BMC Veterinary Research

RESEARCH

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Immune protective, stress indicators, antioxidant, histopathological status, and heat shock protein gene expression impacts of dietary *Bacillus* spp. against heat shock in Nile tilapia, *Oreochromis niloticus*

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Abstract

This research evaluated the efficacy of mixed *Bacillus* strains probiotic supplements in mitigating acute thermal-induced stress in Nile tilapia (*Oreochromis niloticus*). Three experimental fish groups involved 135 Nile tilapia (49 ± 2 g); one control (no added probiotics), 0.5, and 1% of selected *Bacillus* strains (*B. subtilis*, *B. licheniformis*, and *B. pumilus*) for 58 days. After the feeding period, growth parameters, immunological parameters, stress biochemical markers, and antioxidant parameters in addition to genes related to stress and histopathological changes in fish, were assessed; subsequently subjected to heat shock at 36 ± 0.5 ºC for 2 h. Before the heat challenge, our results exhibited a marked increase in the growth efficacy ($P < 0.05$), lower marked serum levels of triglycerides and cholesterol, and tissue malondialdehyde (MDA) levels along with significantly increased superoxide dismutase (SOD) and catalase (CAT) enzymes activity in fish-fed *Bacillus* probiotic at 0.5% concerning the control group ($P < 0.05$). There were no significant changes in the serum levels of glucose, cortisol, lactate, phagocytic activity, respiratory burst (ROS), total immunoglobulin Ig, alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), total protein, albumin, globulin, uric acid, urea, creatinine, as well as *HSP70*, *GST*, and *GPx* mRNA expression in most of the probiotic groups compared to the control group ($P > 0.05$). When Nile tilapia was exposed to heat stress, supplementation with *Bacillus* probiotic in the diet significantly decreased most of the indices related to serum biochemical (ALT ($P < 0.01$; $P < 0.001$), AST ($P < 0.01$), LDH ($P < 0.01$), urea ($P < 0.05$), and creatinine ($P < 0.01$)), triglycerides ($P < 0.001$; ($P < 0.01$)), cholesterol ($P < 0.01$; ($P < 0.05$)), glucose ($P < 0.001$), and cortisol ($P < 0.01$; ($P < 0.05$)), with tissue oxidative stress MDA levels ($P < 0.05$), and *HSP*70 mRNA expression ($P < 0.01$; $P < 0.001$), aligned with the stressed control group. In addition, a notable upsurge in the total protein, albumin, globulin, phagocytic and ROS activities, and total Ig, as well as the enzymatic antioxidant ability (SOD, CAT) ($P < 0.01$), with *GST* and *GPx* mRNA expression ($P < 0.05$; $P < 0.01$), were shown in fish-fed *Bacillus* spp. post-exposure

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compared with the stressed control group. Besides, no histopathological alterations were revealed in the spleen and brain of fish pre- and post-heat exposure. According to our findings, diet supplementation of *Bacillus* species has the potential to combat the suppressive effects of heat shock in Nile tilapia.

Highlights

- Heat exposure altered the phagocytic activity, ROS, and total Ig and hepatic and liver biomarkers.
- Heat exposure induced oxidative damage and pathological alterations of different tissues.
- Heat exposure enhanced the expression of HSP70 in the head kidney of tilapia.
- Mixed *Bacillus* spp., a dietary probiotic, effectively mitigated these heat-induced toxicity.

Keywords *Bacillus* probiotic, Heat shock, Immune response, Gene expression, Nile tilapia

Introduction

Among other environmental factors that affect the metabolism and health of fish, water temperature is one of the major substantial ones. Most biological processes are influenced by water temperature which comprises dissolved oxygen, photosynthesis aerobic respiration, and metabolic functions of aquatic organisms [1–3].

Exposure of aquatic organisms to regional environmental stressors, such as acute changes in temperature leads to many biochemical and physiological changes, which are interposed by the neuroendocrine and cellular stress responses [4, 5]. In general, fish's capacity to confront environmental challenges relies on an effective physiological reaction to stress, which includes the generation and discharge of cortisol and catecholamines facilitated by triggering neuroendocrine pathways: the hypothalamic–pituitary-interrenal axis and the brain–sympathetic–chromaffin cell axis [6, 7].

The cellular stress response induces the synthesis of heat shock-conserved proteins (HSPs), which exist in every living organism [8, 9]. Recently, in the course of studies on the stress tolerance of inducible heat shock protein 70 kDa (HSP70), which is employed as a biological marker to different environmental stressors, such as thermal shock, ammonia toxicity, toxic compounds, and pathogens [2, 10]. Several reports assayed the higher expression of HSPs in fish following thermal shock [1, 2, 6, 8].

Much research related to physiological responses against thermal shock in aquatic organisms has been elucidated; higher metabolism has been generated with increasing oxygen consumption and consequently, active oxygen species (ROS) production (O_2^-, H_2O_2, and OH^-), which can damage the cellular components [11, 12]. The antioxidant enzymes like glutathione peroxidases (GPx) and glutathione transferases (GST) protect against such oxidative stress through the detoxification of ROS [1, 9, 13]. Nevertheless, suppression of the immune system with the consequent disease outbreaks is induced in fish exposed to an acute increase in temperature (heat shock) [6, 14–18].

Within the aquaculture sector, the nutritional requirements of fish must be met to achieve optimum growth and regain a normal physiological state to manage stress effectively; thereby the prospective impact of probiotics has been investigated, particularly under undesirable temperature fluctuations [19–25]. Dietary inclusion of probiotics can alleviate the noxious consequences of environmental stressors and can optimize the immune system of fish because of the modulation of the gut colonization of the probiotic *bacillus* strains, along with the production of antibodies, lysozyme, and anti-microbial peptides [19, 26–28]. *Bacillus* species enhance the water quality and stimulate the anti-heat stress pathways [29, 30]. The *Bacillus* spp. can reinforce the growth capacity and healthy status of aquatic animals [31–34]. Probiotics containing mixed *Bacillus* species have improved the immune and stress responses of many farmed fish and shrimp species, such as Catla (*Catla catla*), Rohu (*Labeo rohita*), and pacific white shrimp (*Litopenaeus vannamei*) [19, 35–37], Nile tilapia [31, 38, 39], grass carp [40], and common carp [41, 42]. Likewise, dietary supplements of *Bacillus* species (i.e., *B. subtilis* and *B. licheniformis*) showed a pronounced enhancement in most immunological and physiological indices against the suppressive impacts of hypoxia and cold stressors exposed to Nile tilapia [43]. Besides, a mixture of *B. amyloliquefaciens* and *B. pumilus* enhanced the immunological indices against ammonia toxicity in striped catfish (*Pangasianodon hypophthalmus*) [44].

Nile tilapia is highly valued farmed fish in semi-intensive and intensive aquaculture practices in Egypt and worldwide as it has palatable flesh, reasonable prices, and wide tolerance to fluctuated environmental water quality [7, 38, 45]. For Optimum growth and reproduction, Nile tilapia needs a temperature of 22–32 °C [46]. In Egypt, higher water temperatures faced in the farming of Nile tilapia have been associated with diminished fish production, higher fish mortalities, and disease outbreaks [47–49]. Therefore, this study explored the influence of a probiotics blend featuring *Bacillus* strains on hematological and biochemical indices, nonspecific immune response, antioxidant activity, histopathological changes,

and gene expression influenced by stress against thermal shock in Nile tilapia.

Materials and methods

Ethical affirmation

Our study was implemented with the consent of the Institutional Ethics Committee at the Faculty of Veterinary Medicine, Mansoura University (MU-ACUC), Egypt, with the assigned sequential authorization code (VM.R.24.01.151). All methods were performed following the relevant IACUC guidelines and regulations. We handled and transported live fish in sealed plastic bags containing oxygen (two-thirds) from a private hatchery farm in Al-Manzala, Dakahlia Province, Egypt, and chose fish for experimental use.

Fish management, diet preparation, and experimental protocol

Healthy Nile tilapia monosex, displaying no signs of disease, and exhibiting an average body weight of 49±2 g, were sourced from a private hatchery farm in Al-Manzala, Dakahlia Province, Egypt, which adheres to strict biosecurity measures. In all, 135 fish were adjusted to laboratory conditions for two weeks and then distributed into 120 L glass aquarium tanks. These tanks, measuring 40×60×70 cm, were equipped with de-chlorinated tap water, filters, and aerators. The fish were separated into three categories in triplicate. Fifteen fish were housed in each of the 9 tanks (45 fish/category). The water quality parameters were regularly monitored every week using standard methods outlined by the American Public Health Association for Water Evaluation [50]. The water quality parameters (pH and ammonia) were evaluated using freshwater master test kits (Aquarium Pharmaceuticals, Inc., code number, RAP01034) following the instructed protocol, whereas the water temperature was checked using a digital aquarium thermometer (AQUANEAT®), as well as the dissolved oxygen (D.O) was measured using portable dissolved oxygen meter (RCYAGO). The DO levels were maintained at 6.6±0.5 mg L^{-1}, water temperature was kept at 26 °C±2 °C, Ammonia NH_3 at 0.02±0.01, and pH was maintained at 7.2±0.5. Additionally, more than half of the aquarium water was replaced daily with clean fresh water from a storage tank, and waste removal was carried out to prevent ammonia toxicity. The fish were administered a commercial basal diet twice per day, 3% of their body weight during the acclimatization period.

