Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 28(5): 149 – 171 (2024)
www.ejabf.journals.ekb.eg

Probiotic, Prebiotic, and Synbiotic effects on Growth Performance, Water Quality, Non-Specific Immune Response, Antioxidant Activity, and Food Safety of the Nile Tilapia (*Oreochromis niloticus*)

Aya G. Abd El-Hamid[1], Omaima M. Ahmed[2], Mohamed M. Said[1]
[1]Department of Aquaculture, Faculty of Fish Resources, Suez University, Suez, Egypt
[2]Department of Fish Processing and Technology, Faculty of Fish Resources, Suez University, Suez, Egypt
***Corresponding author:** g.aya57@yahoo.com

ARTICLE INFO
Article History:
Received: Aug. 2, 2024
Accepted: Aug. 29, 2024
Online: Sep. 1st, 2024

Keywords:
Bacillus probiotic,
Chitosan,
Growth performance,
Innate immunity,
Antioxidants,
Food safety

ABSTRACT
This study aimed to evaluate the effects of diets supplemented with probiotics (*Bacillus* spp.), prebiotics (chitosan), and synbiotics on the growth performance, innate immune system, antioxidant levels, intestinal community, and food quality of the Nile tilapia over 120 days. Experimental fingerlings (15.5 ± 0.352g) were randomly distributed into 12 rectangular polyethylene tanks, with 60 fish per tank. Four treatments, each with three replicates, were tested: Control, probiotic (Sanolife® PRO-F, Pro), prebiotic (chitosan, Pre), and synbiotic (combination of probiotic and chitosan, Syn). Results showed a significant increase in dissolved oxygen concentration and improved pH levels in the probiotic treatment. Unionized ammonia (NH3) levels were reduced in all treatments compared to the control. The prebiotic-supplemented diet significantly improved the final body weight, final length, weight gain, condition factor, average daily weight gain, specific growth rate, and survival rate. Serum lysozyme activity and nitric oxide levels were higher in all treatments supplemented with probiotics. Additionally, superoxide dismutase (SOD) and glutathione peroxidase (GPX) enzyme levels in the liver were significantly higher in the probiotic group, while malondialdehyde (MDA) levels decreased. The addition of probiotics and the presence of synbiotics increased the total bacterial counts in fish intestines and pond water over four months. Pathogenic *Aeromonas hydrophila* was identified only in the control group's water during the first month. *Escherichia coli* and *Salmonella* were identified in the intestines of the control group in the fourth month. 16S rDNA gene sequencing identified *Lysinibacillus sphaericus* in the water of the probiotic treatment and *Citrobacter freundii* in the flesh of the control treatment. Adding *Bacillus* strains and chitosan individually enhances the growth and health of the Nile tilapia (*Oreochromis niloticus*).

INTRODUCTION

Aquaculture, which is the most rapidly growing food producing field globally accounts for half of all fish for human consumption **(Kyule-Muendo *et al*., 2022)**. Fish are regarded as the most expanding food-producing field, efficiently converting feed into biomass **(Elvy *et al*., 2022)**. One of the most commonly distributed freshwater species has been the Nile tilapia (*Oreochromis niloticus*). Intensive high-density fish production resulted in the introduction of numerous bacterial diseases **(Cheng *et al*., 2022)**.

Antibiotics which are used to avoid the transmission of diseases in fish farms, pose a considerable threat toward aquaculture and public health due to their persistence in water and aquatic organisms that will have possibly adverse effects on human health **(Huang *et al*., 2019)**. Adopting alternative feed additives such as probiotics, prebiotics, and synbiotics has gained attention for the tilapia **(del Valle *et al*., 2023; Khanjani *et al*., 2023)**.

Probiotics are living or dead microbe cells that are bio-friendly, and boost fish health when given into culture water or feed by enhancing gut microbial stability. They can tolerate harsh environmental requirements due to their sporulation capabilities. Additionally, their inclusion in fish feed helps balance the intestinal microbial community and suppress pathogenic bacteria **(Soltani *et al*., 2019)**. Probiotics can generate compounds that are antimicrobial, combat infections and diseases in fish by various mechanisms **(Kuebutornye *et al*., 2019)**. Particularly, *Bacillus* spp. showed effective impacts on infectious disorders, maintained survival, and improved fish development **(Adorian *et al*., 2019)**.

Prebiotics refer to non-microbial materials that improve growth and healthy intestinal flora. Chitosan, a long-chain polymer of β-(1,4)-2-deoxy-D-glucose, resulted from the deacetylation of chitin and is effective as a prebiotic in aquaculture **(Kou *et al*., 2021)**. In the aquaculture field, chitosan is of major concern due to growth promotion, immune-stimulating effects, regulation of antioxidant enzyme activities, and reduction of lipid peroxidation **(Aranaz *et al*., 2021)**. Chitosan also has positive benefits on fish in aquaculture since it boosts growth and reduces mortality **(Zaki *et al*., 2015; El-Sayed & Barakat, 2016)**.

Synbiotics comprise a combination of prebiotics and probiotics designed to operate synergistically, increasing their positive impact on host organisms **(Cerezuela *et al*., 2011)**. According to **Huynh *et al*. (2017)**, beneficial bacteria in synbiotics promote their development and activation, while preventing pathogens from attaching by competing on substrates and penetration points. The innate immune system is activated, prebiotic fermentation products are generated, and enzymatic digestion is increased by synbiotics **(Ringø & Song, 2016)**. The importance of careful selection of prebiotics and probiotics in synbiotic preparation was reported by **Cerezuela *et al*. (2013)**, as incorrect combinations can disrupt the physiological processes of animals and diversity of microbial communities.

Microbial imbalance has a profound impact on the immunity of the host and physiology, thus the gut microflora is closely associated with fish health. With the global increase in tilapia production, ensuring microbial safety is crucial for promoting fish health, supporting growth, and preventing diseases. Therefore, this study assessed the effects of *Bacillus* strains and chitosan, both individually and in combination as synbiotics, on growth promotion, feed utilization, body composition, immune response,

antioxidant activity, microbial population, and food safety in the Nile tilapia (*Oreochromis niloticus*).

MATERIALS AND METHODS

1. Experiment site and duration

This experiment took place in the Aquaculture Laboratories Building, Faculty of Fish Resources, Suez University, Suez, Egypt. The experiment was carried out from July 3rd to November 3 rd, 2022.

2. Experimental system

Fish were reared in rectangular polyethylene tanks (2.4 cubic meters each; 100×200×120cm) with a uniform size and stocking density of 60 fish per tank. The water volume used in tanks was 1600L. PVC pipes were attached to air blower (4000W) (Vortex® MODEL: HG-4000) that compresses air to all experimental tanks through a pipe (86cm long, 1.5-inch diameter). Dechlorinated tap water was used to fill an external tank, which was continuously aerated.

3. Experimental design

There were four treatments including control, probiotic, prebiotic, and synbiotic treatments. Each treatment had three replicates. The diet of fish in control group (Con) (basal diet) did not include probiotics or prebiotics. Fish in probiotic treatments consumed diets treated with probiotics mixture of *Bacillus* strains at 2g/ kg feed (Pro) for 120 days, while fish in the prebiotic treatments consumed diets treated with chitosan powder at 4g/ kg feed (Pre) for 120 days. Fish in the synbiotic treatments were fed diets supplemented with a mixture of *Bacillus* strains and chitosan at the same rate for each one separately (Syn) until the end of the experiment (120 days).

4. Experimental fish

The Nile tilapia (*O. niloticus*) male fingerlings were obtained from a private farm in Al Sharqia, Egypt. A specialized transporting car including oxygenated facilities was used to transfer the trial fish. The fingerlings had an initial mean weight of 15.5 ± 0.352g, and length of 6.5± 0.288cm, respectively, when they were received. Fish were acclimated for fourteen days prior to the begining of the trial. Throughout the acclimatization phase, the fish were fed a control diet containing 30% crude protein. Aerated water was used to refill the water volume from a storage tank, and settled fish wastes were discharged on daily basis.

