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RESEARCH

Dietary multi-strains *Bacillus* spp. enhanced growth performance, blood metabolites, digestive tissues histology, gene expression of *Oreochromis niloticus*, and resistance to *Aspergillus flavus* infection

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Abstract
The present study tested the symbiotic effects of dietary multi-strain *Bacillus* probiotics (MSB) (*Bacillus licheniformis*, *B. pumilus*, *and B. subtilis*) in Nile tilapia (*Oreochromis niloticus*) exposed to *Aspergillus flavus* infection. Furthermore, this study investigated water quality, growth performance, blood metabolites, histological morphology, immune regulatory genes, and resistance to *A. flavus* infection. For 70 days, fish ($n=240$) were divided into four groups in triplicate: T0 (control group; MSB0), T1 (1 g/kg, MSB1), T2 (2 g/kg, MSB2), and T3 (3 g/kg, MSB3). The immune response was then assessed by challenging all fish groups with the *A. flavus* pathogen. The results showed that the rearing water quality, fish growth, and blood parameters, as well as total proteins, albumin, globulins, and amylase activity were significantly ($P<0.05$) increased in all MSB-treated groups with the best results in MSB2 and MSB3 groups. Meanwhile, the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol, and glucose levels were significantly ($P<0.05$) modulated, particularly at higher concentrations of the probiotic mixture (MSB3 group). Fish fed with various levels of MSB showed a maintained histological structure of the hepatopancreas, intestine, and spleen tissues. The mRNA expression of growth hormone (*GH*), insulin-like growth factor-1 (*IGF-1*), insulin-like growth factor receptor-1 (*IGF-1R*), and interleukin-8 (*IL-8*) were increased in a dose-dependent manner due to MSB dietary inclusion ($P<0.05$). Conversely, the mRNA expression of interleukin-1β (*IL-1β*) gene was significantly decreased in MSB groups compared to untreated group ($P<0.05$). Surprisingly, supplemented groups in *Bacillus* spp. probiotics exhibited significant modulations in all computed parameters. MSB supplementation improved the pathogenic tolerance of tilapia after change with *A. flavus*. The integration of growth performance, biochemical, and transcriptomic results confirms that the dietary intervention of multi-strain *Bacillus* spp. is symbiotic and enhances the benefits for the maintenance of *O. niloticus'* health, growth, and digestion. This is achieved by supporting growth genes, reducing inflammatory genes, and enhancing immune-antioxidant resistance to combat *A. flavus* infection.

Keywords *Bacillus* spp. · Nile tilapia · Pathogen · Growth · Immunology · Genes

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Introduction

Nile tilapia is one of the most widely cultivated species in aquaculture, and the industry is expanding rapidly around the world (Magbanua and Ragaza 2022). With increased fish production, many diseases emerge and cause mortality (Mahboub et al. 2022a, b; Eissa et al. 2024), particularly bacterial diseases (Alzahrani et al. 2022). Recent studies have attempted to use natural antimicrobial products (Ashour et al. 2020; Abdelnour et al. 2020; Mahboub et al. 2022a, b; Rahman et al. 2022; Abd El-Hack et al. 2022). Among these products, probiotics are safe, eco-friendly, live microorganisms that, when supplied in sufficient amounts, improve the health status of the fish (Eissa et al. 2022a, b) by elevating favorable bacteria, augmenting metabolism, and strengthening the immune system against diseases (Ringø et al. 2022). There are numerous benefits to using probiotics in the fish industry, including disrupting pathogens' stroke by releasing inhibitory materials, improving enzymatic activity, feed utilization and digestibility, and performance (Dawood 2021; Mamun et al. 2019; Monier et al. 2023; Moaheda et al. 2023). Probiotics play an important role in aquaculture as immunostimulants, maintaining gastrointestinal health, stabilizing pathogenic strains, and reducing toxicity (Ahmed et al. 2022; El-Bouhy et al. 2021; Puri et al. 2022; Munir et al., 2016a, b; 2018a, b).

Bacillus spp. is a proven probiotic that has a promising effect on the growth, hematological, and defense mechanisms, intestinal microbiota, biomass gain, and intestinal absorption in several fish species (Aftabgard et al. 2019; Azevedo et al., 2016; Chelladurai et al. 2023; do Veiga et al. 2020). This bacterium could also be the most effective substitute as dietary probiotic for disease control and prevention in animals. *Bacillus subtilis, B. licheniformis, B. pumilus*, and *B. amyloliquefaciens* are among the most commonly used *Bacillus* species as probiotics in aquaculture (Yi et al. 2018). Bacillus supplementation has been associated with increased resistance to *V. parahaemolyticus, Edwardsiella tarda* (Santos et al. 2021), *Aeromona salmonicida, Lactococcus garvieae, Streptococcus iniae* (Cha et al. 2013), A. *hydrophila* (Ramesh and Souissi 2018), and *Acinetobacter* spp. (Kavitha et al., 2018). Furthermore, improved disease resistance through dietary *Bacillus* administration has been reported in various aquatic species, such as rainbow trout (Newaj-Fyzul et al. 2007), tilapia (Aly et al. 2008), and white prawns (Tseng et al. 2009). Furthermore, *Bacillus subtilis* spores work well as an oral vaccination against *Streptococcus agalactiae* infection in *O. niloticus* (Yao et al. 2019). As a result, the current study investigated the efficacy of dietary multistrain *Bacillus* spp. bacteria as antimicrobials against *A. flavus* infection in *O. niloticus*. Thus, the growth pattern, digestive enzyme activity, haemato-biochemical parameters, blood metabolites, tissue assessment, and gene expression were all investigated.

Materials and methods

Fish rearing and experimental design

Nile tilapia healthy fingerlings (20.3 ± 0.6 g) were obtained from a private fish farm in Ismailia, Egypt. The farm has a routing health checks in their stock. They were kept in a 3 m^3 fiberglass tank for 2 weeks to adjust to indoor laboratory conditions. Following the assimilation period, 240 healthy Nile tilapia (*O. niloticus*) were used in this experiment. The fish were divided into four groups (20 fish per tank). Each treatment consists of three tanks, each with 60 fish.

A pure lyophilized culture of *Bacillus* spp. probiotic strains, including *B. pumillis, B. licheniformis*, and *B. subtilis*, was obtained from the Microbiological Resource Centre at Ain Shams University in Cairo, Egypt. The colony forming unit (CFU) was determined using the dilution method described by Munir et al. (2016a) and was 10^8/gm. Multi-strain Bacillus probiotics (MSB) were made with equal amounts of each strain (a combination of *Bacillus licheniformis*, *B. pumillis*, and *B. subtilis*). Probiotics were added to the diets during manufacturing in accordance with the study's protocol. The fish in the tanks were divided into four groups for treatment. Experimental fish were fed basal diets containing varying levels of MSB: 0 (MSB0), 1 (MSB1), 2 (MSB2), and 3 (MSB3) g/kg. The MSB was well mixed with wheat bran and supplemented to the diets according to the protocol of this study. For 70 days, fish were fed experimental diets three times a day at 9:00, 12:00 and 15:00. with 4-h intervals until apparent satiety was achieved. The diet was offered based on body weight, with the fish being fed 6% of their body weight for the first month and 4% for the remainder of the experiment. Every day, the water in the tanks was changed by 25%, along with the fish feces, and replaced with fresh, well-aerated water from a storage tank. Fish were raised in natural light with no artificial lighting. Table 1 contains information on fish dietary ingredients and chemical analysis.