A commercial tilapia pelleted diet was obtained via the National Company for Fisheries and Aquaculture, Ghalioun Company, Egypt. The basal diet contains 30.36% crude protein, carbohydrate 38.48%, 5.3% fiber, 6% lipid, 7.86% ash, and 12% moisture, which meets the appropriate nutritional requirements of Nile tilapia [51], based on our previous study [31]. Sanolife® PRO-F probiotic compound, consisting of a blend of selected *Bacillus* strains (*B. subtilis* 3.25×10^9 CFU/g, *B. licheniformis* 3.50×10^9 CFU/g, and *B. pumilus* 3.25×10^9 CFU/g; totaling 1.0×10^{10} CFU/g), was obtained from INVE Aquaculture, a Benchmark company based in Belgium. The product contains a highly concentrated microbial mixture that allows gut microflora colonization and prohibits pathogenic microbes with prompt disease control [38]. This probiotic compound was directly incorporated into the basal diet, with sunflower oil used as a coating agent (at a ratio of 20 ml/kg diet), at three different inclusion levels: 0 g (B_0), 0.5 g ($B_{0.5}$), and 1 g (B_1) per kg of diet, correspondingly [31]. The formulated feed was subsequently air-dried at room temperature, packaged in polythene bags, and stored at −20 °C until use. The Nile tilapia were fed the prepared diets twice daily for 58 days at a feeding ratio of 3% of their body weight [31].

Time point I: pre-exposure

Following the 58-day feeding period, the final weight (FBW) of Nile tilapia was recorded to calculate the following formulas: weight gain (WG)= (W_t–W_0), weight gain rate (%) ($W_t - W_0$)/$W_0\times100$, specific growth rate (SGR, % body weight/day)=100 [(Ln W_t–Ln W_0)/t], where W_0 and W_t are the initial and final weights of fish (g), respectively, and (t) is the feeding interval in days. The survival rate (SR%) was determined as the number of fish at the end of the feeding experiment/initial number of fish ×100 [52]. Subsequently, ten (10) fish samples were randomly collected utilizing a mesh net from the experimental groups for blood sampling, serum, and tissue samples (as described in Sect. 2.5) to evaluate the innate immune response, biochemical factors, antioxidant enzymes, stress indicators, expression of genes, and histopathological analysis.

Time point II: heat stress exposure

According to the previously mentioned protocol [43], fish that persist in each experimental batch, ten (10) fish were randomly relocated from the previous tanks, which maintained a temperature of 28±2 °C, to 80 L tanks. Each of these tanks contained 40 L of water, with two tanks per group, each housing 10 fish. The water temperature was gradually elevated from 28±2 °C to 36±0.5 °C at a rate of 2 °C per hour using anti-explosion aquarium heaters (200-Watt, RS Electrical, China) alongside continuous aeration. The stability of the temperature was monitored using a digital aquarium thermometer (AQUANEAT®). Subsequently, the fish were not fed and were sampled after 2 h of heat exposure.

Sample collection

Firstly, whole blood, serum, and internal organs were sampled after 58 days of feeding and subsequent heat exposure to investigate stress biochemical indices, immune response, and pathological effects. Fish were rendered unconscious using clove oil (60 mg L^{-1}) [53]. Blood was collected from the caudal veins of five fish ($n=5$) from each group and transferred to Eppendorf tubes containing tri-potassium EDTA for the analysis of the phagocytic index and respiratory burst activity. Subsequently, additional blood samples ($n=5$ fish/aquarium) were obtained and allowed to coagulate for 2 h. After centrifugation at 3500 ×g for 15 min at 4 °C, the serum was separated, recovered, and stored at -20 °C for analysis. Sections of the fish's head kidney and liver were excised, rinsed three times with a cold NaCl solution (0.9%), and then homogenized in a cold phosphate-buffered saline (PBS) solution (pH 7.5). The homogenates were transferred to clean tubes and subsequently centrifuged at 3500 ×g for approximately 15 min at a cold temperature to evaluate the levels of antioxidants and oxidative stress molecules [54]. By following the outlined previous procedures [55], the protein level of the homogenate was measured. The remaining portion of the head kidney ($n=3$) was combined and promptly preserved in RNA later® solution (Sigma) for gene expression analysis. Samples from the spleen and brain were dissected and fixed in 10% neutral buffered formalin for histopathological examinations.

Immunological indices

Phagocytic activity assay

Phagocytic action was assessed subsequent to established protocols [56], with slight modifications. Briefly, latex beads (10^7 beads mL^{-1}, 1.094 L; Poly sciences, USA) were added to the blood and set aside for 30 min at room temperature. Prepared smears were allowed to dry naturally, fixed with 95% ethanol, and stained with Giemsa. Under a microscope, 200 cells engulfing beads were counted. Phagocytic activity was calculated as follows: Phagocytic activity % = (Phagocytic leucocytes/ 200) x 100.

Respiratory burst activity

The oxygen radical production by phagocytes was determined employing the nitroblue tetrazolium (NBT; Sigma-Aldrich, USA).reduction test [57]. NBT (1 mg/ml) was diluted evenly with whole blood and incubated at 25 ° C for 30 min. After adding 1.0 mL of N, N-dimethylformamide solution, a centrifugal force of 3000 ×g for 5 min was applied to the NBT-blood cell suspension. A spectrophotometer (M Co. Germany) was employed to measure the optical density of the supernatant at 540 nm.

Total ig

Using a modified version of the Lowry method, the amount of total serum immunoglobulin (Ig) was calculated. To purify the immunoglobulin from the serum, a polyethylene glycol solution (10.000 kDa) (Sigma-Aldrich, USA) was used. In a nutshell, the serum was combined with 12% polyethylene glycol in the same volume. The mixture was permitted to stand for 2 h at ambient temperature, followed by a 15 min centrifugation at 3000 g. The Ig content is represented by the variation in protein content. Following the manufacturer's instructions, commercial kits (Stanbio laboratory, USA) were used to measure the protein levels.

Serum liver and kidney markers

Utilizing a spectrophotometer (Perkin Elmer), the activities of alanine aminotransferase (ALT, Catalog No.; MBS038444), aspartate aminotransferase ((AST, Catalog No.; EK12276) (from Maryland, USA), and lactate dehydrogenase (LDH, Catalog No.; MBS9902440) were assessed, in addition to the determination of total protein (Catalog No.; MBS9917835), albumin (Catalog No.; MBS019237) levels (from California, USA), urea concentration (Catalog No.; URE120120, from BioMed Co., Cairo, Egypt), creatinine levels (Catalog No.; LS-K207, from Lifespan Biosciences Co., USA), and uric acid levels (REF; 41001, from Spinreact Co., Santa Coloma, Spain) were determined according to the procedures outlined in their respective pamphlets.

Stress markers

Cortisol concentration (Catalog No.; CSB-E08487f) in serum, triglycerides (Catalog No.; AE63342FI-4800), and cholesterol levels (Catalog No.; EK12283) were evaluated utilizing test kits supplied by Cusabio Co. (Texas, USA), CLIN Sciences Co. (Nanterre, France), and Biotrend Co. (Maryland, USA), respectively. Moreover, the serum glucose level was determined using altered methods [58]. The serum lactate (Catalog No.; MET-5012) concentration was evaluated through the lactate oxidase method provided by a commercial kit manufactured by Cell Biolabs Company (based in California, USA).

Oxidative /antioxidant biomarkers

The method of measuring malondialdehyde (MDA) involved the use of thiobarbituric acid. After being detected at 535 nm, the thiobarbituric acid-reactive substances were displayed in the MDA generated, as demonstrated by a former methodology [59]. As well, the previous method [60] was followed to determine the hepatic and renal superoxide dismutase (SOD) activities. This procedure relied on the ability of epinephrine oxidation at pH 10.2, which produced superoxide and adrenochrome radicals. Reducing the absorbance at 480 nm was

used as a basis for investigating the suppression of SOD activity.

Ellman's methods [61] were used to demonstrate the glutathione (GSH) level in the head kidney, and liver, and the produced yellow chroma gene was measured to be 412 nm in length. The catalase activity in the liver and kidney supernatant using the procedure outlined was also estimated [62]. For the evaluation of the catalytic efficiency of catalase, the enzymatic reaction solution (with a total volume of 1 mL) comprised 50 mM potassium phosphate (pH 7.0), 19 mM H_2O_2, and 50 µL of homogenate supernatant. The absorbance of H_2O_2 was measured at 240 nm using a UV-VIS spectrophotometer. Catalase activity was quantified as the amount of enzyme that decomposes H_2O_2 (1 µmol) per minute per milligram of tissue protein (U/mg protein). Plasma total antioxidant capacity (TAC) was assessed spectrophotometrically following the prescribed procedures [63].

