* enhanced diets (10^9 and 10^{10} CFU). These investigations used higher doses than we did, confirming the advantageous role of probiotic bacterium nanoforms in reducing dosage and increasing efficacy. Additionally, earlier research on another nano-probiotic bacterium (selenium nanoparticles-loaded *Lactiplantibacillus plantarum*; LABS14-Se0NPs) reported improved growth of rainbow trout (*Oncorhynchus mykiss*) [63].

Certain blood parameters, such as glucose, can be utilized to gauge a fish's stress and overall health [70–73]. The most significant factor influencing these indices' concentration is the diet formulation [74]. Blood glucose is a trustworthy indicator of stress in fish, as it is the primary energy source to withstand adverse circumstances [75]. In this work, the blood glucose level was significantly reduced by NBA diets compared with the control diet, reflecting the homeostatic state of fish by NBA diets. Dietary *B. amyloliquefaciens* stimulated insulin sensitivity and glucagon-like peptide-1 (GLP-1) signaling pathway. GLP-1 lowers blood glucose levels by promoting insulin release and blocking the gastrointestinal tract's ability to absorb nutrients [67]. It has been demonstrated that feeding *B. amyloliquefaciens* lowers the glucose level in rohu (*Labeo rohita*) and Nile tilapia [67,76].

Fish immunity against all infections is based mostly on nonspecific immunity, which also helps to create the adaptive immune response [77–79]. Essential elements of nonspecific immunity that phagocytize pathogens are phagocytic cells. Blood TP, particularly the GLB, is assumed to reflect the fish's improved nutritional state and immune response [80]. Phagocytosis cannot begin without the activity of another component, LYZ, produced by leucocytes, that lysis the bacterial cell wall [81]. Complement proteins have a

significant role in the action of LYZ by disrupting the outer layer of the bacterium, which allows LYZ to access the peptidoglycan layer of the bacteria [82]. The progress of the inflammatory process and the destruction of microorganisms are influenced by the MPO enzyme [83]. By eliminating free radicals and shielding cellular components from harm, the antioxidant defenses are essential to the health of fish. Dietary additives boosted these defenses, including probiotics [84].

Interestingly, the immune (TP, GLB, LYZ, C3, MPO, PA%, and PI) and antioxidant (SOD, CAT, and TAC) parameters demonstrated a noticeable increase when fish were given NBA-enriched diets over the control. We claim that Nile tilapia responds effectively to both doses (10^2 and 10^4) to trigger immune and antioxidant responses. The immunomodulatory potential of bacillus probiotics can related to the release of a variety of cytokines that have antibacterial properties against pathogens in fish [85]. Moreover, the bacillus bacterium's cell wall components or spores can activate mucosal lymphoid cells [86]. These outcomes confirm that NBA can strengthen the antioxidant capacity and immunological response, most likely via a rise in the local intestine immunity and the immune system as a whole [87]. Furthermore, prior findings have linked the enhancement of liver function assessments to the immune-regulating impact of NBA on hepatocytes, which stimulates hepatocyte anabolism to generate blood proteins and influences hepatocyte integrity maintenance [88].

Comparable findings were observed in Nile tilapia *B. amyloliquefaciens*-enriched meals (10^6, 10^8, and 10^9 CFU) as reported by Selim and Reda [89], Kuebutornye et al. [90], and Al-Deriny et al. [30], respectively, but on higher doses than our doses. Also, the positive charge and small size of NPs make it easy for fish hepatocytes to internalize and boost antioxidant capacity brought on by NBA additions [91]. In recent work, rainbow trout fed on meals supplemented with LABS14-Se0NPs exhibited notable increases in LYZ and glutathione peroxidase [63].

A challenge assay is employed to evaluate the fish's immune response. This investigation demonstrated that dietary NBA protected fish from *A. hydrophila* infection by increasing their SR (%). The positive benefits of NBA on fish survival can be attributed to the enhanced immunological and antioxidant variables, as shown in our study. Also, the generation of antibacterial compounds (bacteriocins and hydrogen peroxide) and competing with pathogens for attachment sites and nutrients, stopping the colonization of the gut by pathogens, are the main causes of probiotics' antibacterial activity [92,93]. These findings were validated by other research [90,94,95].