Water quality parameters

Every day at 15:00, water quality parameters including pH, temperature, dissolved oxygen, salinity, ammonia, and nitrite were measured. Temperature, dissolved oxygen, pH, and salinity of the water were measured using a SensoDirect150 MultiMeter (Lovibond, Tintometer Limited, Amesbury, UK). Total ammonia nitrogen (TAN) was measured using the HANNA HI 96715-11 Ammonia Medium Range photometer (HANNA, Nusfalau, Romania). Unionized ammonia was calculated using the pre-estimated TAN, temperature, and pH values (NH3). Nitrate was measured using the HANNA HI 708 (HANNA, Nusfalau, Romania).

Growth efficacy and survival percentage

Every 2 weeks, fish samples were collected at random to assess weight growth. Final body weight (FW), average daily gain (ADG), weight gain (WG), specific growth rate (SGR), and rate of survival were calculated using the following equations (Fath El-Bab et al. 2022):

Weight gain (WG; g/fish) = final body weight (FBW, g) − initial body weight (IBW, g)
ADG (g/fish /day) = (FBW- IBW) / duration (day)
Specific growth rate (%/day): SGR = 100 × [(ln FBW − ln IBW) / days].
Survival (%) = 100 × (final number of fish / initial number of fish).
Feed utilization: The fish feed utilization was computed depending on the equation illustrated by (Farliana et al. 2022; Wu et al. 2021).
Feed intake (g/fish): Quantity of feed offered or supplied throughout the experimental period /fish (g).
Feed conversion ratio (FCR) = feed intake (g) / weight gain (g)

Table 1 Composition and chemical analysis of experimental diets

Ingredients	g/kg diet
Fish meal (72% CP)	110
Soybean meal (45% CP)	360
Wheat bran	200
Yellow corn	60
Rice bran	200
Baclilus PRO-F	0.00
Fish oil	15
Soybean oil	15
Dicalcium phosphate	10
Vitamins mixture[1]	10
Minerals mixture[2]	10
Carboxymethyl cellulose	10
Total	1000
Proximate chemical analysis	**g/kg diet**
Dry matter	89.7
Crude protein	31.2
Ether extract	8.1
Ash	7.25
Crude fiber	6.62
Nitrogen-free extract[3]	46.6

[1]Vitamin premix (per kg of premix): Vitamin B1, 700 mg; Vitamin B2, 3500 mg; Vitamin B6, 1000 mg; Vitamin B12, 7 mg; Vitamin A,8,000,000 IU; Vitamin D3, 2,000,000 IU; Vitamin E, 7000 mg; Vitamin K3,1500 mg; biotin, 50 mg; folic acid, 700 mg; nicotinic, 20,000 mg; pantothenic acid,7000 mg

[2]Mineral premix (per kg of premix): Calcium carbonate as carrier up to 1 kg for zinc, 40 g; iron, 20 g; copper, 2.7 g; iodine, 0.34 g; manganese,53 g; selenium, 70 mg and cobalt, 70 mg

[3] NFE = 100- (CP%+ EE%+ CF%+ Ash%)]

Analytical protocols for fish and feed

Feed samples were analyzed at the beginning of experiment, and fish samples were collected at the end of each study to evaluate the proximate analyses of commercial diets and fish bodies, such as protein, moisture, lipid, and ash components. On stocking day, five fish ($n = 5$) were selected at random for chemical analysis. The crude protein, moisture, and crude fat compositions of entire fish bodies were calculated on a dry matter basis using (AOAC 1997).

Blood sampling

Blood was extracted from the caudal vertebral vein of six fish from each group (Clark et al. 2011). Whole blood was drawn into sterile tubes and mixed with an anticoagulant before being analyzed. The hemocytometer was used to count leukocytes and erythrocytes and

measure hemoglobin concentrations using the cyanomet hemoglobin protocol, Drabkin's solution, and Natt-Herrik solution (Stoskopf 1993). The packed cell volume (PCV) and differential leukocyte count values were calculated in accordance with Lamas et al. (1991). This formula was used to calculate the total differential leukocytic count (DLC), according to Thrall (2004).

$$\text{Absolute DLC} = \text{No. of each white cell} \times \text{no. of total leukocytic count}/100$$

Blood samples were drained into sterile Eppendorf tubes without anticoagulant, then centrifuged at 1500 × g/15 min to collect clear serum for biochemical analysis. To calculate the content of globulins in the blood, albumin and total proteins were estimated at 540 and 550 nm, respectively. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were colorimetrically measured at 540 nm (Reitman and Frankel 1957). The CHOD-PAP (commercial clinical kit) protocol was used to measure cholesterol (CHL) levels. Glucose concentration (mg/100 ml) was determined using glucose enzymatic PAP (Trinder 1969) kits (Bio-Merieux, France). The amylase activity was calculated three times.

Challenge assays with *Aspergillus flavus*

The pathogen, *Aspergillus flavus* strain was previously isolated from *O. niloticus* and obtained from the Microbiological Unit of the Fish Diseases Department, Animal Health Institute, Dokki, Giza, Egypt. Sabourauds dextrose agar (SDA, Difco) with streptomycin (100 ug/ml) and penicillin (100 UI/ml) was used to culture *A. flavus* for spore suspension, which was then incubated at 25 °C for a week (Eissa et al. 2023b). To collect the mass of conidia, each plate was filled with 20 mL of sterile distilled water, and the suspension was harvested in sterile tubes. The suspension was filtered through two layers of sterilized gauze.

The erythrocyte counting chamber of the hemocytometer was used to calculate and adjust the conidial suspension to 4×10^3 conidia/ml in sterile distilled water. After that, 20 fish from each group were injected with a sterilized needle carrying nearly 0.2 ml of *A. flavus* (4×10^3 conidia/ml) and monitored daily for the next 15 days. Injected fish were given a commercial diet. At the end of the experimental challenge, the cumulative mortality percentage was calculated.

The histological examination

At the end of the study, from the four experimental groups, 3 samples of hepatopancreas, intestine, and spleen were removed, cleaned in saline solution, and then fixed in 10% neutral formalin for 48 h. Next, the tissues were passed through increasing grades of ethyl alcohol and xylene, and finally paraplast blocks were created and cut using a rotating microtome. The processed tissues were then stained with hematoxylin and eosin stains, and finally photographs of the various groups' tissues were taken. In accordance with the protocols described by Eissa et al. (2023a, b, c; Jastaniah et al. (2023), the Nile tilapia's intestinal villi length and width are measured in each experimental group, the means ± standard errors are computed, and a chart graph is created to show the variations between the groups fed MSB and their resistance to *A. flavus* infection.

The total RNA extraction and real-time quantitative PCR (RTqPCR)

Using the RNA purification kit (Thermo Fisher Scientific, USA), total RNA was extracted from 50 mg of each experimental group's hepatic tissues in accordance with the manufacturer's instructions. The O.D. 260 nm/O.D. 280 nm ratio was measured using a nanodrop lite spectrophotometer (Thermo Scientific, USA) to assess the purity of the extracted RNA. Complementary DNAs (cDNAs) were generated from 1 µg of RNA using oligo-dT primers and the SuperScript TM III First-Strand Synthesis System (Invitrogen, USA) in accordance with manufacturer instructions. Following that, the cDNA samples were stored at −20 °C until needed again. The primers used in the current study are listed in Table 2.