Histopathological assay

Fixed spleen and brain samples in 10% neutral buffered formalin for 24 h, were placed in tissue cassettes for further processing. These dissected tissues were dried out with an alcohol solution, cleansed with xylene, and impregnated with paraffin wax. After that, firmed tissues were cut off using a manual microtome (Rankin Biomedical Corporation, Leica RM2235) and sliced into Sect. 5 µm thick. Hematoxylin and eosin (H&E) staining was conducted on sections to facilitate microscopic evaluation (Olympus CX 31, Shinjuku-ku, Tokyo, Japan) [64].

Gene expression

RNA retrieval from tissue samples of each classification pre- and post-heat treatment, commercial total RNA (DNA-free) extraction kits (Easy-Spin™, iNtRON Biotechnology, Inc., Gyeonggi-do, Korea) were employed adhering to the manufacturer's instructions. The RNA concentrations were assessed at 260 nm and the OD260/OD280 ratio was determined utilizing a NanoDrop spectrophotometer (Thermo Scientific, Massachusetts, USA). Commercial DNA complementary strand formation kits (TOPScript™, Enzynomics Co. Ltd., Daejeon, Korea) were utilized for the synthesis of first-strand cDNAs *via* total RNA reverse transcription. Subsequently, gene expression qRT-PCR amplification reactions were conducted using the synthesized cDNAs and TOPreal™ 2× Pre-Mix SYBR Green qPCR master mix with low ROX (Enzynomics Co. Ltd., Daejeon, Korea) on a real-time PCR system (Rotor-Gene Q MDx 6 plex, Qiagen Co., Maryland, USA) [31]. The thermal profile of qRT-PCR program was 40 cycles at 95 ∘C (10 s), 60 ∘C (15 s), and 72 ∘C (30 s), followed by melt curve generation at 72 ∘C–95 ∘C for seconds. The designed primers were used to amplify the selected genes (Heat shock protein 70 (*HSP70*), Glutathione-S-transferase (*GST*), and Glutathione peroxidase (*GPx*)) of Nile tilapia. Beta-actin (*β-actin*) served as a housekeeping marker (Table 1). The comparative gene expression patterns were analyzed in triplicate utilizing the $2^{\wedge(-\Delta\Delta Ct)}$ technique, as described previously [65].

Statistical analysis

Following confirmation of normality through Shapiro-Wilk and Levene's tests, the data underwent analysis utilizing one-way ANOVA alongside post-hoc Tukey's multiple range tests. This examination was performed using GraphPad Prism version 8.0 (GraphPad Software, Inc., San Diego, California, USA), designed to evaluate hematological and biochemical indicators, along with immune response, antioxidant enzyme activities, and gene expression patterns. Results were presented as means ± SE for each parameter. parameter. Significance levels were denoted by different letters for probabilities of ($P<0.05$) when compared to pre-exposure controls, while significance at *; $P<0.05$, **; $P<0.01$, and ***; $P<0.001$ indicated variation compared to post-exposure controls.

Results

Effect of *Bacillus* supplement on growth indices and survivability

The impact of dietary mix of *Bacillus* spp. supplementation on growth metrics in Nile tilapia is listed in Table 2. In comparison with the control B_0 group, both

Table 1 Sequences of primer pairs used in the quantitative real-time PCR reactions

Gene	qPCR primers (5'-3')	GenBank number	Product size (bp)	Primer efficiency (%)	Annealing temperature
Beta actin-F *Beta actin*-R	CAGCAAGCAGGAGTACGATGAG TGTGTGGTGTGTGGTTGTTTTG	XM_003455949.2	143	96.84	58.50
Heat shock protein 70 -F *Heat shock protein 70*-R	TTCAAGGTGATTTCAGACGGAG CTTCATCTTCACCAGGACCATG	XM_019357557.1	85	97.40	58.00
Glutathione peroxidase -F *Glutathione peroxidase -R*	CCAAGAGAACTGCAAGAACGA CAGGACACGTCATTCCTACAC	DQ355022.1	107	94.00	58.00
Glutathione-S-transferase -F *Glutathione-S-transferase -R*	TAATGGGAGAGGGAAGATGG CTCTGCGATGTAATTCAGGA	XM_025897213.1	640	96.70	56.00

Table 2 Impact of dietary *Bacillus* supplement on the growth efficacy of Nile tilapia

	Groups		
Parameters	B_0	$B_{0.5}$	B_1
IBW (g)	49.33 ± 0.30	50.30 ± 0.20	50.67 ± 0.30
FBW(g)	71.00 ± 0.57^{b}	82.67 ± 1.20^{a}	85.00 ± 0.60^{a}
W.G. (g)	21.67 ± 0.34^{b}	32.33 ± 1.30^{a}	34.33 ± 0.40^{a}
WGR (%)	43.92 ± 0.59^{b}	64.26 ± 2.80^{a}	67.76 ± 0.57^{a}
SGR (%/d)	1.90 ± 0.04^{b}	2.40 ± 0.03^{a}	2.6 ± 0.00^{a}
SR (%)	96.67 ± 1.50	98.33 ± 1.70	100 ± 0.00

Note: Values were expressed as means ± SE (n = 3). The different superscript letters in the same row significantly varied when P < 0.05. Abbreviations: IBW, initial body weight; FBW, final body weight; WG; weight gain; WGR, weight gain rate; SGR, specific growth rate; SR: survival rate

supplemented groups with *Bacillus* for 58 days displayed significant (P < 0.05) augmentation in FBW, weight gain, WGR, and SGR. However, the survivability between groups was not markedly changed (Table 2).

Protective effects of *Bacillus* supplement on immune parameters pre- and post-exposure to heat stress

Immune reactions (phagocytic activity, NBT, and total Ig) of Nile tilapia fed experimental diets are illustrated in Fig. 1. Before heat, fish fed the *Bacillus* diet exhibited insignificant differences (P > 0.05) in phagocytic activity, NBT, and total Ig than the control. After heat shock (HS), all above-mentioned parameters substantively reduced (P < 0.05) in fish fed the $B_{0.5}$ and B_1 diets as opposed to the control pre-exposure group. Following heat exposure, the phagocytic activity (P < 0.01), NBT (P < 0.01), and total Ig (P < 0.001) were increased significantly in fish fed the $B_{0.5}$ diet with significant elevation only of NBT (P < 0.01), and total Ig (P < 0.001) levels in the B_1 group relative to the control-stressed group.

Protective effects of *Bacillus* supplement on serum biochemical parameters

Relative to the control-unstressed (pre-exposure) group and the other experimental cohorts, all fish groups consigned to heat exhibited a significant increase (P < 0.05) in all serum liver biomarkers (ALT, AST, and LDH) and kidney biomarkers (urea, creatinine, and uric acid), along with notable reduced total protein and albumin levels (P < 0.05). However, heat-stressed fish fed a probiotic diet ($B_{0.5}$) showed significantly lower levels of ALT (P < 0.001), AST (P < 0.01), LDH (P < 0.01), urea (P < 0.05), creatinine (P < 0.01), and uric acid (P < 0.01), with higher total protein (P < 0.01), albumin (P < 0.01), and globulin levels (P < 0.05) compared to the control-stressed group. Additionally, supplementation with a diet containing *Bacillus* probiotic (1 g kg^{-1}) induced a substantial decrease in serum ALT levels (P < 0.01), AST (P < 0.01), LDH (P < 0.01), urea (P < 0.05), and creatinine (P < 0.01) levels, with elevated total protein (P < 0.01) and albumin (P < 0.05) levels in Nile tilapia compared to control-stressed fish. Unaltered hepatic and renal biomarker profiles were recorded in the serum of Nile tilapia before exposure to heat, implying the innocuousness of probiotic supplementation on liver and kidney function (Table 3).

Data were represented as Mean ± SE (n = 5). a: significant with respect to the control (P ˂ 0.05). *: significant with respect to the heat-stressed untreated group; *; P < 0.05, **; P < 0.01, ***; P < 0.001.