5. Conclusion

In this study, the nutritional importance of NBA in Nile tilapia is being highlighted for the first time. Our study implies that dietary NBA might possess special properties that can modulate the intestinal architecture's digestive/absorptive capacity, reflecting the high growth rate of fish. Moreover, Nile tilapia showed better immune-antioxidant status and a lower stress biomarker (glucose) level in response to dietary NBA intake. Remarkably, NBA increases fish tolerance to *A. hydrophila* by increasing survivability, enabling its application as a natural antibacterial agent. It will take more studies to ascertain how effectively NBA enhances the health and performance of different fish species. As well, its ability to neutralize different types of water pollutants and its different antimicrobial capabilities need to be investigated.

Ethics declarations

This study was reviewed and approved by the Institutional Animal Care and Use Committee of Zagazig University, Egypt (ZU-IACUC/2//F/384/2023).

CRediT authorship contribution statement

Mohammed E. Hassanin: Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Abdelhakeem El-Murr:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Amr R. EL-Khattib:** Writing – review & editing, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Abdelwahab A. Abdelwarith:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Elsayed M. Younis:** Writing – review & editing, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mohamed M.M. Metwally:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sameh H. Ismail:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Simon J. Davies:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Afaf N. Abdel Rahman:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Rowida E. Ibrahim:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Additional information

No additional information is available for this paper.

Data availability statement

All data generated in this study has not been deposited into any publicly available repository. Data included in article/supp. material/referenced in article.

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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References