5. Feeding management

A total of 720 tilapia fingerlings were stocked into twelve rectangular polyethylene tanks, with each tank containing 60 fish. The fish received the tested diets at a feeding rate of 5% of the total body weight within the first two weeks and 4% for the rest of the trial, respectively. Fish were fed six days a week with two feeding intervals in the morning every day (9:00 a.m. and 2:00 p.m.). All fish tanks were sampled and

weighed biweekly using a Citizen® balance (ISO 9001:2000) to determine daily feed input based on the newly acquired fish biomass and their lengths. A commercial fish feed with 30% crude protein (CP), manufactured by Grand Aqua® (New Damietta, Egypt), was used for the experiment (see Table 1). After feeding, any excess food was promptly removed from each tank, and approximately 10% of the water was replaced daily. Sampling occurred after depriving the fish from food for 24 hours, and mortality was documented.

Table 1. Proximate analysis of experimental diets without supplementation

Nutritional index	**%**
Moisture	8.9
Crude protein	31.0
Ether extract	5.7
Crude Fiber	4.6
Total Ash	7.2
Nitrogen free extract	51.5

6. Experimental diets

6.1. Probiotic-supplemented diets

The probiotic powder used in this experiment was a mixture of strains from Sanolife® PRO-F INVE Aquaculture, Thailand; *Bacillus* strains (*B. subtilis* 3.00×10^9 CFU/g, *B. licheniformis* 3.50×10^9 CFU/g, and *B. pumilus* 3.50×10^9 CFU/g), with a total number of 1.0×10^{10} CFU/g. Probiotics were added to the basal diet every day. Probiotic powder was homogenized using sunflower oil at 20ml/ kg diet and sprinkled on feed at 2g/ kg diet according to manufacturer instructions. The dried and mixed feed was stored in polythene bags at -20°C until used.

6.2. Prebiotic-supplemented diets

Prebiotic chitosan powder (M.W.: 3600) used in the experiment was obtained from PIOCHEM LABORATORY CHEMICALS, located in 6th October, Giza, Egypt. Chitosan was mixed daily with the basal diet at a concentration of 4g/ kg of diet. According to **Cavalcante *et al.* (2020)**, this dosage is confirmed to be safe and effective. The chitosan was mixed with sunflower oil for homogeneity with the fish feed (basal diet), then dried and stored in polyethylene bags at -20°C until use.

6.3. Synbiotic-supplemented diets

The synbiotic powder used in this experiment was a mixture of probiotics (Sanolife® PRO-F; *B. subtilis* 3.00×10^9 CFU/g, *B. licheniformis* 3.50×10^9 CFU/g, and *B. pumilus* 3.50×10^9 CFU/g) and prebiotic chitosan powder (PIOCHEM LABORATORY CHEMICALS). Synbiotic was prepared and mixed daily with the basal diet as probiotic and prebiotic with the same rate for each treatment alone, which is 2g/

kg diet for pro plus 4g/ kg diet for pre, then mixed with sunflower oil, until dried and then stored in polythene bags at -20°C until used.

7. Measurements

7.1. Water quality monitoring

Temperature, pH, and dissolved oxygen levels in water samples were monitored on a daily basis, whereas ammonia (NH_3) levels were assessed weekly. HANNA portable DO meter was used to measure dissolved oxygen (DO) and temperature. Moreover, ammonia was tested using Lovibond MD 100 Ammonia, and pH levels were measured using a Milwaukee MW 102 pH meter, while temperature was recorded with a temperature meter.

7.2. Growth performance, and feed utilization of the Nile Tilapia

The sampling interval for growth and feed utilization assessment occurred after fish being starved for 24h at the end of the trial and then harvested, weighed, and counted. These formulae were used to evaluate the growth performance variables: Weight gain (WG) = Wt – Wo, where Wt = final weight, Wo = initial weight **(Goda *et al*., 2007)**; condition factor (K) = FW / FL^3 x 100, where FW: Final body weight (g), FL3: Final body length (cm^3) **(Adeoye *et al*., 2016)**. The specific growth rate (SGR %/day) = (Ln Wt – Ln Wo) / t × 100, where Ln= natural Logarithm, t = Rearing Period in Days **(Adeoye *et al*., 2016)**. Average daily weight gain (ADWG) = (Weight Gain / experimental period) **(Naiel *et al*., 2022)**. Survival rate (SR %) = (Number of fish at the end of the experiment /Number of fish stocked originally) x 100 (**Ayisi *et al*., 2017)**.

Concerning feed utilization parameters, total Feed intake (TFI) = FI / N, where FI is the utilized diets during the feeding trial, and N is the total number of fish **(Naiel *et al*., 2022)**. Feed conversion ratio (FCR) = Feed intake /Weight gain **(Adeoye *et al*., 2016)**. Feed Efficiency ratio (FER) = WG /TFI **(Naiel *et al*., 2022)**. Protein efficiency ratio (PER) = wet weight gain (g) / total protein intake (g) **(Adeoye *et al*., 2016)**.

7.3. Body composition analysis

Ten fingerlings were placed in a deep freezer at -18°C at the start of the experiment for chemical analysis (as zero group). The same method was followed when the experiment was finished (five fingerlings were used as final treatment samples). Samples were chemically analyzed for moisture, ash, protein, and fat in aquaculture lab located in faculty of Fish Resources, Suez University, using the standard procedures described by the Association of official analytical chemists **(AOAC, 1990)**. A Kjeldahl instrument (N x 6.25) was used to determine the crude protein concentration. For moisture content, samples were heated in an oven at 85ºC, and the loss weight was estimated. Crude fats are extracted using the Soxhlet technique, which involves the combustion of samples at 550ºC for 2h in a muffle furnace.

8. Immunological and antioxidant enzymes parameters

The following parameters were carried out in the Aquaculture Research Unit of Sakha in Kafr El-Sheikh, Egypt. Superoxide dismutase (SOD), malondialdehyde (MDA),

and glutathione peroxidase (GPX) activity in fish liver, while serum immune response indexes, including lysozyme and nitric oxide from serum, were determined. Upon the end of the experiment, nine fish from each treatment (three fish in each replicate) were selected randomly after fasting for 24 hours. According to **Adeshina *et al*. (2016)**, the fish individual was anesthetized for 3 minutes with 95mg/ l clove oil to collect blood and liver samples.

8.1. Sampling procedure

8.1.1. Serum samples

Blood samples were obtained from blood vessels of caudal, using 1-ml sterilized syringe without anticoagulant. To separate serum, samples were placed in an eppendorf tube, and blood was allowed for clotting at 4°C for 60 minutes, then centrifuging was condducted at 3000rpm/ 15min at 4°C for separation. Serum samples were stored immediately at -20°C after being collected as supernatants.

8.1.2. Liver samples

Before dissection, PBS (phosphate-buffered saline) solution was used to perfuse the liver, pH value of 7.4, with 0.16mg/ mL of heparin, to get rid of any red blood cells. Fish livers were weighed and mixed in the cold buffer of 5–10ml (i.e., 100 mM potassium phosphate, pH value of 7.0, including 2 mM ethylenediaminetetraacetic acid (EDTA)) per gram of tissue. Liver homogenates were then centrifuged at 4,000rpm for 15 minutes at 4°C. Eppendorf tube was used for gathering the supernatant for the assay and was stored on ice or frozen at -80°C until analyzed.

8.2. Serum immune response

8.2.1. Lysozyme activity

The serum lysozyme was assayed following the ELISA micro-well technique by using a fish lysozyme ELISA kit (CAT. No. SL0050FI Sunlong Biotech co. China.) at a wavelength of 450nm with the microplate ELISA reader following manufacturer's instructions.

8.2.2. Nitric oxide (NO)

NO levels were measured via Biodiagnostic co. Egypt. and determined colorimetrically at a wavelength of 540nm according to **Montgomery and Dymock (1961)**.

8.3. Antioxidant enzymes in the liver

8.3.1. Superoxide Dismutase (SOD)

According to **Nishikimi *et al*. (1972)**, SOD was determined colorimetrically at a wavelength of 560nm via SD 25 21 Biodiagnostic co., Egypt.

8.3.2. Malondialdehyde (MDA)

MDA levels in liver homogenate were measured using a commercial kit (MD 25 29 Biodiagnostic co., Egypt,) and determined colorimetrically with a wavelength of 534nm according to **Satoh (1978)**.

8.3.3. Glutathione Peroxidase (GPX)

According to **Paglia and Valentine (1967)**, GPx was determined colorimetrically with a wavelength of 340nm by using a commercial kit (GP 25 24 Biodiagnostic Co., Egypt).