Protective effects of *Bacillus* supplement on stress markers

Before the heat challenge, lower significant (P < 0.05) levels of triglycerides and cholesterol were observed in fish fed $B_{0.5}$ than in the control group (Fig. 2A and B). After heat stress, triglycerides, cholesterol, glucose, cortisol,

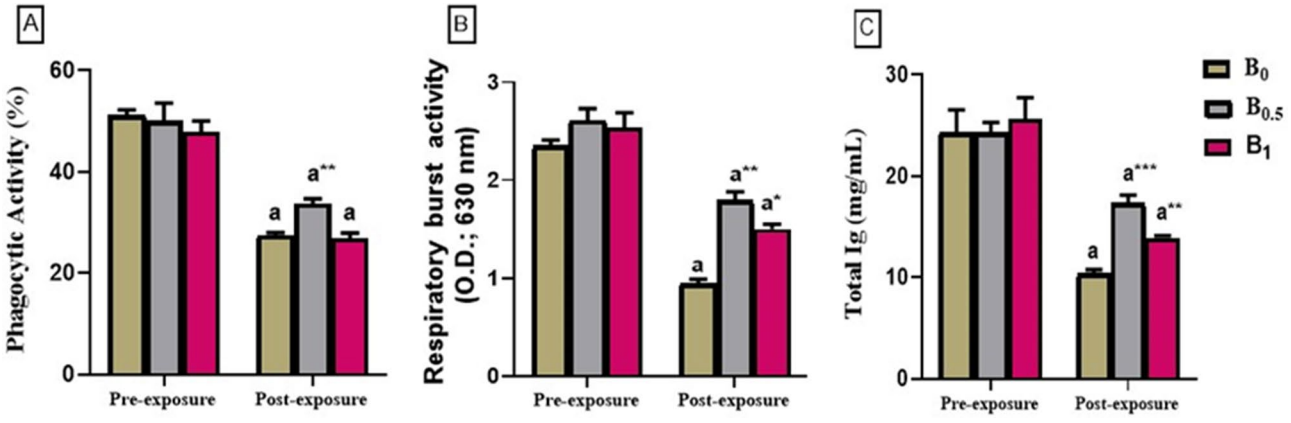

Fig. 1 Protective effects of mixed *Bacillus* spp. supplement on phagocytic activity (**A**), respiratory burst activity (**B**), and Ig level (**C**) of Nile tilapia pre- and post-exposure to heat stress. Three groups, B_0, $B_{0.5}$, and B_1 (pre-exposure) were identified as tested probiotics at 0, 0.5, and 1 g kg^{-1} diet, subsequently, they were exposed to heat stress for 2 hrs at 36 ˚C (post-exposure). Data were represented as Mean ± SE (n = 5). ^{a}P < 0.05 Represents a significant change against the control negative (pre-exposure control (B_0) while *' **' *** Significant variation at P < 0.05, P < 0.01, and P < 0.001, Represents a significant change against the control positive (post-exposure control (B_0)), respectively

Table 3 Protective effects *Bacillus* supplement on serum liver and kidney biomarkers of Nile tilapia pre- and post-exposure to heat stress

	Groups					
	Pre-exposure			Post-exposure		
Parameters	**B_0**	**$B_{0.5}$**	**B_1**	**B_0**	**$B_{0.5}$**	**B_1**
ALT (U/L)	12.46 ± 0.58	13.83 ± 1.76	12.61 ± 1.57	44.03 ± 1.74^{a}	22.66 ± 1.73^{a***}	29.44 ± 1.86^{a**}
AST (U/L)	54.23 ± 6.17	57.19 ± 8.17	61.39 ± 4.08	137.26 ± 3.32^{a}	87.84 ± 3.90^{a**}	96.83 ± 5.22^{a**}
LDH (U/L)	456.68 ± 18.05	446.81 ± 9.93	439.66 ± 9.50	648.84 ± 14.9^{a}	522.53 ± 13.97^{a**}	546.55 ± 16.64^{a**}
Total protein (g/dL)	6.43 ± 0.15	7.06 ± 0.41	6.89 ± 0.90	3.90 ± 0.16^{a}	5.65 ± 0.15^{a**}	4.67 ± 0.08^{a**}
Albumin (g/dL)	3.23 ± 0.06	3.39 ± 0.08	3.56 ± 0.16	1.04 ± 0.06^{a}	2.35 ± 0.23^{a**}	1.64 ± 0.07^{a*}
Globulin (g/dL)	3.19 ± 0.22	3.67 ± 0.48	3.33 ± 0.19	2.85 ± 0.10	3.29 ± 0.09*	3.03 ± 0.14
Urea (mg/dL)	18.79 ± 0.98	17.43 ± 1.18	19.27 ± 1.45	36.19 ± 1.91^{a}	20.80 ± 0.69^{a**}	30.15 ± 1.19^{a*}
Creatinine (mg/dL)	0.33 ± 0.04	0.37 ± 0.06	0.39 ± 0.08	01.04 ± 0.04^{a}	0.72 ± 0.04^{a**}	0.77 ± 0.05^{a**}
Uric acid (mg/dL)	2.34 ± 0.64	2.61 ± 0.34	2.25 ± 0.62	4.69 ± 0.36^{a}	3.56 ± 0.15*	3.86 ± 0.69

Data were represented as Mean ± SE ($n=5$). ^{a}: significant with respect to the control ($P<0.05$). *: significant with respect to the heat-stressed untreated group; *; $P<0.05$, **; $P<0.01$, ***; $P<0.001$

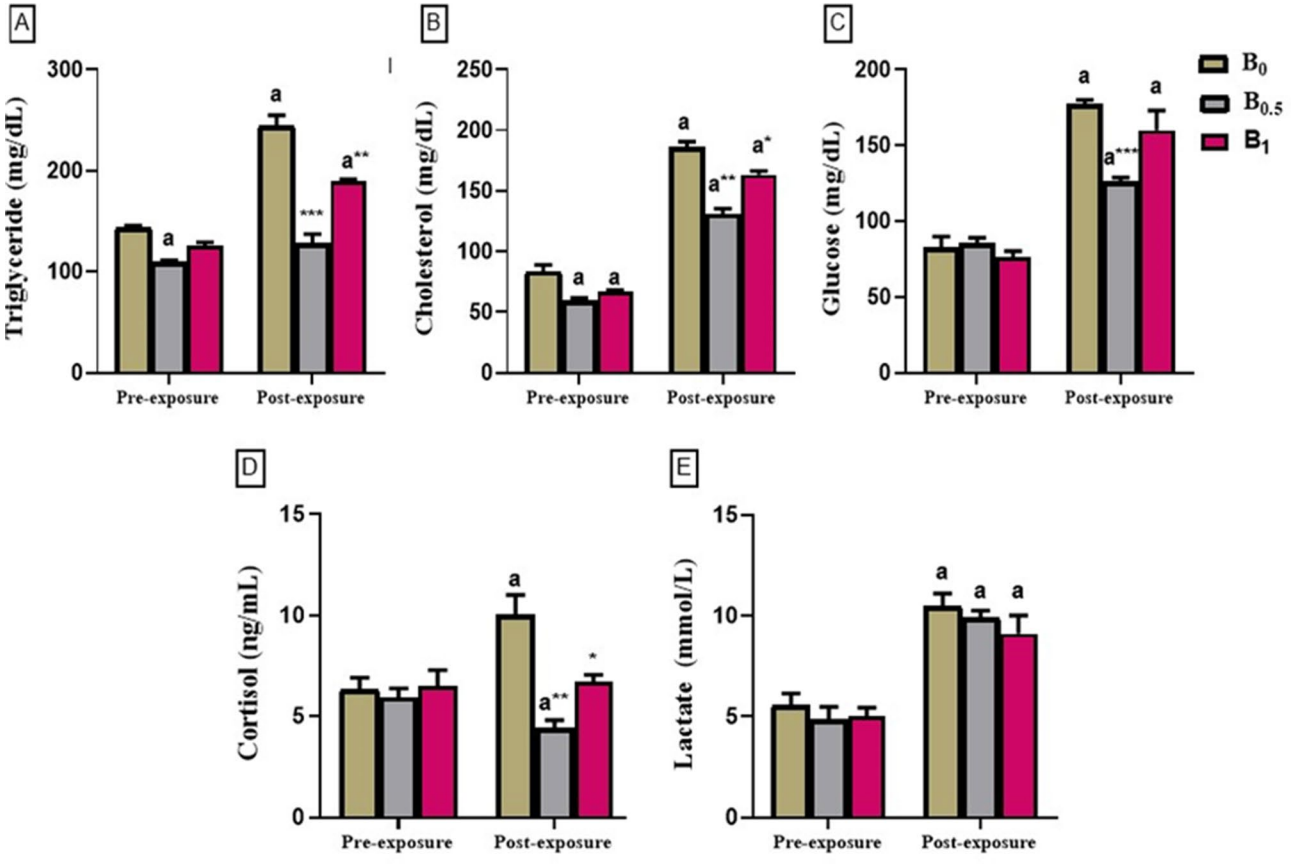

Fig. 2 Protective effects of mixed *Bacillus* spp. supplement on serum triglyceride (**A**), cholesterol (**B**), glucose (**C**), cortisol (**D**), and lactate (**E**) levels of Nile tilapia pre- and post-exposure to heat stress. Three groups, B_0, $B_{0.5}$, and B_1 (pre-exposure) were identified as tested probiotics at 0, 0.5, and 1 g kg^{-1} diet, subsequently, they were exposed to heat stress for 2 hrs at 36 ˚C (post-exposure). Data were represented as Mean ± SE (n = 5). $^{a}P<0.05$ Represents a significant change against the control negative (pre-exposure control (B_0) while */**/*** Significant variation at $P<0.05$, $P<0.01$, and $P<0.001$, Represents a significant change against the control positive (post-exposure control (B_0)), respectively

and lactate levels were statistically increased ($P<0.05$) in fish fed the control, $B_{0.5,}$ and B_1 diets compared to the control-unstressed group. The supplementation of *Bacillus* product in the fish diet resulted in lower triglycerides ($P<0.001$), cholesterol ($P<0.01$), glucose ($P<0.001$), and cortisol ($P<0.01$) levels in the $B_{0.5}$ group, while reduced only triglycerides ($P<0.01$), cholesterol ($P<0.05$) and cortisol ($P<0.05$) than in the control-stressed group.