[1] D.D. Mizuta, H.E. Froehlich, J.R. Wilson, The changing role and definitions of aquaculture for environmental purposes,, Rev. Aquac 15 (2023) 130–141.
[2] FAO. The state of food security and nutrition in the world 2023, Urbanization, agrifood systems transformation and healthy diets across the rural–urban continuum Rome, FAO, 2023.
[3] A.F.M. El-Sayed, Tilapia Culture, second ed., Academic Press, 2019.
[4] H. de Verdal, M. Vandeputte, W. Mekkawy, B. Chatain, J.A.H. Benzie, Quantifying the genetic parameters of feed efficiency in juvenile Nile tilapia *Oreochromis niloticus*, BMC Genet. 19 (2018) 105.
[5] H. Thanh, V. Viet, H. Dinh, P. Sangsuriya, S. Jitrakorn, Naturally concurrent infections of bacterial and viral pathogens in disease outbreaks in cultured Nile tilapia (*Oreochromis niloticus*) farms, Aquaculture 448 (2015) 427–435.
[6] A.N. Abdel Rahman, S.H. Ismail, M.M.S. Fouda, A.A. Abdelwarith, E.M. Younis, S.S. Khalil, M.M. El-Saber, A.E. Abdelhamid, S.J. Davies, R.E. Ibrahim, Impact of *Streptococcus agalactiae* challenge on immune response, antioxidant status and hepatorenal indices of Nile tilapia: the palliative role of chitosan white poplar nanocapsule, Fishes 8 (2023) 199.
[7] M. Shirajum Monir, S.M. Yusoff, A. Mohamad, M.Y. Ina-Salwany, Vaccination of tilapia against motile aeromonas septicemia, A. Review, J. Aquat, Anim, Health 32 (2020) 65–76.
[8] M.S. Monir, S.B.M. Yusoff, Z.B.M. Zulperi, H.B.A. Hassim, A. Mohamad, M.S.B.M.H. Ngoo, M.Y. Ina-Salwany, Haematoimmunological responses and effectiveness of feed-based bivalent vaccine against Streptococcus iniae and *Aeromonas hydrophila* infections in hybrid red tilapia (*Oreochromis mossambicus* x *O. niloticus*), BMC Vet. Res. 16 (2020) 226.
[9] S.A. Kraemer, A. Ramachandran, G.G. Perron, Antibiotic pollution in the environment: from microbial ecology to public policy, Microorganisms 7 (2019) 180.
[10] R.C. Okocha, I.O. Olatoye, O.B. Adedeji, Food safety impacts of antimicrobial use and their residues in aquaculture, Public Health Rev. 39 (2018) 21.
[11] E. Amenyogbe, G. Chen, Z. Wang, J. Huang, B. Huang, H. Li, The exploitation of probiotics, prebiotics and synbiotics in aquaculture: present study, limitations and future directions: a review, Aquac, Int 28 (2020) 1017–1041.
[12] H. Elabd, C. Faggio, H.H. Mahboub, M.A. Emam, S. Kamel, R. El Kammar, N.S. Abdelnaeim, A. Shaheen, N. Tresnakova, A. Matter, Mucuna pruriens seeds extract boosts growth, immunity, testicular histology, and expression of immune-related genes of mono-sex Nile tilapia (*Oreochromis niloticus*), Fish Shellfish Immunol. 127 (2022) 672–680.
[13] H. Elabd, H. Youssuf, H.H. Mahboub, S.M.R. Salem, W.A. Husseiny, A. Khalid, H.S. El-Desouky, C. Faggio, Growth, hemato-biochemical, immune-antioxidant response, and gene expression in Nile tilapia (*Oreochromis niloticus*) received nano iron oxide-incorporated diets, Fish Shellfish Immunol. 128 (2022) 574–581.
[14] E. Ahmadifar, S. Mohammadzadeh, N. Kalhor, M. Yousefi, M.S. Moghadam, W. Naraballobh, M. Ahmadifar, S.H. Hoseinifar, H. Van Doan, Cornelian cherry (*Cornus mas* L.) fruit extract improves growth performance, disease resistance, and serum immune-and antioxidant-related gene expression of common carp (*Cyprinus carpio*), Aquaculture 558 (2022) 738372.