9. Bacteriological Sampling and Analysis

9.1. Collection of samples

A total of 36 fish samples (three random fish from each pond) and 12 pond water samples from the tilapia pond discharge were collected early in the morning. The samples were then transported to the Microbiology Lab in the Faculty of Fish Resources at Suez University for immediate analysis. Flesh portions were aseptically cut and prepared for analysis. After making an incision under the abdomen, the intestines were removed and weighed. These assessments were repeated at the end of each month throughout the study period.

9.2. Aerobic plate count (APC)

9.2.1. Media preparation, dilutions and plate count

The bacteriological media of Nutrient agar (NA; Lab M, UK) was prepared following the manufacturer's instructions. Samples of both flesh and intestine (5g for each) were separately homogenized in sterilized bags for 2min using a stomacher (Seward Stomacher 400 circulator, UK), then they were added to 45ml of sterile 0.1% peptone water (PW; DM185D, MAST, UK). For pond water samples, 1 ml of the water sample was added to 9ml of 0.1% sterile peptone water (PW; DM185D, MAST, UK) and vortexed for 2 minutes. Serial dilutions up to 10^4 were performed using the spread plate technique on Nutrient Agar (NA). These dilutions were incubated at 37°C for 24 hours in an incubator (JSGI-100T). For fish samples, bacterial counts were expressed as log colony-forming units per gram (log CFU/g), while for water samples, they were reported as log colony-forming units per milliliter (log CFU/mL) **(FDA, 2001)**.

10. Bacterial Isolation

10.1. Isolation of Bacillus spp.

Bacillus spp. were identified from water and fish intestine samples on a nutrient agar plate and sub cultured on slants of tryptic soy agar (TSA; LAB M, UK), then incubated for 24h at 37°C. Suspected colonies have been detected with gram staining and biochemical assays (IMVIC) **(FDA, 2001)**.

10.2. Isolation of Aeromonas hydrophila

Samples from pond water and fish intestines were homogenized with 0.1% peptone water and shaken for 5min. A loop filled with suspension has been streaked onto MacConkey Agar (MA; CONDA, Madrid, Spain) plates and incubated for 24h at 37°C. Probable colonies of bacteria were identified via culturing on salnts of tryptic soy agar (TSA), followed by confirmation with gram staining and biochemical assays (IMVIC) **(Tolouei Gilani *et al*., 2021)**.

10.3. Isolation and identification of pathogenic bacteria

Flesh, intestine, and pond water samples were analyzed separately for any *Enterobacteriaceae*.

10.3.1. E. coli

E. coli species was identified from samples using *E. coli* broth (EC), which was incubated for 24h at 37°C. The MacConkey agar (MA) plates have been incubated with a loop full of broth enrichment at 37°C for 24h. After incubation, the colonies were analyzed biochemically by IMVIC test confirmed using API 20E (BioMérieux, France) **(Sugiartha *et al*., 2017)**.

10.3.2. Salmonella

Salmonella spp. were identified from samples using buffered peptone water (BPW) broth and was then cultivated in tetrathionate selective broth (ISO 6579-1:2017). Cultures have been incubated for 24h at 37°C, then cultivated onto xylose lysine deoxycholate agar (XLD; Acumedia, USA) and incubated **(Boukharouba, 2022)**. Selected colonies were biochemically tested and confirmed using IMVIC. Furthermore, triple sugar iron (TSI) was tested and confirmed using API 20E ((BioMérieux, France).

10.3.3. Shigella

Shigella spp. were identified from samples using tryptic soy broth with yeast extract added (TSYE) as the enrichment media. Subsequently, *Salmonella-Shigella* agar was used for the streaking of colonies, then incubated under the same conditions as the rest of *Enterobacteriaceae*. IMVIC and API 20E were used to confirm selected colonies **(FDA, 2013)**.

11. Bacterial isolates identification and characterization

11.1. Gram's staining

This technique was done following the method and steps mentioned by **Sarkar *et al*. (2020)**. The bacteria were identified by examining the slides under a light microscope (Euromex MicroBlue., Arnhem, The Netherlands).

11.2. Biochemical characterization

The isolated bacterial colonies were subjected to the following tests: Indole, Methyl Red test, Voges Proskauer test, and Citrate Utilization test (IMVIC), in addition to triple sugar iron and API 20E (BioMérieux, Marcy-Etoile, France) **(Meiyarasi *et al*., 2017)**.

12. Bacterial identification based on PCR and 16S rDNA gene sequencing

12.1. DNA extraction and PCR amplification

According to **Azwai *et al*. (2016)**, a bacterial DNA preparation kit (Jena Bioscience, Germany) was used to extract DNA, and the manufacturer's instructions were followed. Primers of the universal oligonucleotides forward 5′-AGTTTGATCCTGGCTTAG-3′ and reverse 5′- GGTTACCTTGTTACGACTT-3′ were used to amplify polymerase chain reaction (PCR).

12.2. DNA sequencing

A QIAquick kit (Qiagen, Hilden, Germany) was used for the purification of PCR products. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit. Additional purification was achieved with CENTRI-SEP Columns (Princeton Separations, Freehold, NJ). DNA sequencing was conducted using a 3500 Genetic Analyzer (Applied Biosystems, Massachusetts, USA). The resulting sequences were analyzed using BLAST (Basic Local Alignment Search Tool) and compared with sequences from the GenBank database through the MEGA program (version 7.0.20) **(Kumar *et al.*, 2016)**.

13. Statistical analysis

Analysis of data was conducted using SPSS version 22. (2014). One-way ANOVA and T-test were applied, followed by Duncan's test to identify the significance of differences among treatments.

RESULTS AND DISCUSSION

1. Water quality

Water quality parameters including dissolved oxygen (DO), water temperature, pH and ammonia (NH_3) in the experimental units are presented in Table (2).

Table 2. Effect of probiotic, prebiotic, and synbiotic on water quality parameters of Nile tilapia, *O. niloticus* after 120 days.

Parameters	Con	Pro	Pre	Syn
Dissolved oxygen (mg/l)	7.13 ± 0.033^{c}	7.70 ± 0.058^{a}	7.33 ± 0.088^{b}	7.03 ± 0.033^{c}
Temperature (°C)	23.2 ± 0.600^{a}	23.0 ± 0.288^{a}	23.7 ± 0.145^{a}	23.8 ± 0.440^{a}
pH	7.01 ± 0.043^{a}	6.03 ± 0.040^{c}	6.76 ± 0.066^{b}	6.77 ± 0.014^{b}
NH_3 (mg/L)	0.0010 ± 0.00012^{a}	0.0002 ± 0.00013^{b}	0.0005 ± 0.00006^{b}	0.0005 ± 0.00003^{b}

Data presented as means ± SE. Means within the same row expressing different superscript letters are significantly different ($P<0.05$)

In all experimental treatments, the dissolved oxygen concentration (mg/l) exceeded 5mg/ l. Dissolved oxygen was significantly increased in probiotic group (7.70 ± 0.058) compared to prebiotic, synbiotic, and control with values of 7.33 ± 0.088, 7.03 ± 0.033, and 7.13 ± 0.033, respectively. The water temperature (^{o}C) was without significant differences in all treatments. The pH level was significantly improved by probiotic supplementation (6.03 ± 0.040). The prebiotic (6.76 ± 0.066) and synbiotic (6.77 ± 0.014) groups also showed enhanced pH levels, with no significant differences between them, but they differed significantly from the control group (7.01 ± 0.043). Ammonia (NH_3) levels (mg/L) were significantly reduced in all treatment groups (Probiotic: 0.0002 ± 0.00013, Synbiotic: 0.0005 ± 0.00003, Prebiotic: 0.0005 ± 0.00006) compared to the control group (0.0010 ± 0.00012).

The values of water parameters (PH, DO, and NH_3) reported in this study were preserved within the accepted ranges in all treatment groups and also for control as reported for the tilapia farming **(Boyd & Tucker, 1998)**. The present findings revealed that some water quality parameters (NH_3 and pH) were superior in the probiotic treatment. Our findings agree with previous studies that highlighted the positive impact of probiotic addition (Sanolife PRO-F) resulting in substantial improvements in all water quality parameters compared to the control group **(Elsabagh *et al*., 2018; Kord *et al*., 2021)**. The present results showed that all treated groups enhanced the levels of ammonia. Similar findings were recorded by **Elsabagh *et al*. (2018)** and **Naiel *et al*. (2022)**, who mentioned that ammonia decreased with probiotic addition.