Protective effects of *Bacillus* supplement on antioxidant status

Before HS exposure, MDA levels significantly decreased ($P<0.05$), whereas SOD and catalase levels increased significantly ($P<0.05$) in both the head kidney and liver of fish fed the $B_{0.5}$ diet relative to the control (Fig. 3A, B, and C). However, renal SOD and hepatic catalase were significantly elevated ($P<0.05$) in fish-fed B_1-supplemented diets compared to the control (Fig. 3B and C). Following heat stress, MDA levels remarkably enhanced ($P<0.05$), while antioxidant molecules (SOD, catalase, GSH) exhibited a significant reduction ($P<0.05$) in both the head kidney, and liver among the stressed groups compared to the control-unstressed group.

The introduction of a diet supplemented with *Bacillus* (0.5 g kg^{-1}) significantly suppressed MDA production ($P<0.01$) and boosted SOD ($P<0.01$) and catalase ($P<0.01$) activity in both the liver and kidney compared to the control-stressed group (B0). However, the B_1 supplement only reduced renal MDA ($P<0.05$) while increasing renal SOD ($P<0.01$) and hepatic catalase ($P<0.01$) compared to the control-stressed group. Notably, GSH levels remained unaffected ($P\geq0.05$) in the head kidney, and liver tissues across all groups following heat stress (Fig. 3).

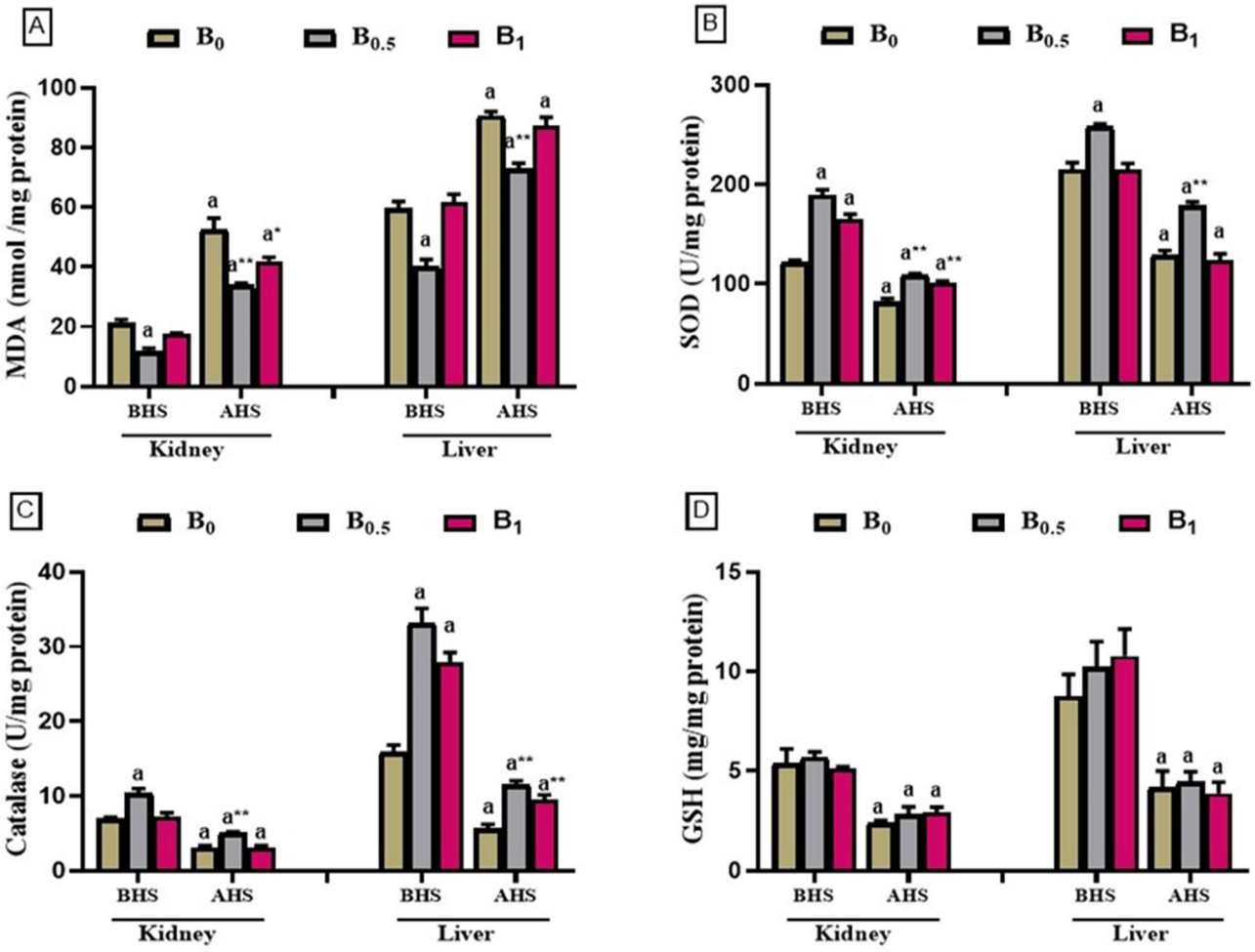

Fig. 3 Protective effects of mixed *Bacillus* spp. supplement on malondialdehyde (MDA, **A**), superoxide dismutase (SOD, **B**) catalase (**C**), and reduced glutathione (GSH, **D**) level in head kidney and liver of Nile tilapia before and after exposure to heat stress. Three groups, B_0, $B_{0.5}$, and B_1 (pre-exposure) were identified as tested probiotics at 0, 0.5, and 1 g kg^{-1} diet, subsequently, they were exposed to heat stress for 2 hrs at 36 °C (post-exposure). Data were represented as Mean ± SE (n = 5). $^{a}P<0.05$ Represents a significant change against the control negative (pre-exposure control (B_0), while $^{*/**/***}$ Significant variation at $P<0.05$, $P<0.01$, and $P<0.001$, Represents a significant change against the control positive (post-exposure control (B_0)), respectively

Effects of *Bacillus* supplement on the expression of HSP70 and antioxidant enzymes mRNAs

HSP70, *GST*, and *GPx* mRNA levels in the head kidney of Nile tilapia investigated pre- and post-exposure to acute thermal stress are shown in Fig. 4. HSP70 mRNA exhibited heightened expression in Nile tilapia subjected to heat compared to the pre-exposure control group ($P<0.05$), however, no significant change in *HSP70* mRNA was shown in probiotic-supplemented groups before heat exposure. In each probiotic group following heat exposure, *HSP70* mRNA expression was significantly decreased compared to the post-exposure control group ($P<0.01$; $P<0.001$). There was no significant change in the expression of *GST* and *GPx* mRNAs in the probiotic groups before heat stress compared to the pre-exposure control group. The probiotic B1 group markedly elevated the activity of *GST* and *GPx* mRNA enzymes ($P<0.05$; $P<0.01$) after exposure to heat stress relative to both the post and pre-exposure control group.

Spleen and brain histopathological findings

The histopathological examination of the spleen revealed typical white and red pulp components with a normal distribution of melanomacrophage centers in the pre-exposure control (B_0) group (Fig. 5A). Besides, there are extensive aggregations of melanomacrophage centers through the splenic tissue of Nile tilapia in the supplemented groups ($B_{0.5}$ & B_1) (Fig. 5B, C). In post heat exposure (B_0) group, characteristic hemorrhagic splenitis was observed with extensive hemorrhage mixed with lymphocytic infiltration and small-sized melanomacrophage center within splenic parenchyma (Fig. 5D). In the post-heat supplemented groups ($B_{0.5}$ & B_1), there was minimal hemorrhage with no necrosis in the spleen, revealing relatively increased melanomacrophage center around the splenic bulb (Fig. 5E, F).

The neuropil, neurons, and nervous parenchyma in the brains of Nile tilapia not exposed to heat showed a standard histological appearance (Fig. 6A-C). Meanwhile, control posts showing diffuse neutrophil spongiosis with inflammatory infiltrations were the most evident alterations in the brain of Nile tilapia exposed to heat (Figure.6D). In the *Bacillus*-supplemented group and exposed to heat, restoration of most nervous tissue architecture with minimal neutrophil vacuolation was revealed (Fig. 6E-F).