[15] S. Yilmaz, E. Yilmaz, M.A.O. Dawood, E. Ringø, E. Ahmadifar, H.M.R. Abdel-Latif, Probiotics, prebiotics, and synbiotics used to control vibriosis in fish: a review,, Aquaculture 547 (2022) 737514.
[16] S. Hoseinifar, Y.-Z. Sun, A. Wang, Z. Zhou, Probiotics as means of diseases control in aquaculture, a review of current knowledge and future perspectives, Front. Microbiol. 9 (2018) 2429.
[17] H. Van Doan, S.H. Hoseinifar, E. Ringø, M. Ángeles Esteban, M. Dadar, M.A.O. Dawood, C. Faggio, Host-Associated Probiotics: a Key factor in sustainable aquaculture,, Rev. Fish. Sci. Aquac 28 (2020) 16–42.
[18] H. Van Doan, P. Prakash, S.H. Hoseinifar, E. Ringø, E. El-Haroun, C. Faggio, R.E. Olsen, H.Q. Tran, V. Stejskal, H.M.R. Abdel-Latif, M.A.O. Dawood, Marine-derived products as functional feed additives in aquaculture: a review, Aquac, Rep 31 (2023) 101679.
[19] M. Mirbakhsh, B. Ghaednia, M.J. Zorriehzahra, F. Esmaeili, C. Faggio, Dietary mixed and sprayed probiotic improves growth performance and digestive enzymes of juvenile whiteleg shrimp (*Litopenaeus vannamei*, Boone, 1931), J Appl Aquac 35 (2023) 823–836.
[20] C. Lumsangkul, N. Vu Linh, F. Chaiwan, M. Abdel-Tawwab, M.A.O. Dawood, C. Faggio, S. Jaturasitha, H. Van Doan, Dietary treatment of Nile tilapia (*Oreochromis niloticus*) with aquatic fern (*Azolla caroliniana*) improves growth performance, immunological response, and disease resistance against *Streptococcus agalactiae* cultured in bio-floc system, Aquac. Rep 24 (2022) 101114.
[21] P. Elumalai, A. Kurian, S. Lakshmi, M.S. Musthafa, E. Ringo, C. Faggio, Effect of *Leucas aspera* against *Aeromonas hydrophila* in Nile Tilapia (*Oreochromis niloticus*): immunity and gene expression evaluation, Turk. J. Fish. Aquat. Sci. 22 (2022). TRJFAS19802.
[22] A. Kuriana, S. Lakshmia, F.J. Fawoleb, C. Faggio, P. Elumalaia, Combined effects of *Leucas aspera*, oxy-cyclodextrin and bentonite on the growth, serum biochemistry, and the expression of immune-related gene in Nile tilapia (*Oreochromis niloticus*), Turk. J. Fish. Aquat. Sci. 21 (2021) 147–158.
[23] H.S. Hamed, S.M. Ismal, C. Faggio, Effect of allicin on antioxidant defense system, and immune response after carbofuran exposure in Nile tilapia, *Oreochromis niloticus*, Comp. Biochem. Physiol. C Toxicol, Pharmacol 240 (2021) 108919.
[24] M.A.O. Dawood, H.G. Abo-Al-Ela, M.T. Hasan, Modulation of transcriptomic profile in aquatic animals: probiotics, prebiotics and synbiotics scenarios, Fish Shellfish Immunol. 97 (2020) 268–682.
[25] A.A. El-Kady, F.I. Magouz, S.A. Mahmoud, M.M. Abdel-Rahim, The effects of some commercial probiotics as water additive on water quality, fish performance, blood biochemical parameters, expression of growth and immune-related genes, and histology of Nile tilapia (*Oreochromis niloticus*),, Aquaculture 546 (2022) 737249.
[26] H. Van Doan, S.H. Hoseinifar, C. Faggio, C. Chitmanat, N.T. Mai, S. Jaturasitha, E. Ringø, Effects of corncob derived xylooligosaccharide on innate immune response, disease resistance, and growth performance in Nile tilapia (*Oreochromis niloticus*) fingerlings, Aquaculture 495 (2018) 786–793.
[27] M.A.M. El-Son, G.E. Elshopakey, S. Rezk, E.A.A. Eldessouki, S. Elbahnaswy, Dietary mixed Bacillus strains promoted the growth indices, enzymatic profile, intestinal immunity, and liver and intestinal histomorphology of Nile tilapia, *Oreochromis niloticus*, Aquac. Rep 27 (2022) 101385.