2. Growth performance

The experimental treatments varied significantly in growth parameters from one another (Table 3). The prebiotic-supplemented diet significantly improved the final body weight (FW), final length (FL), weight gain (WG), average daily weight gain (ADWG) and specific growth rate (SGR) (45.37 ± 1.212, 13.80 ±0.029, 29.8 ± 1.325, 0.250 ± 0.010 and 1.53 ± 0.033, respectively) followed by probiotic (41.30 ± 0.436, 13.65 ±0.058, 25.8 ± 0.416, 0.213 ± 0.003 and 1.44 ± 0.012, respectively), then synbiotic (39.03 ± 0.731, 13.45 ±0.032, 23.4 ± 0.819, 0.193 ± 0.007 and 1.37 ± 0.022, respectively), and finally the control group (37.70 ± 0.458, 13.45 ± 0.058, 22.1 ± 0.59, 0.183 ± 0.003 and 1.34 ± 0.021, respectively). Condition factor (K) showed a significant enhancement in prebiotic (1.72 ±0.037) than control group (1.55 ±0.006). The survival rate (%) was significantly greater in prebiotic group (77.33 ± 0.333) compared to control group (58.33 ± 6.641).

Table 3. Effect of probiotic, prebiotic, and synbiotic on growth performance parameters of Nile tilapia, *O. niloticus* after 120 days.

Parameters	Con	Pro	Pre	Syn
FW (g)	37.70 ± 0.458^{c}	41.30 ± 0.436^{b}	45.37 ± 1.212^{a}	39.03 ± 0.731^{bc}
FL (cm)	13.45 ± 0.058^{c}	13.65 ± 0.058^{b}	13.80 ± 0.029^{a}	13.45 ± 0.032^{c}
CF (%)	1.55 ± 0.006^{c}	1.63 ± 0.003^{b}	1.72 ± 0.037^{a}	1.61 ± 0.019^{b}
WG (g)	22.1 ± 0.59^{c}	25.8 ± 0.416^{b}	29.8 ± 1.325^{a}	23.4 ± 0.819^{bc}
ADWG (g)	0.183 ± 0.003^{c}	0.213 ± 0.003^{b}	0.250 ± 0.010^{a}	0.193 ± 0.007^{bc}
SGR (% BW/ day)	1.34 ± 0.021^{c}	1.44 ± 0.012^{b}	1.53 ± 0.033^{a}	1.37 ± 0.022^{bc}
SR (%)	58.33 ± 6.641^{c}	73.33 ± 4.91^{ab}	77.33 ± 0.333^{a}	69.33 ± 3.480^{ab}

Data presented as means ± SE. Means within the same row expressing different superscript letters are significantly different ($P<0.05$)

The findings of the current investigation concerning growth performance revealed that the dietary treated groups significantly enhanced the growth parameters than the control group except for synbiotic-supplemented diets. In this study, chitosan and probiotic Sanolife® PRO-F were significantly effective in promoting the growth of the tilapia when utilized as separate supplements. Interestingly, our findings indicate that dietary supplementation with chitosan at 4g/ kg of diet exhibited a considerably improved

growth performance of the tilapia in the following terms: FW, FL, WG, ADWG, SGR. These findings agree with results reported by **Chen *et al*. (2014)** and **Cavalcante *et al*. (2020)**, suggesting chitosan actively promotes growth. Previous studies used different doses of chitosan and detected an increased growth performance for *O. niloticus* **(Abd El-Naby *et al*., 2019; El-Naggar *et al*., 2022; Hossam-Elden *et al*., 2024)**. This observation was recorded in different species **(Zaki *et al*., 2015; El-Sayed & Barakat, 2016; Salam *et al*., 2021)**. This might be due to stimulating digestive enzymes, preventing potential infections, and encouraging growth of beneficial microorganisms **(Qin *et al*., 2014)**.

Our results also showed growth increment with probiotic-supplemented diets than the control, which aligns with the following studies fed with *Bacillus* spp. **(Elsabagh *et al*., 2018; Kord *et al*., 2021; El-Son *et al*., 2022)**. In the current study, synbiotic-supplemented diets did not significantly improve growth parameters. These results agree with the results of **Sîrbu *et al*. (2022)** and **Say *et al*. (2023)**. Although **Geng *et al*. (2011)** documented that Cobia (*Rachycentron canadum*) grew best on a diet supplied with 6.0g of chitosan per kg combined with 1.0g of *Bacillus subtilis* per kg, while the higher levels of *Bacillus subtilis* (2.0 g kg^{-1}) significantly restricted cobia growth. This might be due to different fish species having varying responses to the same dietary supplements. The results found by **Politis *et al*. (2023)** is similar to the current growth performance results.

From the present findings, the prebiotic-supplemented diets at 4g/ kg diet, respectively, achieved the highest survival rates, consistent with previous studies using chitosan at different doses **(El-Sayed & Barakat, 2016; Ahmed *et al*., 2020; Sathish *et al*., 2021)**. The same findings were recorded by **Cavalcante *et al*. (2020)**, who mentioned diets supplied with chitosan at 4g/ kg diet showed a greater survival rate. Survival rates were also enhanced in probiotic and synbiotic treatments than the control. Furthermore, the enhanced effect on survival rate through probiotic and synbiotic supplementation was studied by several researchers. **de Araújo *et al*. (2018)** demonstrated that the use of symbiotic comprised of Active-MOS® + Bioplus2BC® had improvement in the survival rate after the bacterial challenge. **Guimarães *et al*. (2019)** reported that the inclusion of probiotic composed of *Bacillus subtilis* and *Lactobacillus plantarum* in feed had a survival rate of 73.33%, which aligns with our results.

3. Feed utilization parameters

Feed utilization parameters including feed conversion ratio (FCR), feed intake (FI), feed efficiency (FE), and protein efficiency ratio (PER) were significantly better in prebiotic treatments (Table 4). There was no significant difference reported in the feed utilization parameters between all treatments except for feed intake (FI). The results showed that the best of feed conversion ratio (FCR) values were achieved by prebiotic treatment (1.99 ± 0.038). The prebiotic treatment registered the highest feed intake (59.3

± 2.832), while the lowest value was recorded at the control treatment (47.9 ± 0.104). No significant difference was recorded concerning feed efficiency and protein efficiency ratio between all treated groups and the control. Considering our results of feed utilization, it can be noted that the best FCR values were observed in the chitosan treatments. These findings align with the results of **Chen *et al*. (2014)** and **Abd El-Naby *et al*. (2019)**, who used chitosan at different doses in the diets of the tilapia.

Table 4. Effect of probiotic, prebiotic, and synbiotic on feed utilization parameters of Nile tilapia, *O. niloticus* after 120 days.

Parameters	Con	Pro	Pre	Syn
FI (g)	47.9 ± 0.104^{c}	53.2 ± 2.052^{ab}	59.3 ± 2.832^{a}	49.4 ± 4.708^{ab}
FCR (g/g)	2.17 ± 0.050^{a}	2.06 ± 0.047^{a}	1.99 ± 0.038^{a}	2.10 ± 0.134^{a}
FE	0.460 ± 0.010^{a}	0.487 ± 0.012^{a}	0.503 ± 0.009^{a}	0.480 ± 0.029^{a}
PER (g/g)	1.54 ± 0.037^{a}	1.61 ± 0.038^{a}	1.68 ± 0.032^{a}	1.69 ± 0.098^{a}

Data presented as means ± SE. Means within the same row expressing different superscript letters are significantly different (P<0.05)

4. Chemical analysis of fish

No significant difference was shown between the tilapia chemical composition among all treatments in terms of moisture and ash. Prebiotic treatment resulted in significantly higher protein contents (52.23 ± 2.766) compared to the lowest initial protein content (21.55 ± 0.195). Prebiotic treatment (18.98 ± 1.518) recorded the lowest content of lipids. While the initial group had the highest content of lipids (37.03 ± 0.041). The findings of our study revealed that improved fish carcass composition occurred through chitosan addition treatment, which increased protein while decreasing fat levels. The current results align with previous studies **(Zaki *et al*., 2015; Abd El-Naby *et al*., 2019; Ahmed *et al*., 2020; Salam *et al*., 2021)**.