In order to quantify the folds of gene transcription, qPCR (SensiFast SYBR Lo-Rox kit, Bioline, London, UK) was used to determine the mRNA expressions of genes associated with growth (growth hormone (*GH*), insulin-like growth factor-1 (*IGF-1*), insulin-like growth factor receptor-1 (*IGF-1R*), inflammatory genes including interleukin one beta (*IL-1β*), and interleukin 8 (*IL-8*). Following were the conditions of the reaction's heat cycle that were observed: 10 min at 95 °C, 40 brief cycles at 95 °C, 30 min at 60 °C, and finally 5 min at 85 °C. The $2-^{\Delta\Delta CT}$ protocol was used to standardize the transcription levels to the *β-actin* gene (Livak and Schmittgen 2001).

Table 2 The genes name, primer sequence, product size of some genes used in this experiment

Gene	Primer sequence	Product size bp	Slope	Efficiency	Tm°C	Accession number
GH	F: ACATCATCAGCCCGA TCGAC R: TCAGCAGCAAGATTC CCGTT	183	-3.33	99.66	F:53.8 R:51.8	XM_003442542.5
IGF-1	F: GGACGAGTGCTGCTT CCAAAGC R: TGCTCTTGGCATGTC TGTGTGC	121	-3.35	99.25	F:58.6 R:56.7	XM_019346352.2
IGF1-R	F: GCGACCCAAAGAGCA ACAGTGG R: TGCCAGATCTCGGTG GACAAAC	130	-3.34	98.84	F:58.6 R: 56.7	XM_013276852.3
IL-8	F: CTGTGAAGGCATGGG TGTGGAG R: TCGCAGTGGGAGTTG GGAAGAA	111	-3.37	98.03	F:58.6 R:56.7	NM_001279704.1
Il-1β	F: CAAGGATGACGACAA GCCAACC R: AGCGGACAGACATGA GAGTGC	149	-3.31	100.50	F:56.7 R:56.3	XM_019365844.2
B-actin	F: CAGCAAGCAGGAGTA CGATGAG R: TGTGTGGTGTGTGGT TGTTTTG	136	-3.35	98.84	F:56.7 R:53	XM_003443127.5

Growth hormone (*GH*), insulin-like growth factor (*IGF-1*), insulin-like growth factor receptor-1 (*IGF-1R*), interleukin-8 (*IL-8*), and interleukin-1β (*IL-1β*)

Data analysis

Bartlett and Kolmogorov-Smirnov tests were used to verify that the results were homogeneous and normal. Then, using the SPSS software (Version 21.0, IBM Corp., Armonk, NY, USA), the data was analyzed using a one-way ANOVA. To find the significant mean differences at a probability level of $P < 0.05$, Tucky's B test was employed. We present the data as means ± standard error.

Results

Effects on water quality

Table 3 illustrates how dietary inclusion of multi-strain Bacillus (MSB) probiotics affected the quality of the water. All groups that added MSB showed no significant effect ($P > 0.05$) in both salinity temperature compared to control. When compared to other groups, the pH was slightly lower in the MSB2 and MSB1 groups. In comparison to the other groups, the MSB1 group had the lowest dissolved oxygen (DO) values ($P < 0.05$). The inclusion of dietary MSB did not significantly affect the values of NH4, NO2, or NO3 ($P > 0.05$).

Effects of MSB on growth and feed utilization

Supplementing with dietary probiotics (Table 4) significantly increased fish final weight, weight gain, SGR, feed intake, ADG, survival, fish biomass, and fish final number ($P < 0.05$). The fish fed with MSB3 had the highest values for all growth performance and feed utilization ($P < 0.05$). Fish final number and biomass per 1 m^3 were higher in the MSB2 and MSB3 groups than in the other groups ($P < 0.05$). Regarding survival, feed intake, and final body weight, all MSB-treated groups showed comparable results ($P > 0.05$), with a notable difference compared to the untreated group ($P < 0.05$).

Table 3 Effect of various levels of multi-strains *Bacillus* probiotics (MSB) on water quality after 70 days of treatment

Item	Experimental groups[1]			
	MSB0	MSB1	MSB2	MSB3
Temperature (C^o)	26.58 ± 0.06^a	26.56 ± 0.04^a	26.53 ± 0.03^a	26.62 ± 0.03^a
Salinity (ppt)	2.05 ± 0.01^a	2.04 ± 0.01^a	2.03 ± 0.00^a	2.03 ± 0.01^a
Ph	8.00 ± 0.00^a	7.98 ± 0.00^b	7.90 ± 0.00^c	8.00 ± 0.01^a
DO (mg/l)	6.00 ± 0.00^{ab}	5.10 ± 0.00^b	6.01 ± 0.00^{ab}	6.01 ± 0.00^a
NH_4	0.32 ± 0.00^a	0.32 ± 0.00^a	0.32 ± 0.00^a	0.31 ± 0.00^a
NO_2	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
NO_3	0.22 ± 0.00^a	0.21 ± 0.00^a	0.21 ± 0.00^a	0.21 ± 0.00^a

[1]Fish given multi- strains Bacillus probiotics (MSB) at various levels 0 (MSB0), 1 (MSB1), 2 (MSB2), and 3 (MSB3) g /kg diet for 70 days. Results are expressed as mean ± SE.

Table 4 Effect of various levels of multi-strains *Bacillus* probiotics (MBS) on growth performance parameters of Nile tilapia, *O. niloticus* after 70 days

Item	Experimental groups			
	MSB0	MSB1	MSB2	MSB3
Initial fish weight (g)	20.07 ± 0.09^{a}	20.07 ± 0.09^{a}	20.07 ± 0.09^{a}	19.97 ± 0.19^{a}
Final fish weight (g)	53.20 ± 1.51^{c}	62.57 ± 1.19^{b}	66.70 ± 1.42^{ab}	70.83 ± 1.07^{a}
Weight gain	33.13 ± 1.43^{c}	42.50 ± 1.11^{b}	46.63 ± 1.33^{ab}	50.87 ± 0.88^{a}
Specific growth rate (SGR; %/ fish/day)	1.39 ± 0.03^{c}	1.62 ± 0.02^{b}	1.71 ± 0.02^{b}	1.81 ± 0.01^{a}
Feed intake	58.12 ± 0.64^{b}	63.14 ± 0.48^{a}	64.64 ± 0.78^{a}	65.39 ± 0.50^{a}
Feed conversion ratio (FCR)	1.76 ± 0.06^{a}	1.49 ± 0.03^{b}	1.39 ± 0.02^{bc}	1.29 ± 0.01^{c}
Average daily gain	0.48 ± 0.02^{c}	0.61 ± 0.02^{b}	0.67 ± 0.02^{ab}	0.73 ± 0.01^{a}
Weight gain %	165.06 ± 6.40^{d}	211.76 ± 4.71^{c}	232.34 ± 5.66^{b}	254.72 ± 2.13^{a}
Initial fish number	20.00 ± 0.00	20.00 ± 0.00	20.00 ± 0.00	20.00 ± 0.00
Fish final number	18.67 ± 0.33^{b}	19.33 ± 0.33^{ab}	20.00 ± 0.00^{a}	19.67 ± 0.33^{ab}
Fish biomass per $1m^3$	992.93 ± 31.80^{c}	1208.83 ± 2.92^{b}	1334.00 ± 28.38^{a}	1392.90 ± 28.23^{a}
Survival	93.33 ± 1.67^{b}	96.67 ± 1.67^{ab}	100.00 ± 0.00^{a}	98.33 ± 1.67^{ab}