[28] V.M. Shija, K. Amoah, J. Cai, Effect of Bacillus probiotics on the immunological responses of nile tilapia (*Oreochromis niloticus*): a review,, J. Fishes 8 (2023) 366.
[29] G. Cai, D. Wu, X. Li, J. Lu, Levan from *Bacillus amyloliquefaciens* JN4 acts as a prebiotic for enhancing the intestinal adhesion capacity of *Lactobacillus reuteri* JN101, Int. J. Biol. Macromol. 146 (2020) 482–487.
[30] S.H. Al-Deriny, M.A.O. Dawood, A.A.A. Zaid, W.F. El-Tras, B.A. Paray, H. Van Doan, R.A. Mohamed, The synergistic effects of *Spirulina platensis* and *Bacillus amyloliquefaciens* on the growth performance, intestinal histomorphology, and immune response of Nile tilapia (*Oreochromis niloticus*), Aquac, Rep 17 (2020) 100390.
[31] H.R. Ghalwash, A.S. Salah, A.M. El-Nokrashy, A.M. Abozeid, V.H. Zaki, R.A. Mohamed, Dietary supplementation with *Bacillus species* improves growth, intestinal histomorphology, innate immunity, antioxidative status and expression of growth and appetite-regulating genes of Nile tilapia fingerlings, Aquac. Res. 53 (2022) 1378–1394.
[32] T.K. Das, S. Pradhan, S. Chakrabarti, K.C. Mondal, K. Ghosh, Current status of probiotic and related health benefits, Appl. Food Res. 2 (2022) 100185.
[33] J. Jampilek, J. Kos, K. Kralova, Potential of nanomaterial applications in dietary supplements and foods for special medical purposes, Nanomater 9 (2019) 296.
[34] R.E. Ibrahim, S.A. Amer, K.Y. Farroh, N.A. Al-Gabri, A.I. Ahmed, D.A. El-Araby, S.A.A. Ahmed, The effects of chitosan-vitamin C nanocomposite supplementation on the growth performance, antioxidant status, immune response, and disease resistance of Nile tilapia (*Oreochromis niloticus*) fingerlings,, Aquaculture 534 (2021) (2021) 736269.
[35] G. Rashidian, C.C. Lazado, H. Mahboub, R. Mohammadi-Aloucheh, M. Prokić, H. Nada, C. Faggio, Chemically and green synthesized zno nanoparticles alter key immunological molecules in common carp (*Cyprinus carpio*) skin mucus, Int. J. Mol. Sci. 22 (2021) 3270.
[36] P. Paulpandian, I.S. Beevi, B. Somanath, R.K. Kamatchi, B. Paulraj, C. Faggio, Impact of *Camellia sinensis* Iron oxide nanoparticle on growth, hemato-biochemical and antioxidant capacity of Blue Gourami (*Trichogaster trichopterus*) fingerlings, Biol. Trace Elem. Res. 201 (2023) 412–424.
[37] J. Jeyavani, A. Sibiya, J. Sivakamavalli, M. Divya, E. Preetham, B. Vaseeharan, C. Faggio, Phytotherapy and combined nanoformulations as a promising disease management in aquaculture: a review, Aquac Inter 30 (2022) 1071–1086.
[38] S. Vijayakumar, B. Vaseeharan, R. Sudhakaran, J. Jeyakandan, P. Ramasamy, A. Sonawane, A. Padhi, P. Velusamy, P. Anbu, C. Faggio, Bioinspired zinc oxide nanoparticles using lycopersicon esculentum for antimicrobial and anticancer applications, J. Clust Sci. 30 (2019) 1465–1479.
[39] NRC, Nutrient Requirements of Fish and Shrimp; Wash, National Academies Press, 2011.
[40] AOAC, Official methods of analysis of the association of official analytical chemists, sixteenth, in: Washington (Ed.), Arlington, VA, Association of Official Analytical Chemists, Inc., 2003.
[41] CCAC. Guidelines on: the Care and use of fish in researchteaching and testing, Canadian council on animal Care, 2005.
[42] APHA, American Public Health Association, Standard Methods for Examination of Water and Wastewater, 21th Ed. New York, 2005.
[43] J. Castell, K. Tiews, Report of the EIFAC, IUNS and ICES working group on standardization of methodology in fish nutrition research, hamburg, Federal Republic of Germany (1980) 21–23.
[44] J.S. Stuart, S.S. Hung, Growth of juvenile white sturgeon (*Acipenser transmontanus*) fed different proteins, Aquaculture 76 (1989) 303–316.
[45] A. Mozsár, G. Boros, P. Sály, L. Antal, S. Nagy, Relationship between F ulton's condition factor and proximate body composition in three freshwater fish species, J. Appl. Ichthyol. 31 (2015) 315–320.
[46] D.L. Neiffer, M.A. Stamper, Fish sedation, anesthesia, analgesia, and euthanasia:considerations, methods, and types of drugs, ILAR J. 50 (2009) 343–360.
[47] M.A. Sáenz de Rodrigáñez, P. Díaz-rosales, M. Chabrillón, H. Smidt, S. Arijo, J.M. León-rubio, F.J. Alarcón, M.C. Balebona, M.A. Moriñigo, J.B. Cara, F. J. Moyano, Effect of dietary administration of probiotics on growth and intestine functionality of juvenile Senegalese sole (*Solea senegalensis*, Kaup 1858), Aquac. Nutr 15 (2009) 177–185.
[48] N.J. Kruger, The Bradford Method for Protein Quantitation, 2009.
[49] P. Bernfeld, Amylase α and β methods in enzymology 1, in: S.P. Colswick, NOK (Eds.), Academic Press Inc, New-York, 1995.
[50] V. Worthington. Worthington enzyme manual: enzymes and related biochemicals, Worthingthon Chemical, 1993, p. 399.