5. Immunological and antioxidant enzymes parameters

5.1. Serum immune responses

Probiotic, prebiotic, and synbiotic effects on serum immune responses are presented in Table (5). The findings showed that the most elevated serum lysozyme values were obtained from probiotic group (3.36±0.158) which differs significantly from other treatments. Nitric oxide levels displayed a significant increase in probiotic group (8.70±0.188) compared to synbiotic (5.90±0.300) and prebiotic (4.75±0.268). However, the lowest No levels were recorded in the control group (4.11±0.271), without a significant difference from other treatments. In fish, innate immunity serves as the major defense against diseases caused by infections and toxins.

In the present investigation, serum lysozyme is significantly greater in probiotic-supplemented diet than in other treatments. These findings align with the results of **Adeoye *et al.* (2016)**, **Kord *et al*. (2021)**, **El-Kady *et al*. (2022)** and **El-Son *et al*. (2022)**. This might be attributed to increased phagocytes secreting lysozyme **(Saurabh & Sahoo,**

2008). The findings also indicated that synbiotic treatment showed no significant differences from control in lysozyme activity. In contrast, other studies documented that synbiotic treatment showed significant differences in lysozyme activity **(Lin *et al*., 2012; Sîrbu *et al*., 2022; Say *et al*., 2023)**. Current results further indicate that lysozyme activity was not influenced by dietary chitosan, similar to the results of **Esteban *et al*. (2001)**. This might be explained as a result of the administration of prebiotics and probiotics in feed must take into account amount administered, time of feeding, sources, and content since responses differ based on animal species, age, size, and physiological situation **(Panase *et al*., 2023)**.

The current findings revealed a significant major improvement in serum nitric oxide in a probiotic-supplemented diet. These findings agree with **Selim and Reda (2015)** and **Naiel *et al*. (2022)**. In contrast, **El-Son *et al*. (2022)** observed no significant differences between probiotic Sanolife PRO-F® supplemented group and control in NO levels. The prebiotic-supplemented diet had no significant variation in NO levels from the control group, which aligns with **El-Gawad *et al*. (2016)**.

5.2. Antioxidant enzymes in the liver

Probiotic, prebiotic, and synbiotic effects on liver antioxidant index are shown in Table (5). Superoxide dismutase levels was significantly enhanced in the probiotic treatment (11.80±0.314), followed by synbiotic, prebiotic, and then the control with (10.50±0.107, 9.83±0.089 and 9.49±0.413, respectively). Considering MDA levels, the control treatment demonstrated the highest results (14.36±0.583), which differed significantly from the remaining treatments. The probiotic group achieved significantly higher GPX levels (10.11±0.037) than the control group (5.86±0.297).

Table 5. Effect of probiotic, prebiotic, and synbiotic on serum immune responses and liver antioxidant activity of Nile tilapia, *O. niloticus* after 120 days.

Analysis from	Parameters	Con	Pro	Pre	Syn
Serum	Lysozyme (µg/ml)	2.51 ± 0.400^{b}	3.36 ± 0.158^{a}	2.70 ± 0.109^{b}	2.72 ± 0.110^{b}
	NO (µmol /L)	4.11 ± 0.271^{c}	8.70 ± 0.188^{a}	4.75 ± 0.268^{c}	5.90 ± 0.300^{b}
Liver	SOD (U/gm)	9.49 ± 0.413^{c}	11.80 ± 0.314^{a}	9.83 ± 0.089^{bc}	10.50 ± 0.107^{b}
	MDA (nmol/g)	14.36 ± 0.583^{a}	9.26 ± 0.130^{b}	10.10 ± 0.427^{b}	9.33 ± 0.109^{b}
	GPX (U/gm)	5.86 ± 0.297^{c}	10.11 ± 0.037^{a}	7.50 ± 0.320^{b}	7.43 ± 0.315^{b}

Data presented as means ± SE. Means within the same row expressing different superscript letters are significantly different (P<0.05)

The current investigation found that the antioxidant capacity of liver SOD enzymes were enhanced significantly when probiotics were provided. Similarly, **El-Son *et al*. (2022)**, documented that liver SOD enzymes showed significant enhancement in the probiotic treatments Sanolife® PRO-F supplemented with 0.5 and 1g kg^{-1} diet compared to the control. Similar findings were reported **(Midhun *et al*., 2019; Kord *et al*., 2021)** when probiotic were added. This might be due to *Bacillus* spp producing the soluble polysaccharide fraction in addition to metal-ion absorption and ROS-scavenging activities **(Hoseinifar *et al*., 2020)**. The findings also indicated that synbiotic-supplemented group displayed a significantly enhanced SOD activity compared to control group. These results agree with **Lin *et al*. (2012)**, who found that combination of *Bacillus coagulans* and chitosan oligosaccharides was higher in koi diets which included synbiotics. Our findings showed no significant difference between synbiotic and prebiotic treatment in SOD activity. Similarly, **Taherpour *et al*. (2023)** reported that feeding fish with 2% butyric acid and combined diets of 2% butyric acid and probiotic (*Bacillus licheniformis*) had no significant difference in SOD enzyme in liver among tested groups of the rainbow trout.

A diminished level of MDA was achieved in a probiotic-supplemented diet. The current results are similar to the findings found by **El-Son *et al*. (2022)**, who claimed that feeding a diet with Sanolife® PRO-F at a dose of 1g kg^{-1} significantly inhibited the production of MDA. Our findings coincide with the findings of **Weifen *et al*. (2012)** and **Midhun *et al*. (2019)**. In the same trend, the study of **Ghaly *et al*. (2023)** highlighted that synbiotic treatment exhibited a significant decrease in liver malondialdehyde (MDA). In the current study, probiotic-supplemented diet showed the highest GPX level as compared to control group. These current findings are in agreement with **Weifen *et al*. (2012)**, **Midhun *et al*. (2019)**, **Xue *et al*. (2020)** and **El-Son *et al*. (2022)**, where liver GPx was significantly improved with probiotic-supplemented diets.

6. Bacterial analysis and aerobic plate count (APC)

Bacillus spp. were identified and counted from fish intestine and pond water samples in the four treatments with replicate during 4-month feeding on experimental diets. APC results were presented as means ± SD. The APC of different samples (water and fish intestine) of probiotic treatments in different periods (1- 4 months) ranged from 4.51±0.85 to 9.05 ± 0.11 log CFU/g from the intestine and ranged from 2.31±0.21 to 5.01±0.30 log CFU/ml from water. The means of APC in fish samples from both intestine and water were significantly higher compared to other treatments. Probiotic treatment in this study had an increased intestinal bacterial count means which was followed by a descending order with prebiotics (4.06 ±0.34 - 8.23 ±0.13 log CFU/g) then synbiotic treatment (4.23 ±0.96 - 8.63 ±0.49 log CFU/g) compared to control (3.92 ±0.43 - 7.68 ±0.33 log CFU/g). Results of the current study indicated that probiotics increased the total bacterial count. These findings are in agreement with **Guimarães *et al*. (2019)**,

Sookchaiyaporn *et al*. (2020) and **Samson *et al*. (2022)**, which revealed that *Bacillus* spp. increased the total bacterial count.

Aquatic water APC feeding on experimental diets with probiotics ranged from 2.31 ±0.21 - 5.01 ±0.30 log CFU/ml from the first to the fourth month of the experiment. This range had an increment in bacterial population compared to control group which ranged from 1.88 ±0.13 - 3.82 ±0.86 Log CFU/ml of the same period. This might be due to the added probiotic increasing the water bacterial population and succeeding in flourishing in the aquatic water. Similarly, **Said *et al*. (2022)** mentioned that the addition of probiotics enhanced the total bacterial population in fish and water.

The APC in the gut of the tilapia supplied with chitosan as prebiotic at the end of the experiment was documented as 8.23 ±0.13log CFU/g, which was less than probiotic group and higher than the control at the same time. Therefore, prebiotics improved the bacterial population in the intestine compared to the control and were very close to the effect of probiotics. **Salam *et al.* (2021)** emphasized the effect of chitosan to improve beneficial bacteria growth by using different concentrations of dietary chitosan fed to *Barbonymus gonionotus* juveniles. The APC in water with prebiotic ranged from 2.91 ±0.19 to 4.33 ±0.39.