[1]Fish given multi-strains Bacillus probiotics (MSB) at various levels 0 (MSB0), 1 (MSB1), 2 (MSB2), and 3 (MSB3) g /kg diet for 70 days. Results are expressed as mean ± SE

Effects on blood hematology

The findings showed that after adding MSB, hematocrit, RBCs, WBCs, heterophils, and Hb were all significantly ($P < 0.05$) elevated in a dose-dependent manner (Table 5). The

Table 5 Effect of various levels of multi-strains *Bacillus* probiotics on hematological parameters of Nile tilapia, *O. niloticus* after 70 days

Item	Experimental groups[1]			
	MSB0	MSB1	MSB2	MSB3
Hematocrit (%)	27.88 ± 0.16^{d}	29.39 ± 0.08^{c}	31.35 ± 0.09^{b}	32.12 ± 0.103^{a}
RBCs (10^6/mm^3)	3.16 ± 0.04^{c}	3.36 ± 0.01^{b}	3.58 ± 0.05^{a}	3.73 ± 0.05^{a}
Hb (g/ dl)	9.45 ± 0.05^{d}	10.09 ± 0.07^{c}	10.55 ± 0.06^{b}	11.69 ± 0.07^{a}
MCV (FL)	88.17 ± 1.45^{a}	87.38 ± 0.53^{a}	87.69 ± 1.04^{a}	86.15 ± 1.16^{a}
MCH (g/dL)	29.88 ± 0.25^{ab}	29.99 ± 0.31^{ab}	29.50 ± 0.28^{b}	31.36 ± 0.57^{a}
MCHC (g/dL)	33.90 ± 0.34^{b}	34.32 ± 0.15^{b}	33.64 ± 0.12^{b}	36.40 ± 0.34^{a}
WBCs (10^3/mm^3)	29.48 ± 0.39^{c}	31.17 ± 0.47^{b}	32.22 ± 0.19^{ab}	33.12 ± 0.13^{a}
Heterophils (%)	10.68 ± 0.20^{d}	11.64 ± 0.21^{c}	12.81 ± 0.19^{b}	13.75 ± 0.25^{a}
Lymphocytes (%)	75.59 ± 1.48^{a}	61.16 ± 0.87^{b}	58.35 ± 1.61^{b}	48.71 ± 1.44^{c}
Monocytes (%)	8.96 ± 0.36^{c}	11.66 ± 0.42^{b}	13.06 ± 0.16^{a}	14.03 ± 0.32^{a}
Eosinophils (%)	0.41 ± 0.02^{d}	0.50 ± 0.01^{c}	0.60 ± 0.01^{b}	0.71 ± 0.02^{a}
Basophils (%)	0.50 ± 0.01^{a}	0.38 ± 0.02^{b}	0.32 ± 0.01^{bc}	0.29 ± 0.01^{c}

[1]Fish given multi- strains Bacillus probiotics (MSB) at various levels 0 (MSB0), 1 (MSB1), 2 (MSB2), and 3 (MSB3) g /kg diet for 70 days

MSB3 group had the highest values of MCHC, MCH, and eosinophils. Additionally, the MSB2 and MSB3 groups had significantly higher levels of monocytes and basophils than the other groups ($p < 0.05$). The lymphocyte, MCHC, and MCH values of fish fed probiotics at 1–2 g/kg were comparable to those of the control group. Compared to other groups, fish in the MSB3 group had fewer monocytes ($P < 0.05$). Overall, the hematological variables showed better results for fish fed on MSB3.

Effects on blood metabolites

Table 6 shows the impact of different MSB dosages on Nile tilapia, *O. niloticus*, serum blood metabolites after 70 days. The blood protein fraction (total protein, albumin, and globulins) improved significantly ($P < 0.05$) with dietary MSB inclusion following MSB addition in a dose-dependent manner. In contrast, fish fed diets containing MSB (1, 2, and 3 g/kg diet) showed significantly lower serum levels of triglycerides, uric acid, and liver enzymes (AST, ALT, and ALP) ($P < 0.05$). Serum digestive enzyme levels were considerably higher, and creatinine levels were significantly lower across all MSB treatments, with the MSB3 group showing the best results. When comparing the cholesterol values of the MSB2 group to those of the MSB1 and MSB0 groups, there was a significant decrease ($P < 0.05$). Compared to the MSB1 and control groups, MSB2 and MSB3 had significantly lower serum levels of urea and glucose ($P < 0.05$).

Table 6 Effect of various levels of multi-strains *Bacillus* probiotics on blood metabolites of Nile tilapia, *O. niloticus* after 70 days

Item	Experimental groups			
	MSB0	MSB1	MSB2	MSB3
ALT (U/L)	10.22 ± 0.16^{a}	8.25 ± 0.21^{b}	7.65 ± 0.32^{bc}	7.04 ± 0.26^{c}
AST (U/L)	13.92 ± 0.73^{a}	11.45 ± 0.36^{b}	10.92 ± 0.15^{b}	10.16 ± 0.18^{b}
ALP (U/L)	40.70 ± 0.47^{a}	34.98 ± 0.23^{b}	31.88 ± 0.78^{c}	29.70 ± 0.25^{d}
Albumin (g/dl)	1.99 ± 0.08^{d}	2.33 ± 0.03^{c}	2.63 ± 0.07^{b}	2.92 ± 0.08^{a}
Globulin (g/dl)	2.18 ± 0.03^{c}	2.26 ± 0.07^{bc}	2.44 ± 0.05^{b}	2.84 ± 0.05^{a}
Total protein (g/dl)	4.17 ± 0.08^{d}	4.59 ± 0.10^{c}	5.07 ± 0.05^{b}	5.77 ± 0.08^{a}
Creatinine (mg/dl)	0.54 ± 0.01^{a}	0.47 ± 0.01^{b}	0.44 ± 0.01^{b}	0.44 ± 0.01^{b}
Glucose (mg/dl)	12.87 ± 0.16^{a}	12.46 ± 0.10^{a}	11.29 ± 0.20^{b}	10.62 ± 0.26^{b}
Amylase (mg/dl)	13.65 ± 0.30^{d}	16.71 ± 0.51^{c}	20.12 ± 0.27^{b}	23.13 ± 0.73^{a}
Lipase (mg/dl)	28.66 ± 0.52^{d}	33.65 ± 0.62^{c}	36.92 ± 0.42^{b}	38.91 ± 0.27^{a}
Triglyceride (mg/dl)	167.12 ± 2.88^{a}	152.08 ± 4.10^{b}	141.24 ± 1.60^{bc}	129.69 ± 2.93^{c}
Urea (mg/dl)	3.54 ± 0.05^{a}	3.58 ± 0.09^{a}	3.10 ± 0.03^{b}	2.94 ± 0.06^{b}
Uric acid (mg/dl)	1.95 ± 0.03^{a}	1.83 ± 0.00^{b}	1.58 ± 0.02^{c}	1.48 ± 0.03^{d}
Cholesterol (mg/dl)	86.37 ± 0.44^{a}	85.26 ± 0.34^{a}	80.67 ± 1.89^{b}	83.44 ± 0.30^{ab}

[1]Fish given multi-strains Bacillus probiotics (MSB) at various levels 0 (MSB0), 1 (MSB1), 2 (MSB2), and 3 (MSB3) g /kg diet for 70 days. Aspartate aminotransferase (*AST*); alkaline phosphatase (*ALP*); and alanine transaminase (ALT)

Effects on body composition

Figure 1 illustrates the effects of multi-strain *Bacillus* probiotics on the body composition of Nile tilapia, *O. niloticus*, after 70 days. MSB1 and MSB2 had higher moisture content; MSB-fed fish had higher protein content compared to the untreated group ($P < 0.05$). The percentage of lipid in the body composition decreases gradually with an increase in MSB level ($P < 0.05$). MSB2 and MSB3 had higher Ash percentage compared to the other groups.