[51] Z.X. Wu, X. Feng, L.L. Xie, X.Y. Peng, J. Yuan, X.X. Chen, Effect of probiotic Bacillus subtilis Ch9 for grass carp, *Ctenopharyngodon idel*la (Valenciennes, 1844), on growth performance, digestive enzyme activities and intestinal microflora, J. Appl. Ichthyol. 28 (2012) 721–727.
[52] J.M. Lugo, A. Rodriguez, Y. Helguera, R. Morales, O. Gonzalez, J. Acosta, V. Besada, A. Sanchez, M.P. Estrada, Recombinant novel pituitary adenylate cyclase-activating polypeptide from African catfish (*Clarias gariepinus*) authenticates its biological function as a growth-promoting factor in low vertebrates, J. Endocrinol. 197 (2008) 583.
[53] P. Trinder, Determination of blood glucose using 4-amino phenazone as oxygen acceptor, J. Clin. Pathol 22 (1969) 246.
[54] A. Kaplan, J. Savory, Evaluation of a cellulose-acetate electrophoresis system for serum protein fractionation, Clin. Chem. 11 (1965) 937–942.
[55] M. Ghareghanipoor, P. Akbary, M. Akhlaghi, M. Fereidouni, Nonspecific ımmune responses and ımmune related genes expression of rainbow trout (*Oncorhynchus mykiss*, Walbaum) fed *Zataria multiflora* boiss extract, BEPLS 3 (2014) 140–146.
[56] D. Palić, L.S. Beck, J. Palić, C.B. Andreasen, Use of rapid cytochemical staining to characterize fish blood granulocytes in species of special concern and determine potential for function testing, Fish Shellfish Immunol. 30 (2011) 646–652.
[57] W, -q. Cai, S.-f. Li, J.-y. Ma, Diseases resistance of Nile tilapia (*Oreochromis niloticus*), blue tilapia (*Oreochromis aureus*) and their hybrid (female Nile tilapia×male blue tilapia) to *Aeromonas sobria*, Aquaculture 229 (2004) 79–87.
[58] A.N. Abdel Rahman, G.E. Elshopakey, A. Behairy, D.E. Altohamy, A.I. Ahmed, K.Y. Farroh, M. Alkafafy, S.A. Shahin, R.E. Ibrahim, Chitosan-*Ocimum basilicum* nanocomposite as a dietary additive in *Oreochromis niloticus*: effects on immune-antioxidant response, head kidney gene expression, intestinal architecture, and growth, Fish Shellfish Immunol. 128 (2022) 425–435.
[59] S.K. Suvarna, C. Layton, J.D. Bancroft, Bancroft's theory and practice of histological techniques, 8th ed. England. Churchill Livingstone, Elsevier, 2018.
[60] F. Wilson, T. Cummings, T. Barbosa, C. Williams, P. Gerard, E. Peebles, Comparison of two methods for determination of intestinal villus to crypt ratios and documentation of early age-associated ratio changes in broiler chickens, Poult Sci. 97 (2018) 1757–1761.
[61] D. Finney, A statistical treatment of the sigmoid response curve. Probit Analysis vol. 633, Cambridge University Press, London, 1971.
[62] A. Ahmad, S.R.S. Abdullah, H.A. Hasan, A.R. Othman, N.I. Ismail, Aquaculture industry: supply and demand, best practices, effluent and its current issues and treatment technology, J. Environ. Manag. 287 (2021) 112271.
[63] F. Yanez-Lemus, R. Moraga, C.T. Smith, P. Aguayo, K. Sánchez-Alonzo, A. García-Cancino, A. Valenzuela, V.L. Campos, Selenium nanoparticle-enriched and potential probiotic, *Lactiplantibacillus plantarum* S14 Strain, a diet Supplement beneficial for rainbow trout, Biology 11 (2022) 1523.
[64] A.N. Abdel Rahman, H. Van Doan, H.M. Elsheshtawy, A. Dawood, S.M.R. Salem, N.I. Sheraiba, S.R. Masoud, N.S. Abdelnaeim, T. Khamis, M. Alkafafy, H. H. Mahboub, Dietary *Salvia officinalis* leaves enhances antioxidant-immune-capacity, resistance to *Aeromonas sobria* challenge, and growth of *Cyprinus carpio*, Fish Shellfish Immunol. 127 (2022) 340–348.
[65] H. Liu, S. Wang, Y. Cai, X. Guo, Z. Cao, Y. Zhang, S. Liu, W. Yuan, W. Zhu, Y. Zheng, Dietary administration of Bacillus subtilis HAINUP40 enhances growth, digestive enzyme activities, innate immune responses and disease resistance of tilapia, Oreochromis niloticus Fish shellfish immunol 60 (2017) 326–333.
[66] C.P. Velloso, Regulation of muscle mass by growth hormone and IGF-I Br J Pharmacol 154 (2008) 557–568.
[67] M. Xue, Y. Wu, Y. Hong, Y. Meng, C. Xu, N. Jiang, Y. Li, W. Liu, Y. Fan, Y. Zhou, Effects of dietary *Bacillus amyloliquefaciens* on the growth, immune responses, intestinal microbiota composition and disease resistance of yellow catfish, *Pelteobagrus fulvidraco*, Front. Cell Infect, Microbiol. 12 (2022) 1047351.
[68] N. Van Hai, Research findings from the use of probiotics in tilapia aquaculture: a review,, Fish Shellfish Immunol. 45 (2015) 592–597.
[69] T. Tabassum, A.G.M. Sofi Uddin Mahamud, T.K. Acharjee, R. Hassan, T. Akter Snigdha, T. Islam, R. Alam, M.U. Khoiam, F. Akter, M.R. Azad, M.A. Al Mahamud, G.U. Ahmed, T. Rahman, Probiotic supplementations improve growth, water quality, hematology, gut microbiota and intestinal morphology of Nile tilapia, Aquac. Rep 21 (2021) 100972.
[70] E. Sula, V. Aliko, E. Marku, A. Nuro, C. Faggio, Evaluation of kidney histopathological alterations in Crucian Carp, Carassius carassius, from a pesticide and PCB-contaminated freshwater ecosystem, using light microscopy and organ index mathematical model, Int. J. Aquat. Biol. 8 (2020) 154–165.