The APC in the the tilapia fish intestine of synbiotic group was recorded as 8.63 ±0.49 log CFU/g, which was very close to the bacterial count of prebiotic treatment (8.23 ±0.13 log CFU/g) when the experiment was finished. Synchronized effect of probiotics and prebiotics did not influence bacterial population as much as the probiotic alone. These results are not parallel with other research. **Cavalcante *et al.* (2020)** reported that *Lactobacillus* spp. were found in high numbers in the digestive tracts of fish in the probiotic, prebiotic, and synbiotic groups when using commercial probiotics. The symbiotic effect of probiotics with chitosan in aquatic water was recorded as 4.67 ± 0.32 log CFU/g.

Bacillus subtilis was identified in the intestines of fish from the probiotic and synbiotic treatments from the start of the experiment. *Bacillus pumilus* and *Bacillus licheniformis* were also detected in these groups by the final month. After 24 hours of incubation, colonies appeared on nutrient agar, and bacteria were identified based on their morphology, Gram staining, and biochemical assays (IMVIC). The isolated bacteria were Gram-positive, rod-shaped, and had a white or grayish-white appearance.
Aeromonas spp. grew as pale colonies on MacConkey agar and was identified biochemically as *Aeromonas hydrophila*. This bacterium was found only in the control group's water during the first month and confirmed with biochemical tests (IMVIC) and TSI. Enteric bacteria, such as *Escherichia coli* and *Salmonella*, were identified in the intestines of the control group by the fourth month and confirmed with biochemical tests and TSI. Similarly, **Sarkar *et al.* (2020)** documented the identification of some

Enterobacteriaceae in fish samples. Additionally, *Proteus penneri* was identified in the flesh of the control group using API tests in the fourth month.

Based on 16S rDNA gene sequencing, *Lysinibacillus sphaericus* (GenBank ACC# OR995193) was identified from the water of the probiotic treatment, and *Citrobacter freundii* (GenBank ACC# OR995194) was identified from the flesh of the control treatment. Studies have shown that *C. freundii* is a species known to cause infections in fish **(Junior *et al.*, 2018)**. Previous studies have indicated that *L. sphaericus* is frequently isolated from the gastrointestinal tract and recognized for its probiotic activity, suggesting it could be used as a feed supplement **(Yao *et al.*, 2024)**.

Our study found that probiotics and prebiotics effectively prevented pathogenic bacteria in fish flesh at harvest, thereby improving food safety. These results align with **Karssa *et al.* (2023)**, who reported the presence of *E. coli, Salmonella* spp., *Shigella* species, and *Proteus mirabilis* only before the application of probiotics. Regarding prebiotics, no pathogenic bacteria were identified in the water, intestines, or flesh of the tilapia, while they promoted the bacterial populations in the water and intestines.

CONCLUSION

The current study demonstrated that adding chitosan to tilapia diets at a concentration of 4 g/kg significantly enhances growth, water quality, and modulates intestinal microbiota, thereby promoting better gut health. Additionally, supplementing with a mixture of *Bacillus* strains at 2 g/kg boosts innate immune responses and increases antioxidant levels in the liver of *Oreochromis niloticus*, helping to manage oxidative stress and increase beneficial bacteria. These findings highlight the effectiveness of dietary supplements like chitosan and *Bacillus* strains in improving the performance and health of the Nile tilapia, suggesting their potential for use in aquaculture nutrition.

REFERENCES

Abd El-Naby, F. S.; Naiel, M. A.; Al-Sagheer, A. A. and Negm, S. S. (2019). Dietary chitosan nanoparticles enhance the growth, production performance, and immunity in *Oreochromis niloticus*. Aquaculture, 501, 82-89.

Adeoye, A. A.; Yomla, R.; Jaramillo-Torres, A.; Rodiles, A.; Merrifield, D. L. and Davies, S. J. (2016). Combined effects of exogenous enzymes and probiotic on Nile tilapia (*Oreochromis niloticus*) growth, intestinal morphology, and microbiome. Aquaculture, 463, 61-70.

Adeshina, I.; Jenyo-Oni, A. and Emikpe, B. (2016). Use of eugenia cayrophyllata oil as anaesthetic in farm raised african catfish *clarias gariepinus* juveniles. Egypt. J. Exp. Biol.(Zool.), 12, 71-76.

Adorian, T. J; Jamali, H.; Farsani, H. G.; Darvishi, P.; Hasanpour, S.; Bagheri, T. and Roozbehfar, R. (2019) Effects of probiotic bacteria *Bacillus* on growth

performance, digestive enzyme activity, and hematological parameters of Asian sea bass, *Lates calcarifer (Bloch).* Probiotics and antimicrobial proteins, 11, 248-255.

Ahmed, K. M.; Ibrahim, M. A. and Mounes, H. A. M. (2020). Effect of using low molecular weight Chitosan on water quality, quality indices and stress reduction of Nile tilapia. Egyptian Journal for Aquaculture, 10(2), 47-65.

AOAC. (1990). Association of official analytical chemists. Official methods of analysis.

AOAC: Official Methods of Analysis, 1: 69-90

Aranaz, I.; Alcántara, A. R.; Civera, M. C.; Arias, C.; Elorza, B.; Heras Caballero, A. and Acosta, N. (2021). Chitosan: An overview of its properties and applications. Polymers, 13(19), 3256.

Ayisi, C. L.; Zhao, J. and Rupia, E. J. (2017). Growth performance, feed utilization, body and fatty acid composition of Nile tilapia (*Oreochromis niloticus*) fed diets containing elevated levels of palm oil. Aquaculture and Fisheries, 2 (2): 67-77.

Azwai, S. M.; Alfallani, E. A.; Abolghait, S. K.; Garbaj, A. M.; Naas, H. T.; Moawad, A. A.; Gammoudi, F. T.; Rayes, H. M.; Barbieri, I. and Eldaghayes, I. M. (2016). Isolation and molecular identification of Vibrios by sequencing of 16S rDNA from seafood, meat, and meat products in Libya. Open veterinary journal, 6: 36-43.

Boukharouba, A. (2022). Isolation and identification of foodborne pathogens of special interest in food safety (Doctoral dissertation, Universitat Politècnica de València).

Boyd, C. E. and Tucker, C. S. (1998). Pond aquaculture water quality management (p. 700). Boston, MA: Kluwer Academic Publishers.

Cavalcante, R. B.; Telli, G. S.; Tachibana, L.; de Carla Dias, D.; Oshiro, E.; Natori, M. M. and Ranzani-Paiva, M. J. (2020). Probiotics, Prebiotics and Synbiotics for Nile tilapia: Growth performance and protection against *Aeromonas hydrophila* infection. Aquaculture Reports, 17, 100343.

Cerezuela, R.; Fumanal, M.; Tapia-Paniagua, S. T.; Meseguer, J.; Moriñigo, M. Á. and Esteban, M. Á. (2013). Changes in intestinal morphology and microbiota caused by dietary administration of inulin and *Bacillus subtilis* in gilthead sea bream (*Sparus aurata L.*) specimens. Fish and shellfish immunology, 34(5), 1063-1070.

Cerezuela, R.; Meseguer, J. and Esteban, M. A. (2011). Current knowledge in synbiotic use for fish aquaculture: a review. Journal of Aquaculture Research and Development S, 1, 1-7.

Chen, Y.; Zhu, X.; Yang, Y.; Han, D.; Jin, J. and Xie, S. (2014). Effect of dietary chitosan on growth performance, haematology, immune response, intestine morphology,

intestine microbiota and disease resistance in gibel carp (*C arassius auratus gibelio*). Aquaculture nutrition, 20(5), 532-546.

Cheng, C.; Park, S. C. and Giri, S. S. (2022). Effect of Pandanus tectorius extract as food additive on oxidative stress, immune status, and disease resistance in Cyprinus carpio. Fish and shellfish Immunology, 120:287–294.

de Araújo, E. R. L.; Barbas, L. A. L.; Ishikawa, C. M.; de Carla Dias, D.; Sussel, F. R.; de Almeida Marques, H. L. and Tachibana, L. (2018). Prebiotic, probiotic, and synbiotic in the diet of Nile tilapia post-larvae during the sex reversal phase. Aquaculture international, 26, 85-97.

del Valle, J. C.; Bonadero, M. C. and Fernández-Gimenez, A. V. (2023). *Saccharomyces cerevisiae* as probiotic, prebiotic, synbiotic, postbiotics and parabiotics in aquaculture: An overview. Aquaculture, 569, 739342.