Histological results

The histological interpretations revealed that the hepatopancreas tissues in the control group (MSB0) exhibited degraded hepatic tissue cells scattered throughout the hepatic strands surrounding the hepatic central vein engorged with blood, and Kuffer immunocytes were present within the hepatic sinusoids in a swollen form and in large numbers, as well as the presence of lymphatic immunocytes in the hepatic pancreas' ducts and erosion of certain parts of the hepatopancreas tissues and the activity of fibroblasts to restore the tissue's shape and give the appearance of hepatic cirrhosis caused by *Aspergillus flavus* infection. Hepatic structures, including the hepatic pancreatic ducts and the hepatic cells inside the hepatic strands, were seen to be improving. Furthermore, there was a noticeable improvement in the quantities of immunocytes, similar to those found in the hepatic sinusoids, in groups MSB3 (Fig. 2D), MSB2 (Fig. 2C), and MSB1 (Fig. 2B), respectively. These cells have a reduced number and a normal oval shape.

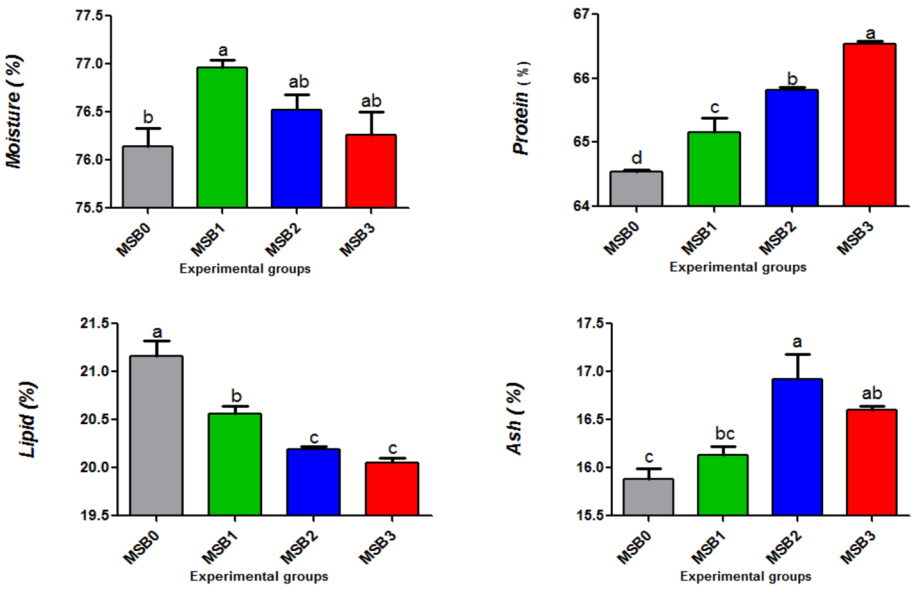

Fig. 1 Effect of various levels of multi-strains Bacillus (MSB) probiotics on body composition including moisture %, protein %, lipid %, and ash % of Nile tilapia, *O. niloticus*, after 70 days. Fish given multi-strains Bacillus probiotics (MSB) at various levels 0 (MSB0), 1 (MSB1), 2 (MSB2), and 3 (MSB3) g /kg diet for 70 days

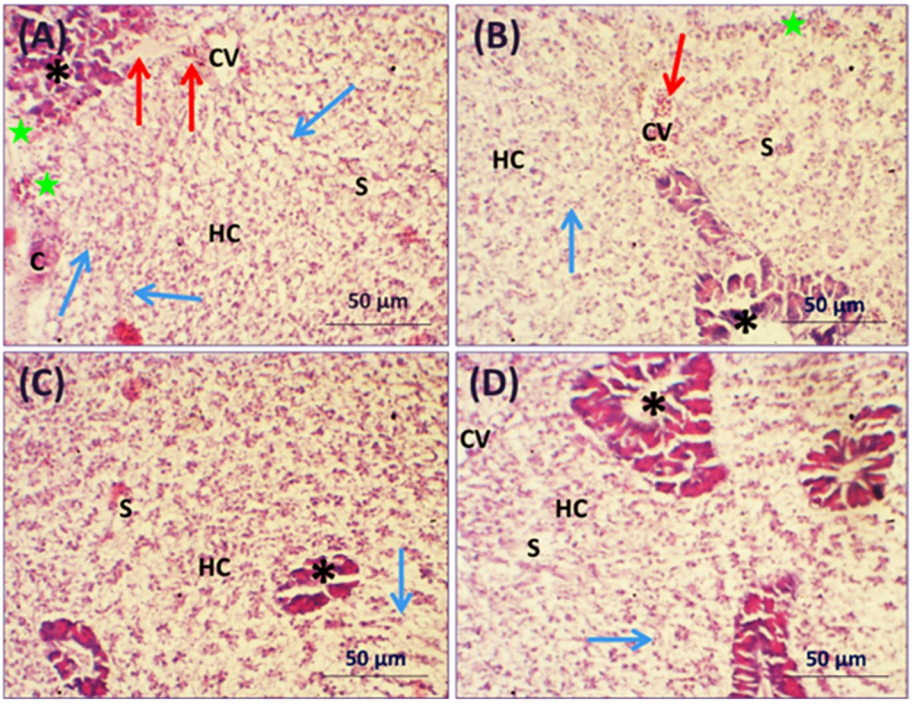

Fig. 2 Photomicrograph sections of hepatopancreatic slices (**A**–**D**) for different experimental groups. Nile tilapia given multi-strains *Bacillus* probiotics (MSB) g /kg diet for 70 days at various levels 0 (MSB0): **A**, 1 (MSB1): **B**, 2 (MSB2): **C**, and 3 (MSB3): **D**. HC, hepatocytes; S, hepatic sinusoids; CV, hepatic central vein; C, hepatic cirrhosis. Asterisks: pancreatic duct. Green stars: inflammatory lymphocytes aggregations. Red arrows: blood accumulation. Blue stars: Kuffer macrophage cells [H&E and scale bar: 50 µm]

The impact of varying MSB dosages on the intestinal tissues of Nile tilapia (*O. niloticus*) after 70 days revealed that, in comparison to the other groups, the addition of dietary MSB greatly improved ($P \leq 0.01$) the average length of the intestinal tissue layers and significantly improved ($P < 0.05$) the width of the intestinal villi. The third experimental group (MSB3) exhibited the best results for bacillus on intestinal tissues, as depicted in Figs. 3D and 4.