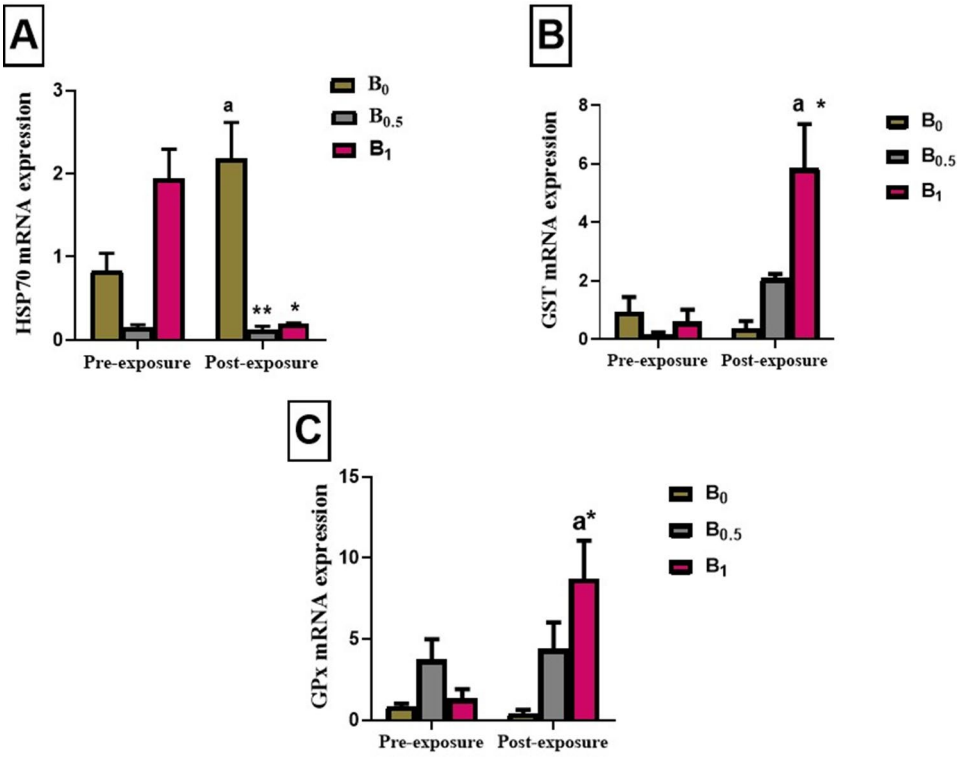

Fig. 4 The mRNA expression of heat shock protein-70 (*HSP70*, **A**), glutathione S-transferase (*GST*, **B**), and glutathione peroxidase (*GPx*, **C**) genes in the head kidney of Nile tilapia were fed diets supplemented with different levels of tested *Bacillus* probiotic mixture for 8 weeks. Three groups, B_0, $B_{0.5}$, and B_1 (pre-exposure) were identified as tested probiotics at 0, 0.5, and 1 g kg^{-1} diet, subsequently, they were exposed to heat stress (post-exposure). Data in the same row assigned with the different superscripts are significantly different ($p<0.05$). Data were represented as mean ± SE of triplicate assays and were normalized to the β-actin mRNA levels. $^{a}P<0.05$ indicates a significant change as compared with pre-exposure control (B_0); $^{*/**}$ Significant variation at $P<0.01$ and $P<0.001$, as compared to the post-exposure control (B_0), respectively

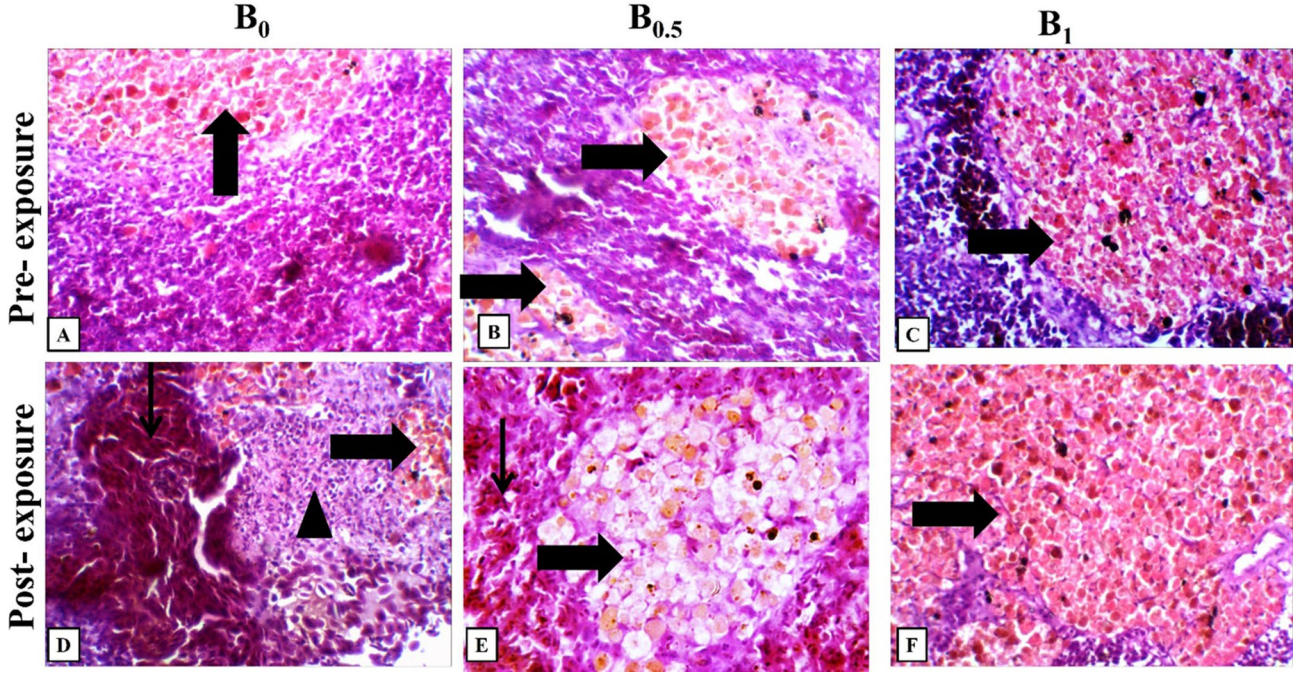

Fig. 5 Representative photomicrograph of spleen sections of Nile tilapia fed with *Bacillus* spp. and followed by exposure to heat stress. **A**) Pre-exposure control group (B_0) showing mixed red and white pulp with presence of melanomacrophage center (thick arrow). **B**) Pre-exposure group ($B_{0.5}$) showing multifocal aggregation of small-sized melanomacrophages (thick arrows). **C**) Pre-exposure group (B_1) showing extensive expansion of melanomacrophages (thick arrow). **D**) Post-exposure control group (B_0) showing marked hemorrhagic splenitis characterized by replacement of splenic parenchyma by extensive hemorrhage (thin arrow) admixed with high numbers of cellular infiltrates (arrowhead) and small-sized melanomacrophage center (thick arrow). **E**) Post-exposure group ($B_{0.5}$) showing moderate to severe expansion of melanomacrophage (thick arrow) with minimal parenchymal hemorrhage (thin arrow). **F**) Post-exposure group (B_1) showing marked expansion of activated melanomacrophages replacing the splenic pulps (thick arrow). Image magnification = 400x

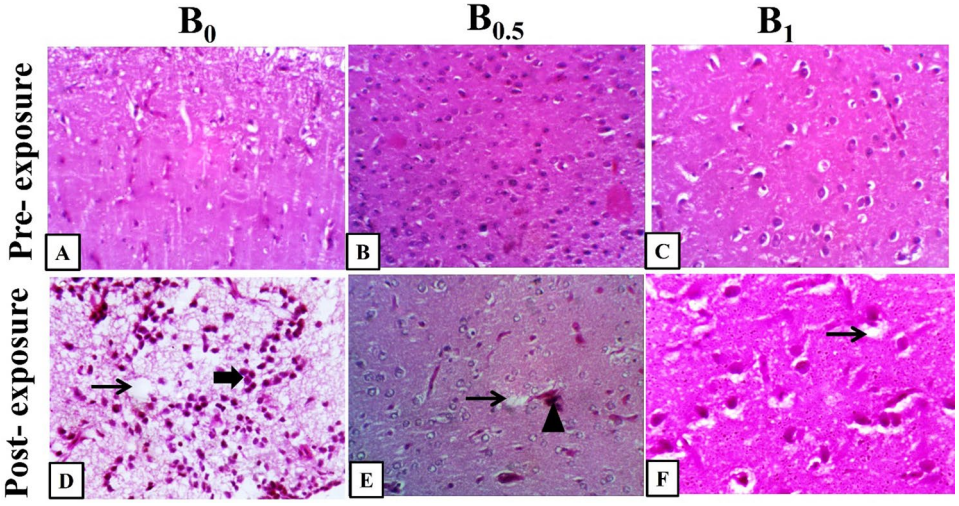

Fig. 6 Representative photomicrograph of brain sections of Nile tilapia fed with *Bacillus* spp. and followed by exposure to heat stress. **A**, **B**) Pre-exposure control (B_0) and pre-exposure group ($B_{0.5}$) groups showing normal histological appearance of neuropil and neurons. **C**) Pre-exposure group (B_1) showing normal histological appearance of nervous parenchyma. **D**) Post-exposure control group (B_0) showing diffuse neutrophil spongiosis (thin arrow) with numerous invading cellular infiltrates (thick arrow). **E**) Post-exposure group ($B_{0.5}$) showing minimal neuropil vacuolation (thin arrow) with congested blood vessels (thick arrow). F) Post-exposure group (B_1) showing restoration of most nervous tissue architecture with few neuropil vacuolation (thin arrow). Image magnification = 400x except A = 100X

Discussion

Many investigations have verified the potential role of probiotics in aquatic systems against the noteworthy losses of environmental high-temperature stress [19, 66]. In this regard, we explored the consequences of administering dietary *Bacillus* strains to prohibit heat stress-stimulated physiological and pathological change in Nile tilapia.