El-Gawad, E. A. A.; El-Latif, A. M. A. and Shourbela, R. M. (2016). Enhancement of antioxidant activity, non-specific immunity, and growth performance of Nile tilapia, *Oreochromis niloticus* by dietary fructooligosaccharide.

El-Kady, A. A.; Magouz, F. I.; Mahmoud, S. A. and Abdel-Rahim, M. M. (2022). 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, 737249.

El-Naggar, M.M.; El-Shabaka, H. A.; Abd El-Rahman, F. A. A.; Abou- Elmagd, W.S.I.; Khalil, M. T. and Suloma, A. (2022). Effects of dietary supplementation of chitosan and chitosan nanoparticles on growth performance, liver histology and fatty acids profile of the Nile tilapia (*Oreochromis niloticus*) fed fish meal-free diets. Egypt. J. Aquat. Biol. Fish., 26(4): 427-448. Doi: https://dx.doi.org/10.21608/ejabf.2022.251297

Elsabagh, M.; Mohamed, R.; Moustafa, E. M.; Hamza, A.; Farrag, F.; Decamp, O. and Eltholth, M. (2018). Assessing the impact of *Bacillus* strains mixture probiotic on water quality, growth performance, blood profile, and intestinal morphology of Nile tilapia, *Oreochromis niloticus*. Aquaculture nutrition, 24(6), 1613-1622.

El-Sayed, H. S. and Barakat, K. M. (2016). Effect of dietary chitosan on challenged Dicentrarchus labrax post larvae with *Aeromonas hydrophila*. Russian Journal of Marine Biology, 42, 501-508.

El-Son, M. A.; Elshopakey, G. E.; Rezk, S.; Eldessouki, E. A. and Elbahnaswy, S. (2022). Dietary mixed *Bacillus* strains promoted the growth indices, enzymatic profile, intestinal immunity, and liver and intestinal histomorphology of Nile tilapia, *Oreochromis niloticus*. Aquaculture Reports, 27, 101385.

Elvy, J. E.; Symonds, J. E.; Hilton, Z.; Walker, S. P.; Tremblay, L. A.; Casanovas, P. and Herbert, N. A. (2022). The relationship of feed intake, growth, nutrient retention, and oxygen consumption to feed conversion ratio of farmed saltwater Chinook salmon (*Oncorhynchus tshawytscha*). Aquaculture, 554, 738184.

Esteban, M. A.; Cuesta, A.; Ortuno, J. and Meseguer, J. (2001). Immunomodulatory effects of dietary intake of chitin on gilthead seabream (*Sparus aurata L.*) innate immune system. Fish & shellfish immunology, 11(4), 303-315.

FDA, Bacteriological analytical manual. Andrews, W. H. and Jacobson, A. (2013). BAM Chapter 6: *Shigella*.

FDA, Food and Drug Administration (2001). Bacteriological analytical manual, edition 8, chapter 3: aerobic plate count.

Geng, X.; Dong, X. H.; Tan, B. P.; Yang, Q. H.; Chi, S. Y.; Liu, H. Y. and Liu, X. Q. (2011). Effects of dietary chitosan and *Bacillus subtilis* on the growth performance, non-specific immunity and disease resistance of cobia, *Rachycentron canadum*. Fish and shellfish immunology, 31(3), 400-406.

Ghaly, F. M.; Hussein, S. H.; Awad, S. M. and El-Makhzangy, A. A. (2023). Growth promoter, immune response, and histopathological change of prebiotic, probiotic, and synbiotic bacteria on Nile tilapia. Saudi Journal of Biological Sciences, 30(2), 103539.

Goda, A. M.; El-Haroun, E. R. and Kabir Chowdhury, M. A. (2007). Effect of totally or partially replacing fish meal by alternative protein sources on growth of African catfish *Clarias gariepinus* (Burchell, 1822) reared in concrete tanks. Aquaculture Research, 38(3), 279-287.

Guimarães, M. C.; Dias, D. C. and de Araujo, F. V. A. (2019). Probiotic *Bacillus subtilis* and *Lactobacillus plantarum* in diet of Nile tilapia. Bol do Inst Pesca 45.

Hoseinifar, S. H.; Yousefi, S.; Van Doan, H.; Ashouri, G.; Gioacchini, G.; Maradonna, F. and Carnevali, O. (2020). Oxidative stress and antioxidant defense in fish: the implications of probiotic, prebiotic, and synbiotics. Reviews in Fisheries Science & Aquaculture, 29(2), 198-217.

Hossam-Elden, N.; Abu-Elala, N.; Ali, S. E.; Khattab, M. S. and Marzouk, M. S. (2024). Dietary Immune Nutritive Effect of Chitosan/Chitosan Nanoparticles on the Nile Tilapia: Short-term Exposure. Egyptian Journal of Aquatic Biology & Fisheries, 28(1).

Huang, C.; Yang, X.; Huang, J.; Liu, X.; Yang, X.; Jin, H.; Huang, Q.; Li, L. and Zhou, R. (2019). Porcine Beta-Defensin 2 Provides Protection Against Bacterial Infection by a Direct Bactericidal Activity and Alleviates Inflammation via Interference with the TLR4/NF-κB Pathway. Frontiers in Immunology, 10(JULY).

Huynh, T. G.; Shiu, Y. L.; Nguyen, T. P.; Truong, Q. P.; Chen, J. C. and Liu, C. H. (2017). Current applications, selection, and possible mechanisms of actions of synbiotics in improving the growth and health status in aquaculture: a review. Fish and shellfish immunology, 64, 367-382.

Junior, G. B.; Dos Santos, A. C.; de Freitas Souza, C.; Baldissera, M. D.; dos Santos Moreira, K. L.; da Veiga, M. L. and Baldisserotto, B. (2018). *Citrobacter freundii* infection in silver catfish (*Rhamdia quelen*): hematological and histological alterations. Microbial pathogenesis, 125, 276-280.

Karssa, T.; Sodeno, M.; Workagen, K.; Pavanasam, N. and Gada, T. (2023). Application of Probiotics in Aquaculture to Improve Growth and Health Performances of Nile tilapia.

Khanjani, M. H.; da Silva, L. O. B.; Fóes, G. K.; do Nascimento Vieira, F.; Poli, M. A.; Santos, M. and Emerenciano, M. G. C. (2023). Synbiotics and aquamimicry as alternative microbial-based approaches in intensive shrimp farming and biofloc: Novel disruptive techniques or complementary management tools? A scientific-based overview. *Aquaculture*, *567*, 739273.

Kord, M. I.; Srour, T. M.; Omar, E. A.; Farag, A. A.; Nour, A. A. M. and Khalil, H. S. (2021). The immunostimulatory effects of commercial feed additives on growth performance, non-specific immune response, antioxidants assay, and intestinal morphometry of Nile tilapia, *Oreochromis niloticus*. Frontiers in Physiology, 12, 627499.

Kou, S. G.; Peters, L. M. and Mucalo, M. R. (2021). Chitosan: A review of sources and preparation methods. International Journal of Biological Macromolecules, 169, 85-94.

Kuebutornye, F. K. A.; Abarike, E. D. and Lu, Y. (2019). A review on the application of *Bacillus* as probiotics in aquaculture. Fish Shellfish Immunology., 87: 820-828.

Kumar, S.; Stecher, G. and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular biology and evolution, 33(7), 1870-1874.

Kyule-Muendo, D.; Otachi, E.; Awour, F.; Ogello, E.; Obiero, K.; Abwao, J.; Muthoni, C. and Munguti, J. (2022) Status of fish health management and biosecurity measures in fish farms, cages and hatcheries in Western Kenya. CABI Agric Biosci 3:18–27.

Lin, S.; Mao, S.; Guan, Y.; Luo, L.; Luo, L. and Pan, Y. (2012). Effects of dietary chitosan oligosaccharides and *Bacillus coagulans* on the growth, innate immunity, and resistance of koi (*Cyprinus carpio koi*). Aquaculture, 342, 36-41.

Meiyarasi, R.; Mohanapriya, T.; Monika, B.; Shravanthika, M.; Nithyapriya, S.; Johney, J. and Ragunathan, R. (2017). Isolation and PCR Amplification of *E. coli* from Freshwater Fish (*Cirrhinus cirrhosis*) and its PCR Amplification of SHV Gene. International Journal of Current Microbiology and Applied Sciences, 6(4), 2467-2476.