Figure 5A–D shows tilapia infected with *A. flavus* and fed varying amounts of MSB. Figure 5D, C, and B illustrate how the histological morphology of the spleen improved in all MSB-treated groups for MSB3, MSB2, and MSB1. The MSB3 group's lymphocyte immunocytes and the splenic histological structure, including the red and white pulp, showed the greatest enhancement (Fig. 5D).

Growth and pro-inflammatory related genes

The results of relative mRNA expression revealed that dietary supplementation of Bacillus probiotics significantly upregulated the expression of growth-related genes, such as *GH* (, *IGF-1* (Fig. 6B), and *IGF-1* (Fig. 6C) in a dose-dependent manner, meaning that MSB3 shows better progress in growth performance than MSB2 and MSB1 when the levels of MSB in diets were increased ($P < 0.05$). Conversely, the

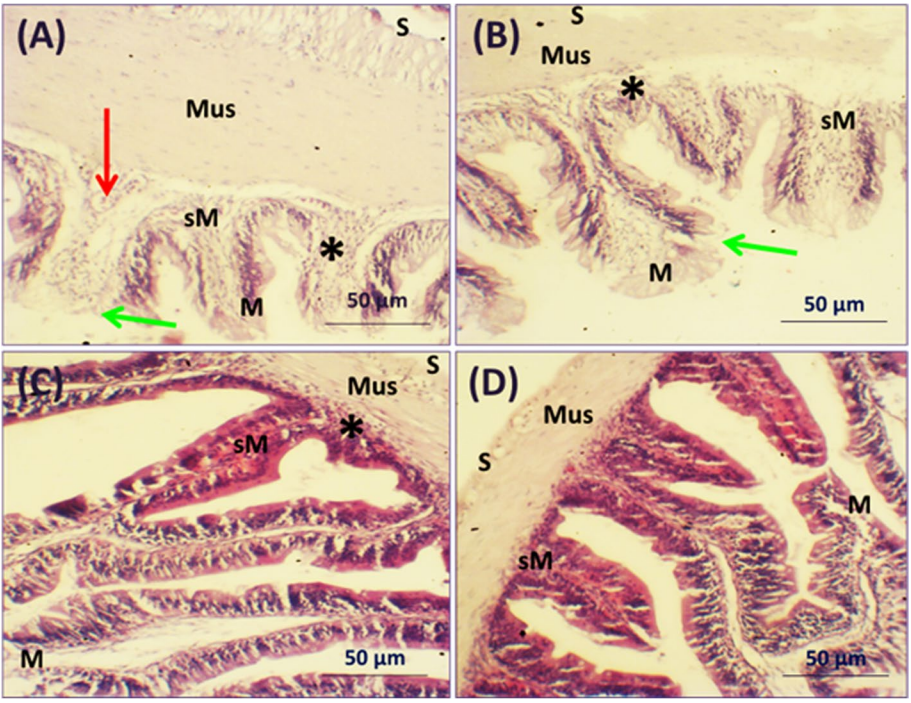

Fig. 3 Photomicrograph sections of intestinal slices (**A**–**D**) for different experimental groups. Nile tilapia given multi- strains *Bacillus* probiotics (MSB) g /kg diet for 70 days at various levels 0 (MSB0): Fig. 2A, 1 (MSB1): Fig. 2B, 2 (MSB2): Fig. 2C, and 3 (MSB3): Fig. 2D. Four intestinal layers; S, serosa; Mus, muscularis; sM, mucosa; M, mucosa; Asterisks: lympho-immunocytes infiltrations. Green arrows: erosion of tips for some intestinal villi. Red arrows: macrophagocytes scattered on the fish villi which infects with *A. flavus* [H&E, and scale bar: 50 µm]

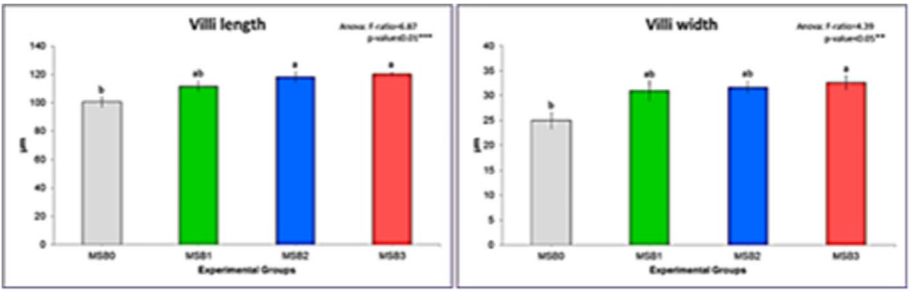

Fig. 4 The impact charts of various levels of multi-strains *Bacillus* probiotics (MSB): 0 (MSB0), 1 (MSB1), 2 (MSB2), and 3 (MSB3) g /kg diet on the intestinal villi parameters including villi length ($P \leq 0.01$) and villi width ($P < 0.05$) of Nile tilapia, *O. niloticus*. Means ± standards errors for each column, the different letters (a-b) which represented the significant between the experimental four groups by Tukey B test after 70 days

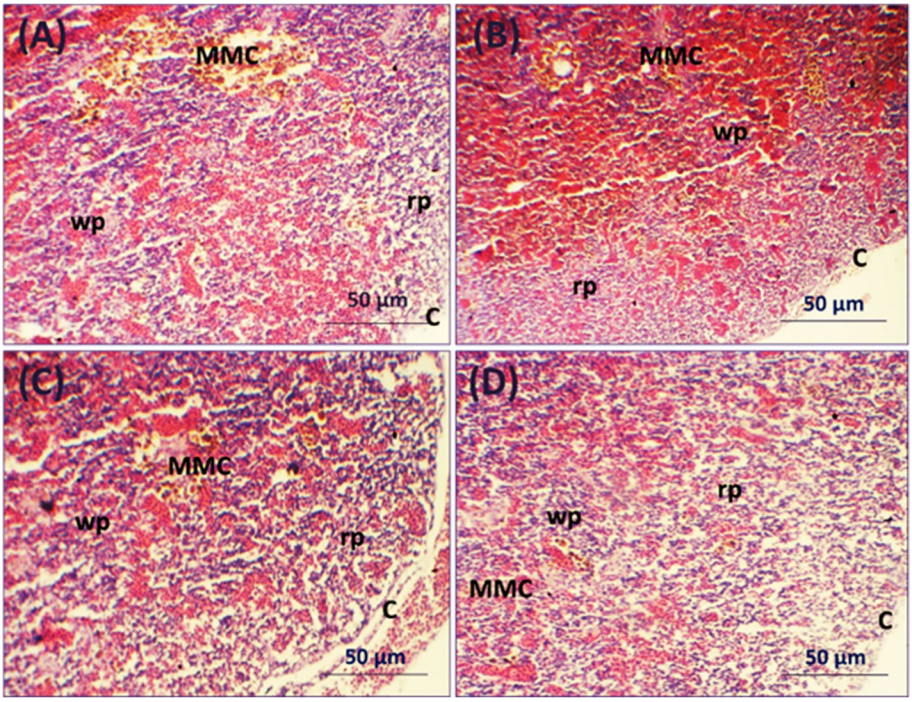

Fig. 5 Photomicrograph sections of splenic slices (**A**–**D**) for different experimental groups. Nile tilapia given multi-strains *Bacillus* probiotics (MSB) g /kg diet for 70 days at various levels 0 (MSB0): Fig. 2A, 1 (MSB1): Fig. 2B, 2 (MSB2): Fig. 2C, and 3 (MSB3): Fig. 2D. Red pulp (rp) and white pulp (wp), they are distributed in the splenic tissues; C, capsule; MMC,, centers of the melanomacrophage which infects with *A. flavus* [H&E and scale bar: 50 µm]

effect of MSB on immunity genes was contradictory, meaning that fish given MSB showed a dose-dependent reduction in the *IL-1β* gene, with the MSB3 group showing the lowest expression, followed by the MSB2 and MSB1 groups (Fig. 6D). Furthermore, the expression of the *IL-8* gene was different from *IL-1β* in all MSB-treated groups. The expression of *IL-1β* showed the lowest significant expression ($P < 0.05$) in the MSB2 group, followed by the MSB3 and MSB1 groups, with the highest expression noticed in the MSB0 group (control).