An evident rise in the values of FWB, WG, WG%, and SGR was noted across all supplemented groups in contrast to the control group. Similarly, notable enhancement in growth was observed in Nile tilapia fingerlings receiving 0.2 g/kg of mixed *Bacillus* spp. for 10 weeks [38]. The growth indices were also increased in Gilthead sea bream (*Sparus aurata*) larvae supplemented with three *Bacillus* strains (*B. subtilis*, *B. licheniformis*, and *B. pumilus*) [67]. This suggests that a higher dosage of *Bacillus* strains probiotic supplementation could effectively enhance the growth and feed utilization of tilapia through the activation of digestive enzymes and promoting the absorption of nutrients from the feed by the fish [31].

The essential enzymes AST, ALT, and LDH support protein metabolism in fish liver [68, 69]. However, when these enzymes are prominently released into the blood or serum, they can indicate organ failure or tissue damage induced by stress [70]. In our results, following exposure to heat stress, a substantial rise in all serum ALT, AST, and LDH levels as well as, urea, creatinine, and uric acid biomarkers along with lower T. protein, albumin, and globulin levels. Interestingly, there have been considerable increases in AST and ALT activities assayed in the serum of yellow perch (*Perca flavescens*) and Nile tilapia following exposure to heat stressors [71, 72]. The elevated ALT, AST, and LDH levels could be related to oxidative stress and liver impairment [73]. Furthermore, fish experiencing heat stress exhibited elevated levels of renal tissue-related indices [74, 75]. Elevated creatinine levels result from the breakdown of creatinine in the muscles of fish, which is subsequently excreted through the kidney [76]. Urea also shows that fish bodies under stress have a high metabolic rate and an excessive frequency of damaged tissues [77]. Conversely, fish exposed to heat and fed a probiotic diet ($B_{0.5}$ & B_1) had notably lower ALT, AST, LDH, urea, creatinine, and uric acid besides higher T. protein, albumin, and globulin levels than in the control-stressed group. Similarly, it has been noted that giving probiotic-enriched diets to tilapia significantly lowers their AST, ALT, and LDH levels [78]. Nile tilapia exposed to higher temperature had moderately lower levels of AST, ALT, and LDH activities in the supplemented *Bacillus* groups than in the control one. Additionally, there was a substantial decrease in ALT, urea, and creatinine whereas there was a marked increase in total protein, globulin, and albumin with the addition of *B. amyloliquefaciens* (1×10^{10} CFU/g) probiotics in *V. anguillarium*- infected Nile tilapia [79]. Renal dysfunction, a decline in probiotic groups, and their contributions to improving renal histology could be the cause of this. These outcomes are consistent with those authors, who demonstrated that Nile tilapia fingerlings, displayed a notable decrease in all probiotics-treated groups' urea, creatinine, ALT, and AST in contrast to the control group [80]. The level of ALP enzyme was increased after the addition of mixed *Bacillus* species (*B. licheniformis* and *B. amyloliquefaciens*) to live feed of Common snook larva (*Centropomus undecimalis*) following transportation stress [81]. Administration of probiotic supplements notably enhanced the levels of liver function biomarkers, suggesting that dietary *Bacillus* spp. could protect hepatocyte integrity and alleviate liver damage under heat conditions, as evidenced by decreased liver enzyme activity (ALT and AST). This phenomenon could be attributed to the supplements' ability to significantly boost the antioxidant defenses in fish, along with reducing MDA levels in the liver. This, in turn, protects the liver from damage caused by reactive oxygen species (ROS) and decreases the activities of ALT and AST in the fish serum [82].

Cortisol and glucose levels are frequently valid markers for the investigation of fish's physiological response to many environmental stressors [7] through regulating the organism's growth, metabolic energy, hemostasis, and immune capability [10], subsequently, elevated cortisol levels may have adverse effects on these previously mentioned responses [83]. To lessen the negative impacts of stress on aquatic species, blood glucose levels are a secondary response that is often sequestered for further energy generation [84]. Based on our findings, the serum triglycerides, cholesterol, glucose, cortisol, and lactate levels were statistically increased after the heat stress. The elevated levels of triglycerides and total cholesterol following heat stress have similarly been seen in Striped catfish [85], Wuchang bream, *Megalobrama amblycephala Yih* [86], and channel catfish, *Ictalurus punctatus* [87]. Heat stress causes lipid metabolism to fail, which leads to a major reinforcement of lipids in the blood and elevated levels of triglycerides and cholesterol [88]. Like our results, there was a rise in the plasma concentrations of lactate, glucose, and cortisol between 0 and 12 h after the heat stress was applied [89]. Due to those measurements being generally accurate indicators of different types of stress, it was revealed that heat exposure caused metabolic stress in Rohu fish [66, 90]. Additionally, cortisol and glucose levels of African catfish cultured in high heat stress conditions were significantly elevated [91]. As well, higher glucose and cortisol levels were seen in Striped catfish cultivated in hyper-salinity and high temperatures [92]. The increase in glucose levels might be

associated with stress, as stress has been demonstrated to trigger the release of catecholamines, leading to the accelerated breakdown of glycogen and the subsequent rise in blood glucose levels [93]. The rise in lactate levels could be attributed to the immediate post-stress glycolysis of muscles [94]. This is consistent with the previously documented findings that heat stress caused higher production of lactate in *Labeo rohita* fingerlings [95], and in Olive flounder, *Paralichthys olivaceus* and Turbot, *Scophthalmus maximus* [96].

In our results, lower cholesterol and triglyceride level was noticed in fish fed the $B_{0.5}$ and B_1 diets. This corresponds to the production of short-chain fatty acids in the gut, which occurs as probiotic bacteria ferment indigestible carbohydrates from food. These fatty acids can subsequently decrease systemic lipid formation in the blood by preventing cholesterol synthesis in the liver and/or facilitating the transfer of cholesterol from plasma to the liver [97]. Previous reports also illustrated that the probiotic-fed groups (*Lactobacillus plantarum*) had significantly lower total cholesterol and triglycerides than the control common carp (*Cyprinus carpio*) [98]. Consistent with our results, lower cortisol and glucose levels were observed in Gilthead seabream (*Sparus auratus*) and Porthole livebearer (*Poecilopsis gracilis*) following the consumption of *lactobacillus casei* probiotic formulations [99, 100]. Moreover, it was found that supplementing Nile tilapia with *Aspergillus oryzae* under hypoxia stress led to decreased levels of glucose, and cortisol levels [45].

Fish phagocytosis is a crucial part of their cellular immune system [101]. Moreover, fish defense mechanisms often rely on respiratory bursts (NBT), which have been shown to enhance phagocyte oxidation levels when triggered by foreign substances [102]. Blood immunoglobulins (total Ig) serve as an additional marker of the strengthened immune system in fish [103]. Our data revealed that phagocytic activity, NBT, and total Ig of Nile tilapia decreased significantly after exposure to HS. Similarly, Striped catfish [85] and Nile tilapia [104] exposed to heat stress had impaired phagocytic activity. It also clarified that the fish exposed to heat stress had a clear decrease in their phagocytic index alongside phagocytic and lysozyme activities [45]. According to W Cheng, S-M Chen, F-I Wang, P-I Hsu, C-H Liu and J-C Chen [105], *Macrobrachium rosenbergii*, a large freshwater shrimp, showed a decreased respiratory burst activity in response to higher temperature exposure. The lower immune response in heat-stressed fish is attributed to the ROS production during heat exposure, subsequently leading to impairments in their immune system, blood biochemistry, and physiological functions [85]. Additionally, Nile tilapia had lower levels of IgM after exposure to 40 °C [106]. The decrease in the blood immunoglobulins may be due to stress and can also have an impact on the values of the blood protein profile including globulins which can lead to immune suppression [107].

Our findings also showed that the fish fed the $B_{0.5}$ and B_1 diets displayed considerably increased phagocytic activity, NBT, and total Ig of after-exposure HS compared to heat-stressed fish. Similarly, Nile tilapia fed on probiotic diets showed effective bactericidal and phagocytosis properties, according to N Pirarat, K Pinpimai, M Endo, T Katagiri, A Ponpornpisit, N Chansue and M Maita [108]. Additionally, a study found that adding *B. licheniformis* Dahb1 to Striped catfish diets boosted the enzymatic and non-enzymatic antioxidant responses and, subsequently, humoral and cellular immunological responses [109]. The fish fed the *Aspergillus oryzae*-containing diets also showed significantly higher IgM levels, and phagocytic and lysozyme activity in Nile tilapia after HS when compared to the control group [45]. Increased phagocytic activity, NBT, and total Ig indicate that probiotics may have an immunomodulatory impact on Nile tilapia after heat shock.