[71] I.N.B. Bussons, E.d.S. Sousa, P.H.R. Aride, W.L.P. Duncan, J. Pantoja-Lima, W.M. Furuya, A.T.d. Oliveira, M.R.F.M. Bussons, C. Faggio, Growth performance, hematological responses and economic indexes of *Colossoma macropomum* (Cuvier, 1818) fed graded levels of glycerol, Comp. Biochem. Physiol. C Toxicol, Pharmacol 249 (2021) 109122.
[72] G. Rashidian, K. Shahin, G.E. Elshopakey, H.H. Mahboub, A. Fahim, H. Elabd, M.D. Prokić, C. Faggio, The dietary effects of nutmeg (*Myristica fragrans*) Extract on growth, hematological parameters, immunity, antioxidant status, and disease resistance of common carp (*Cyprinus carpio*) against *Aeromonas hydrophila*,, J. Mar. Sci. Eng. 10 (2022) 325.
[73] S. Saha, K. Dhara, A.V. Chukwuka, P. Pal, N.C. Saha, C. Faggio, Sub-lethal acute effects of environmental concentrations of inorganic mercury on hematological and biochemical parameters in walking catfish, *Clarias batrachus*, Comp. Biochem. Physiol. C Toxicol, Pharmacol 264 (2023) 109511.
[74] S.T. Abarra, S.F. Velasquez, K.D.D.C. Guzman, J.L.F. Felipe, M.M. Tayamen, J.A. Ragaza, Replacement of fishmeal with processed meal from knife fish *Chitala ornate* in diets of juvenile Nile tilapia *Oreochromis niloticus*, Aquac. Rep 5 (2017) 76–83.
[75] S. Polakof, S. Panserat, J.L. Soengas, T.W. Moon, Glucose metabolism in fish: a review, J. Comp. Physiol. B. 182 (2012) 1015–1045.
[76] S. Mohapatra, T. Chakraborty, A. Prusty, K. PaniPrasad, K. Mohanta, Beneficial effects of dietary probiotics mixture on hemato-immunology and cell apoptosis of Labeo rohita fingerlings reared at higher water temperatures, PLoS One 9 (2014) 1–9.
[77] A.N. Abdel Rahman, S.A. Amer, S.R. Masoud, M.M. El-Saber, A. Osman, E.M. Younis, A.A. Abdelwarith, S.J. Davies, T. Khamis, R.E. Ibrahim, Neem seed protein hydrolysate as a fishmeal substitute in Nile tilapia: effects on antioxidant/immune pathway, growth, amino acid transporters-related gene expression, and *Aeromonas veronii* resistance, Aquaculture 573 (2023) 739593.
[78] A. Vazirzadeh, A. Marhamati, R. Rabiee, C. Faggio, Immunomodulation, antioxidant enhancement and immune genes up-regulation in rainbow trout (*Oncorhynchus mykiss*) fed on seaweeds included diets, Fish Shellfish Immunol. 106 (2020) 852–858.
[79] H.H. Mahboub, C. Faggio, B.M. Hendam, S.A. Algharib, M. Alkafafy, M. Abo Hashem, Y.K. Mahmoud, T. Khamis, H.M. Abdel-Ghany, S.R. Masoud, A.N. Abdel Rahman, Immune-antioxidant trait, *Aeromonas veronii* resistance, growth, intestinal architecture, and splenic cytokines expression of *Cyprinus carpio* fed Prunus armeniaca kernel-enriched diets, Fish Shellfish Immunol. 124 (2022) 182–191.
[80] S. Sahoo, H. Banu, A. Prakash, G. Tripathi, Mmune System of Fish: an Evolutionary Perspective, 2021.
[81] S. Saurabh, P.K. Sahoo, Lysozyme: an important defence molecule of fish innate immune system, Aquac. Res. 39 (2008) 223–239.
[82] L. Bavia, L.E. Santiesteban-Lores, M.C. Carneiro, M.M. Prodocimo, Advances in the complement system of a teleost fish, *Oreochromis niloticus*, Fish Shellfish Immunol. 123 (2022) 61–74.