Survival during challenge with pathogen

Figure 7 illustrates the infection of tilapia fish with the pathogen *A. flavus* at different levels of MSB. The control group had a 40% infection survival rate, which was lower than that of the other groups. More fish survived in all MSB-treated groups; for MSB0, MSB1, MSB2, and MSB3, the corresponding survival rates were 40%, 55%, 65%, and 70%. It was found that MSB3 had the highest survival rate.

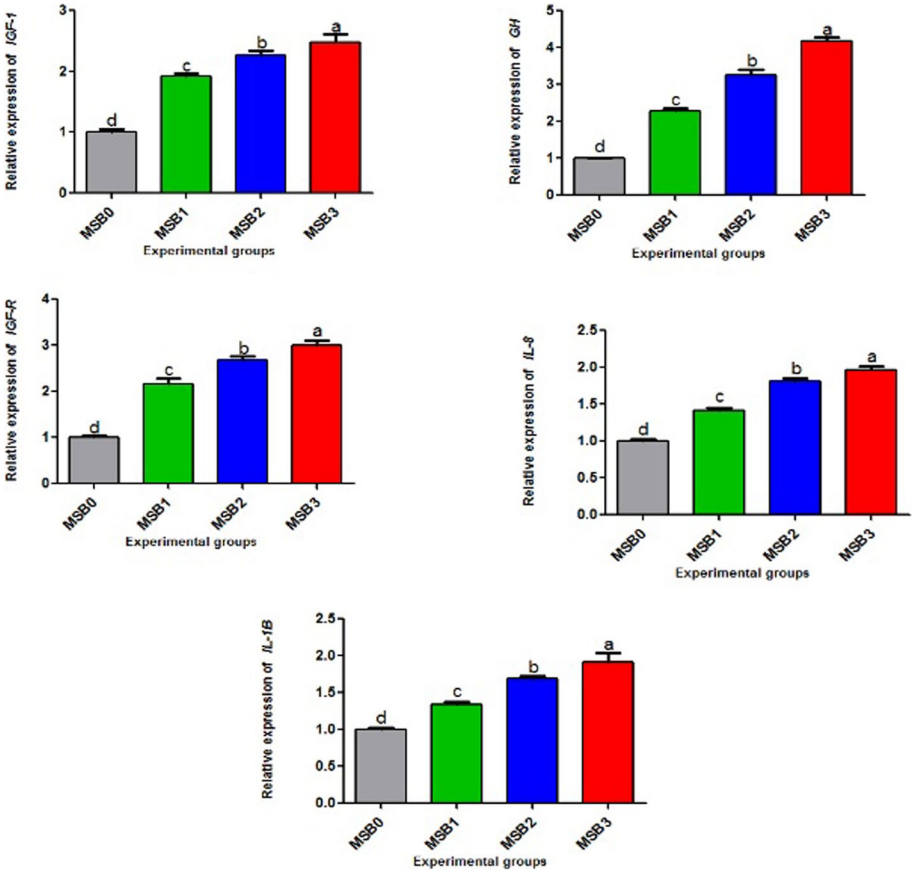

Fig. 6 The impacts of various levels of multi-strains *Bacillus* probiotics (MSB): 0 (MSB0), 1 (MSB1), 2 (MSB2), and 3 (MSB3) g /kg diet for 70 days on the growth-related genes including *IGF-1*, *GH*, *IGF-R,* and pro-inflammatory-related genes including *IL-8* and *IL-1β* of Nile tilapia, *O. niloticus* after 70 days

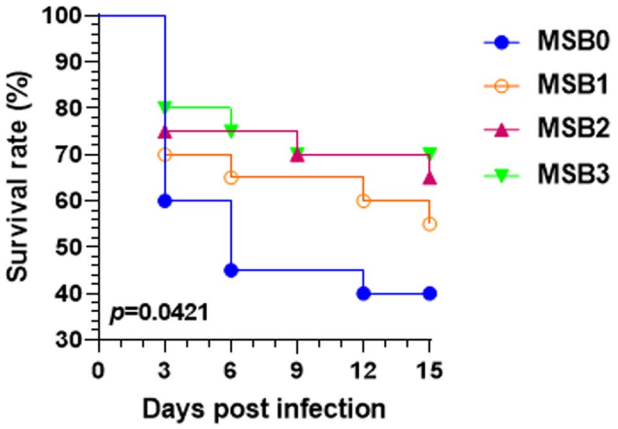

Fig. 7 Tilapia fish infected with *A. flavus* and fed with various levels of MSB in their diets. Fish were given various levels of multi-strains *Bacillus* probiotics (MSB): 0 (MSB0), 1 (MSB1), 2 (MSB2), and 3 (MSB3) g / kg diet

Discussion

Probiotics, according to FAO/WHO, can facilitate the growth and activity of beneficial bacteria in hosts, allowing them to remain alive and active in the gut (Zendeboodi et al. 2020). This article explains the importance of combining a probiotic mixture of Bacillus species (B. *pumilus*, *B. licheniformis*, and *B. subtilis*) to determine its effectiveness in treating *A. flavus* infection in *O. niloticus*. Therefore, an analysis was conducted on the blood picture, growth profile, digestion, biochemical indices, and gene expression.

This study was conducted to assess the immune-stimulating and growth-promoting effects of adding a Bacillus probiotic mix to the water in Nile tilapia rearing systems, as well as the fish's vulnerability to *A. flavus* infection. The effects of this probiotic blend were examined by analyzing a variety of factors, such as the expression of genes linked to growth and immunity, growth performance, blood biochemical parameters, and water quality. Healthy water quality is essential for aquaculture production. To achieve optimal growth, survival, and productivity, a thorough understanding of the relationship between aquatic productivity and water quality is necessary (Dalia et al. 2023; Soundarapandian and Babu 2010). The current investigation demonstrated a definite improvement in water quality, as evidenced by a significant decrease in both total and toxic ammonia levels in the probiotic-treated groups. These findings are consistent with those of Basma et al. (2023), who found that adding probiotics to fish rearing water reduced the levels of harmful compounds (NH^3, NO_2, and NO^3). Probiotics have also been shown to reduce unionized ammonia (NH^3) and other nitrogenous waste in fish rearing water.