Midhun, S. J.; Neethu, S.; Arun, D.; Vysakh, A.; Divya, L.; Radhakrishnan, E. K. and Jyothis, M. (2019). Dietary supplementation of *Bacillus licheniformis* HGA8B improves growth parameters, enzymatic profile, and gene expression of *Oreochromis niloticus*. Aquaculture, 505, 289-296.

Montgomery, H. A. and Dymock, J. F. (1961). The determination of nitrite in water. Analyst, 86, 414- 416.

Naiel, M. A.; Abdelghany, M. F.; Khames, D. K.; Abd El-hameed, S. A.; Mansour, E. M.; El-Nadi, A. S. and Shoukry, A. A. (2022). Administration of some probiotic strains in the rearing water enhances the water quality, performance, body chemical analysis, antioxidant and immune responses of Nile tilapia, *Oreochromis niloticus*. Applied Water Science, 12(9), 209.

Nishikimi, M.; Rao, N. A. and Yagi, K. (1972). The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochemical and biophysical research communications, 46(2), 849-854.

Paglia, D. E. and Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. The Journal of laboratory and clinical medicine, 70(1), 158-169.

Panase, A.; Thirabunyanon, M.; Promya, J. and Chitmanat, C. (2023). Influences of *Bacillus subtilis* and fructooligosaccharide on growth performances, immune responses, and disease resistance of Nile tilapia, *Oreochromis niloticus*. Frontiers in Veterinary Science, 9, 1094681.

Politis, S. N.; Benini, E.; Miest, J. J.; Engrola, S.; Sørensen, S. R.; Syropoulou, E. and Tomkiewicz, J. (2023). First assessment of prebiotics, probiotics, and synbiotics affecting survival, growth, and gene expression of European eel (*Anguilla anguilla*) larvae. Aquaculture Research, 2023(1), 1260967.

Qin, C.; Zhang, Y.; Liu, W.; Xu, L.; Yang, Y. and Zhou, Z. (2014). Effects of chito-oligosaccharides supplementation on growth performance, intestinal cytokine expression, autochthonous gut bacteria, and disease resistance in hybrid tilapia *Oreochromis niloticus*♀× *Oreochromis aureus*♂. Fish and shellfish immunology, 40(1), 267-274.

Ringø, E. and Song, S. K. (2016). Application of dietary supplements (synbiotics and probiotics in combination with plant products and β-glucans) in aquaculture. Aquaculture Nutrition, 22(1), 4-24.

Said, M. M.; Zaki, F. M. and Ahmed, O. M. (2022). Effect of the Probiotic (*Bacillus spp*.) on Water Quality, Production Performance, Microbial Profile, and Food Safety of the Nile Tilapia and Mint in Recirculating Aquaponic System. Egyptian Journal of Aquatic Biology and Fisheries, 26(6).

Salam, M. A.; Rahman, M. A.; Paul, S. I.; Islam, F.; Barman, A. K.; Rahman, Z. and Islam, T. (2021). Dietary chitosan promotes the growth, biochemical composition, gut microbiota, hematological parameters, and internal organ morphology of juvenile *Barbonymus gonionotus*. PLoS One, 16(11), e0260192.

Samson, J. S.; Choresca Jr, C. H. and Quiazon, K. M. A. (2022). Probiotic effect of *Bacillus spp*. isolated from African nightcrawler (*Eudrilus eugeniae*) on the performance of Nile Tilapia (*Oreochromis niloticus L*.). Archives of Microbiology, 204(4), 235.

Sarkar, S.; Dey, S. K.; Nipu, M. A. I.; Brishti, P. S. and Billah, M. B. (2020). Microbiological assessment of Nile tilapia *Oreochromis niloticus* collected from different super shops and local market in Dhaka. Bangladesh. Journal of Fisheries, 8(2), 784-791.

Sathish, V.; Sundaresan, A. and Deivasigamani, B. (2021). Immunomodulatory effects of dietary intake of chitosan on non-specific immune response of Mugil cephalus challenged with *Aeromonas hydrophila*. Journal of Advanced Scientific Research, 12(02), 33-39.

Satoh, K. (1978) Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. Clinica Chimica Acta 90: 37–43.

Saurabh, S. and Sahoo, P. K. (2008). Lysozyme: an important defence molecule of fish innate immune system. Aquaculture research, 39(3), 223-239.

Say, P.; Nimitkul, S.; Bunnoy, A.; Na-Nakorn, U. and Srisapoome, P. (2023). Effects of the combination of chitosan and *Acinetobacter* KU011TH on the growth and health performances and disease resistance of juvenile hybrid catfish (*Clarias gariepinus*× *C. macrocephalus*). Fish and Shellfish Immunology, 142, 109177.

Selim, K. M. and Reda, R. M. (2015). Improvement of immunity and disease resistance in the Nile tilapia, *Oreochromis niloticus*, by dietary supplementation with *Bacillus amyloliquefaciens*. Fish and shellfish immunology, 44(2), 496-503.

Sîrbu, E.; Dima, M. F.; Tenciu, M.; Cretu, M.; Coadă, M. T.; Țoțoiu, A. and Patriche, N. (2022). Effects of dietary supplementation with probiotics and prebiotics on growth, physiological condition, and resistance to pathogens challenge in Nile Tilapia (*Oreochromis niloticus*). Fishes, 7(5), 273.

Soltani, M.; Ghosh, K.; Hoseinifar, S. H.; Kumar, V.; Lymbery, A. J.; Roy, S. and Ringø, E. (2019). Genus *Bacillus*, promising probiotics in aquaculture: aquatic animal origin, bio-active components, bioremediation and efficacy in fish and shellfish. Reviews in Fisheries Science and Aquaculture, 27(3), 331-379.

Sookchaiyaporn, N.; Srisapoome, P.; Unajak, S. and Areechon, N. (2020). Efficacy of *Bacillus spp*. isolated from Nile tilapia *Oreochromis niloticus* Linn. on its growth and immunity, and control of pathogenic bacteria. Fisheries science, 86, 353-365.

Sugiartha, I. E.; Semedi, B. P.; Wardhani, P. and Rejeki, I. P. S. (2017). Comparison results of analytical profile index and disc diffusion antimicrobial susceptibility test to Technical Dedicated Reasonable 300B method. Indonesian Journal of Clinical Pathology and Medical Laboratory, 23(2): 131-137.

Taherpour, M.; Roomiani, L.; Islami, H. R. and Mehrgan, M. S. (2023). Effect of dietary butyric acid, *Bacillus licheniformis*(probiotic), and their combination on hemato-biochemical indices, antioxidant enzymes, immunological parameters, and growth performance of Rainbow trout (*Oncorhynchus mykiss*). Aquaculture Reports, 30, 101534.

Tolouei Gilani, J.; Goudarztalejerdi, A.; Yavari, M. and Nouri Kalourazi, M. (2021). Isolation and identification of *Aeromonas hydrophila* from Cyprinidae suspected with hemorrhagic septicemia in pools of warm water fishes in Gilan Province. International Journal of Nutrition Sciences, 6(1), 52-58.

Weifen, L.; Xiaoping, Z.; Wenhui, S.; Bin, D.; Quan, L.; Luoqin, F. and Dongyou, Y. (2012). Effects of *Bacillus* preparations on immunity and antioxidant activities in grass carp (*Ctenopharyngodon idellus*). Fish physiology and biochemistry, 38, 1585-1592.

Xue, J.; Shen, K.; Hu, Y.; Hu, Y.; Kumar, V.; Yang, G. and Wen, C. (2020). Effects of dietary *Bacillus cereus*, *B. subtilis*, *Paracoccus marcusii*, and *Lactobacillus plantarum* supplementation on the growth, immune response, antioxidant capacity, and intestinal health of juvenile grass carp (*Ctenopharyngodon idellus*). Aquaculture Reports, 17, 100387.

Yao, Y.; Wang, X.; Lin, X.; Wu, J.; Wang, P.; Zhu, C. and Yan, Q. (2024) Isolation and characterization of probiotic *Lysinibacillus* species from the gastrointestinal tract of large yellow croaker (*Larimichthys crocea*).

Zaki, M. A.; Salem, M.; Gaber, M. and Nour, A. M. (2015). Effect of Chitosan Supplemented Diet on Survival, Growth, Feed Utilization, Body Composition and Histology of Sea Bass (*Dicentrarchus labrax*). World Journal of Engineering and Technology 03(04):38-47.