[83] J. Arnhold, The dual role of myeloperoxidase in immune response, Int. J. Mol. Sci. 21 (2020) 8057.
[84] S.H. Hoseinifar, S. Yousefi, H. Van Doan, G. Ashouri, G. Gioacchini, F. Maradonna, O. Carnevali, Oxidative stress and antioxidant defense in fish: the implications of probiotic, prebiotic, and synbiotics, Rev. Fish. Sci. Aquac 29 (2021) 198–217.
[85] E. Amenyogbe, G. Chen, Z. Wang, J. Huang, B. Huang, H. Li, The exploitation of probiotics, prebiotics and synbiotics in aquaculture: present study, limitations and future directions: a review, Aquac, Int 28 (2020) 1017–1041.
[86] S. He, W. Liu, Z. Zhou, W. Mao, P. Ren, T. Marubashi, Evaluation of probiotic strain *Bacillus subtilis* C-3102 as a feed supplement for koi carp (*Cyprinus carpio*), J. Aquac. Res. Dev. S1 5 (2011).
[87] Y. Cao, H. Liu, N. Qin, X. Ren, B. Zhu, X. Xia, Impact of food additives on the composition and function of gut microbiota: a review, Trends Food Sci. Technol. 99 (2020) 295–310.
[88] R.M. Reda, M. El-Hady, K.M. Selim, M.E. Hassanin, Comparative study of three predominant gut Bacillus strains and a commercial B. amyloliquefaciens as probiotics on the performance of *Clarias gariepinus*, Fish Shellfish Immunol. 80 (2018) 416–425.
[89] K.M. Selim, R.M. Reda, Improvement of immunity and disease resistance in the Nile tilapia, Oreochromis niloticus, by dietary supplementation with Bacillus amyloliquefaciens, Fish Shellfish Immunol. 44 (2015) 496–503.
[90] F.K.A. Kuebutornye, Z.W. Wang, Y.S. Lu, E.D. Abarike, M.E. Sakyi, Y. Li, C.X. Xie, V. Hlordzi, Effects of three host-associated *Bacillus species* on mucosal immunity and gut health of Nile tilapia, *Oreochromis nil*oticus and its resistance against Aeromonas hydrophila infection, Fish Shellfish Immunol. 97 (2020) 83–95.
[91] E. Ji&macute;enez-Ferńandez, A. Ruyra, N. Roher, E. Zuasti, C. Infante, C. Ferńandez-Díaz, Nanoparticles as a novel delivery system for vitamin C administration in aquaculture, Aquaculture 432 (2014) 426–433.
[92] D.M. Saulnier, J.K. Spinler, G.R. Gibson, J. Versalovic, Mechanisms of probiosis and prebiosis: considerations for enhanced functional foods, Curr. Opin. Biotechnol. 20 (2009) 135–141.
[93] P. Shokryazdan, C. Sieo, R. Kalavathy, J. Liang, N. Alitheen, M. Jahromi, Y. Ho, Probiotic potential of lactobacillus strains with antimicrobial activity against some human pathogenic strains, BioMed Res. Int. 2014 (2014) 927268.
[94] F. Saputra, Y. Shiu, Y. Chen, A. Puspitasari, R. Danata, C. Liu, S. Hu, Dietary supplementation with xylanase-expressing *B. amyloliquefaciens* R8 improves growth performance and enhances immunity against *Aeromonas hydrophila* in Nile tilapia (*Oreochromis niloticus*), Fish Shellfish Immunol. 58 (2016) 397–405.
[95] F.K. Kuebutornye, J. Tang, J. Cai, H. Yu, Z. Wang, E.D. Abarike, Y. Lu, Y. Li, G. Afriyie, In vivo assessment of the probiotic potentials of three host-associated *Bacillus species* on growth performance, health status and disease resistance of *Oreochromis niloticus* against *Streptococcus agalactiae*, Aquaculture 527 (2020) .735440.