Probiotics can significantly alter gut microbiota, drive metabolic processes, improve digestion, activate the immune system, and protect against illnesses and exogenous bacteria, according to earlier studies (Falcinelli et al. 2018; Li et al. 2019; Munir et al. 2016a, 2018a, b). These benefits have been linked to probiotics' critical roles in digestion, metabolism, and growth performance. The results of this study made it clear that the probiotic mixture significantly increased growth parameters, growth hormone (*GH*), insulin-like growth factor-1 (*IGF-1*), and insulin-like growth factor receptor-1 (*IGF-1R*) genes. The capacity of the probiotics to colonies and adhere to the intestinal wall (Munir et al. 2018a) may account for this, as it increases the secretion of digestive enzymes, which in turn increases metabolism and growth rate. A recent study (Zhao et al. 2020) confirmed that *Bacillus spp.* have the ability to adhere to fish intestines, improving digestion, which lends support to this finding. Furthermore, as partially accepted in comparison with our data, *B. coagulans*, either by itself or in conjunction with β-glucan added to the tilapia diets, could decrease the expression of the *IL-1β* gene while upregulating the expression of the *GH* and *IL-8* genes (Fath El-Bab et al. 2022).

Furthermore, according to Ringø et al. (2022), probiotic microorganisms can colonize the gastrointestinal tract by releasing a variety of digestive and degrading enzymes that allow the body to use a wide range of nutritional components, thereby improving digestion. Comparably, Zibiene and Zibas (2019) showed that commercial probiotics can unquestionably enhance European catfish (*Silurus glanis*) growth performance. According to a recent study, *Bacillus subtilis* can enhance the expression of genes related to appetite and somatic growth in zebrafish (Santos et al. 2020). In addition, Zou et al. (2016) discovered that common carp (*Cyprinus carpio*) had increased intestinal cytokine gene expression in response to probiotic powder containing *Agaricus bisporus*. Furthermore, Fath El-Bab et al. (2022) discovered that adding Bacillus coagulants to Nile tilapia diets greatly enhanced the fish's growth and feed utilization.

In terms of evaluating hepato-renal function, the current data showed a significant alteration in creatinine levels and liver enzymes. Consistent with earlier research, different fish species' AST, ALT, and creatinine levels can be regulated by adding *Bacillus* sp. to their diets (Hassaan et al. 2018; Yu et al. 2018). This may be explained by the immune-modulatory effects of the *Bacillus* spp. mixture on the hepatic cells, which promote hepatocyte anabolism and the release of blood proteins along with a positive outcome regarding the preservation of liver cell integrity (Eladawy 2019).

Probiotics have been shown in earlier studies to play a critical role in growth, metabolism, digestion, and absorption as well as to boost immune system function, improve digestion and absorption, and protect against infectious diseases (Munir et al. 2016a). Additionally, the present study demonstrated that the probiotic mixture greatly accelerated the process of enhancing the tissue structures of the intestines and hepatopancreas, which are home to *Bacillus* probiotic bacteria like *Bacillus subtilis* and *Bacillus cereus*. It lessens the absorption of aluminum, which lessens tissue damage brought on by the buildup of aluminum in tilapia fish tissues (Arun et al. 2021). Furthermore, Lazado et al. (2014) demonstrated that the bacteria produce mucous secretions that function to shield the body's tissues from infectious diseases by boosting the activity of immune lymphocytes that are supportive of the mucous tissue. In 2023, Shija et al. observed that including *Bacillus* probiotics in the diet of various fish species strengthens fish immunity and prevents the need for antibiotics by protecting fish against a variety of infectious diseases, enhancing autoimmunity, and promoting improved growth in farmed fish. Recently, Gao et al. (2024) reported that the immune function and hepatic health of flounder (*Paralichthys olivaceus*) were improved by dietary supplementation with Bacillus in their diets. Probiotic mixture supplements have a number of advantages over *(A) flavus*, as evidenced by their strong antibacterial activity, reduced mortality, improved blood picture, and immune-promoting effects. Elevations in blood parameters, immune-related indices (total protein, albumin, phagocytosis), and immune (interleukin 8 (*IL-8*) and interleukin one beta (*IL-1β*) associated genes were indicative of these results. This may be primarily due to the interaction between probiotic bacteria (*Bacillus* spp.) and various immune system cell types (monocytes, lymphocytes, macrophages, granulocytes, neutrophils, and natural killer cells), as reported by Mazziotta et al. (2023). A recent study by Tachibana et al. (2021) that fed Nile tilapia enriched diets containing *Bacillus* species revealed increased granulocyte and intraepithelial lymphocyte proliferation, supporting our results. It is possible that recent reports have indicated that adding *Bacillus* probiotics, such as *(B) amyloliquefaciens*, *B. licheniformis*, *B. subtilis*, and *B. pumilus*, to the diet of Nile tilapia improves phagocytosis (Eissa et al. 2023a; Ghalwash et al. 2022). According to Wu et al. (2021), feeding Nile tilapia with *Bacillus* (NPUST1) for 8 weeks improved immune biomarkers in head kidney leukocytes (e.g., phagocytic activity and respiratory burst activity), lysozymes, and expression of immune-related genes in the head kidney and spleen. This is in line with previous reports (Chien et al. 2020) that found that *B. subtilis* E20 boosted the immune response in white shrimp by improving the metabolism of glutamine and the hexosamine biosynthesis pathway. In line with Dutta and Ghosh's (2021) findings, this study reported that immune genes (*TNF-1α* and *TNF-2α*) were up-regulated in goldfish (*Carassius auratus gibelio*) when exposed to dietary *Bacillus* spp. Tang et al. (2017) provided support for these results by observing that juvenile tilapia challenged with virulent *(A) hydrophila* exhibited enhanced antibacterial and anti-inflammatory activities following dietary intervention with *(B) subtilis*. Supplementation of *B. subtilis* as a probiotic improved growth pattern, antioxidant response, protein content, and immunocompetency against *S. aureus* in striped catfish (Liaqat et al. 2024). Moreover, a recent study of Neissi et al. (2024) found that adding *B. subtilis* to the diet can enhance the health and blood profile of rainbow trout (*Oncorhynchus mykiss*) under acute hypoxia stress.

Conclusion

This current research confirmed the synergistic effects of probiotics (*Bacillus subtilis*, *B. pumilus*, and *B. licheniformis*) as immune stimulants, growth promoters, enhancers of histological structures, and antifungal agents against *A. flavus* infection in tilapia fish. In *O. niloticus*, this probiotic mixture resulted in improved growth genes expression and helped combat anemia. Combining probiotics from the Bacillus species can work together to maximize their benefits, especially at higher concentrations. Further research is needed to explore the additional antimicrobial effects of the Bacillus species mixture and their impact on other fish species.

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Author contribution H.S.D., S.E.S., Conceptualization, visualization, methodology, E.N.A.A., O.F.A., W.E.A., S.A.H., N.M.A. and Y.M.A.El-A., software, validation, formal analysis, N.A., H.H.M., M.B.M., E.H.E., A.A.E. and Y.M.A.El-A.; investigation, data curation, S.A.H., N.M.A., N.A. and Y.M.A.El-A.; histological processing, examination, and histological data interpretation; M.B.M., Y.M.A.El-A. writing—original draft preparation, H.H.M., E.H.E., A.A.E., M.B.M, and Y.M.A.El-A.; writing—review and editing, W.E.A., S.A.H., N.M.A., N.A., and Y.M.A.El-A.; supervision, E.H.E.; project administration, E.H.E. All authors have read and approved the published version of the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethical approval Ethical approval was granted by the Research ethical of Arish university, with research code: Agri-11.

Consent to participate Not applicable.

Consent for publication All authors review and approve the manuscript for publication.

Competing interests The authors declare no competing interests.

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