Elevated secretion of malondialdehyde (MDA) signifies increased levels of lipid peroxidation. In response to this condition, cells develop anti-oxidant activities to degrade excess free radicals and ROS [110]. Key biomarkers that alleviate the impacts of oxidative stress throughout an organism's body include GSH, catalase, and SOD [111]. The stress conditions cause the generation of ROS, which oxidizes lipids in membranes and impairs cellular function [112]. A previous study revealed suppressed antioxidative responses (SOD, CAT, and GPx) with higher MDA levels in Striped catfish after heat stressors [85]. As well, increased MDA levels with lower SOD and catalase activities were reported after exposure to Blunt snout bream (*Megalobrama amblycephala*) to heat stress [89]. Increased formation of ROS can be attributed to higher water temperatures, which also accelerate oxygen consumption [113]. Mainly, oxidative stress and lipid peroxidation induced by heat stress were the primary factors contributing to the rapid decrease in SOD, CAT, and GPx activities, along with the significant increase in MDA levels observed in Nile tilapia [114]. Antioxidant enzyme depletion during the process of combating created ROS to keep the stabilized-state level of generated free radicals may be the reason for the drop in the antioxidant enzyme activities during heat stress exposure [115].

Our findings indicated that the inclusion of *Bacillus* supplements in the diet (0.5 and 1 g kg^{-1}) markedly suppressed the production of MDA while enhancing the efficacy of SOD and catalase in both the liver and kidney. Studies have reported the dietary utilization of probiotic *B. licheniformis Dahb1* at 10^7 cfu g^{-1}, in *Oreochromis mossambicus* [29] resulted in elevated antioxidant enzyme activities (CAT and GPx) following ammonia

toxicity. Similarly, recent research has shown that Nile tilapia supplemented with a mixed diet containing Chinese herbs and a *Bacillus* species exhibited increased actions of total antioxidant capacity (T-AOC), SOD, and CAT enzymes, which suggests that herbal-probiotic supplements can boost fish's antioxidant capacity both over the environmental stress conditions [43]. Moreover, supplementing Nile tilapia with *B. subtilis* DSM 32,315 under higher ammonia stress led to marked decreased levels of MDA as well as increased levels of T-AOC efficacy of Nile tilapia compared to the control [116].

Heat-stress responses are associated with higher expression of heat shock proteins (*HSPs*) to maintain the structures of damaged proteins of aquatic organisms in response to various ecological stressors (e.g., cold, heat, toxins, microbes, nutritional deficiency, Ammonia toxicity, hypoxia) [111, 114]. Under stress conditions, molecular chaperones *HSPs* to restore the protein misfolding deformities from cellular damage [9, 10]. HSPs are assorted into many families, such as *HSP100*, *HSP90*, *HSP70*, *HSP60*, *HSP40* [117]. In aquatic animals, the relative expression of *HSP70* is a potent stress indicator, promoting thermotolerance and protection from heat stress [114]. In this study, it is evident that heat shock motivated the expression of head kidney *HSP70* in Nile tilapia to boost hypoxic endurance conditions and anaerobic energy production; thus *HSP* gene elevates the oxygen demand to interact with other signaling genes against oxidative damage and cellular apoptosis during stress [68]. Besides, significantly decreased expression of this gene in the probiotic-fed fish after heat challenge, suggests an anti-stress response. However, induced expression of *HSP70* was observed in Nile tilapia fed with commercial probiotic *Bacillus* species before and after the heat challenge [43]. In another study, the dietary supplementation of mixed *Bacillus* probiotics (*B. licheniformis* MAt32, *B. subtilis* MAt43 and *B. subtilis* subsp. *subtilis* GAtB1) reduced the mRNA expression of *HSP70* in the hepatopancreas of *L. vannamei* infected with white spot syndrome virus (WSSV) [118]. A significant down-regulation of the mRNA expression of hepatic *HSP70* was noted in Nile tilapia supplemented with *Aspergillus oryzae* probiotics [119]. It deduced that dietary supplementation of *Bacillus* probiotics could enhance heat tolerance by alleviating the deleterious effect of heat stress in Nile tilapia. Stress-reducing factors generated by probiotics could have a role in lowering the levels of HSP in fish-fed probiotic mixture (*B. subtilis*, *Lactococcus lactis*, and *Saccharomyces cerevisiae*) at higher temperature [36].

Thermal stress in fish farming can elevate metabolic activity and contribute to dissolved oxygen depletion. This, in turn, leads to increased fabrication of ROS, facilitated by SOD into hydrogen peroxide (H_2O_2), which is further broken down into water molecules by either GPx or CAT enzymes [120]. Both GST and GPx are integral components of the antioxidant defense system, playing crucial roles in detoxifying hydroperoxides into water (H_2O) and hydroxyl molecules [121]. Nile tilapia experiencing heat stress and fed diets enriched with *Bacillus* exhibited elevated expression levels of antioxidant enzymes, *GST*, and *GPx*, suggesting a significant enhancement in the antioxidative capability of the head kidney. This improvement was further evidenced by heightened activity levels of SOD and CAT enzymes in both the liver and head kidney, accompanied by reduced levels of MDA. Previous research similarly demonstrated that Nile tilapia fed with 0.1% and 0.3% *B. subtilis* probiotics diminished the reactive oxygen species produced by ammonia stress via upregulation of *SOD* mRNA gene expression [116]. Similarly, the up-regulation of *SOD* gene expression was shown in shrimp infected with WSSV and fed with *B. licheniformis* MAt32, *B. subtilis* MAt43 and *B. subtilis* subsp. *subtilis* GAtB1 [118]. Thus, dietary mixed *Bacillus* spp. probiotic displayed a robust protective effect against oxidative stress induced by heat shock that is related to the response of *Bacillus* to increase the phagocytic and respiratory burst activities during environmental stressors.

The histopathological findings further corroborated all aforementioned results, shedding light on the pathological changes resulting from diverse environmental stressors [122, 123]. Precisely, monitoring of structural modifications reveals the sub-lethal negative damage caused by heat stress [124]. We also observed similar results with functional pathological modifications in fish brains and other immune organs, such as the spleen and liver caused by elevated water temperature in the rearing water [7, 71, 125–127]. Higher water temperature provokes excessive ROS production which can damage the phospholipid membrane of cellular components, causing metabolic and inflammatory dysfunctions in the tissues [128]. Of note, the addition of *Bacillus* mixture probiotics to the Nile tilapia's diet improved the degenerative changes associated with heat stress in the examined spleen and brain tissues. In the same context, a marked improvement in the spleen structure of the Nile tilapia fed on a natural fed on symbiotic mixture containing *Lactobacillus plantarum* probiotic under deltamethrin toxicity and cold water temperature [129]. The ameliorative impact of *Bacillus* spp. probiotic is proposed to increase the immunomodulatory capabilities of lymphoid splenic tissue via the improvement of erythro-phagocytic activity after an increase in the lymphocyte population [19], as well as to enhance the attachment and migration of neutrophils to other immune cells, thus boosting the immune system [130]. Moreover, the addition of *Bacillus* spp. to tilapia diets bolstered the performance of particular immune cells, including lymphocytes, granulocytes,

macrophages, and mast cells, through the stimulation of various specific cytokines' release [131].

Conclusion

Combining the above findings, dietary multispecies of *Bacillus* probiotic supplementation resulted in better physiological and immunological indices and antioxidant activity of the Nile tilapia juveniles cultured at higher water temperature. Overall, the application of a lower dose of *Bacillus* probiotic showed the most stated improvement in these physiological and anti-stress reactions and pathological alterations. So, the low level of the *Bacillus* probiotic ($B_{0.5}$) revealed better effects than B_1 in most assented parameters except the gene expression. These enhanced findings suggest that mixed *Bacillus* species may be useful as a feed supplement to mitigation of heat stressors in commercial tropical tilapia farms prone to high temperatures.

Acknowledgements
The authors thank the staff of Aquatic Animal Medicine Department for their kind cooperation.

Author contributions
Samia Elbahnaswy, Gehad E. Elshopakey, and Mai A.M. El-Son wrote the main manuscript, performed the experiment, and prepared the tables and figures. Abdelwahab A. Abdelwarith, Elsayed M. Younis, and Simon J. Davies investigated and reviewed the manuscript.

Funding
Open access funding is provided by The Science, Technology & Innovation Funding Authority (STDF) in cooperation with The Egyptian Knowledge Bank (EKB). Also, this research was supported by the Researchers Supporting Project Number; RSPD2024R700, King Saud University, Riyadh, Saudi Arabia.

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

Declarations

Ethics approval and consent to participate
Ethical approval for the current study has been obtained from the Institutional Ethics Committee at the Faculty of Veterinary Medicine, Mansoura University (MU-ACUC), Egypt, with the assigned sequential authorization code (VM.R.24.01.151). All authors have participated in this work. We obtained informed consent from the owners of the fish involved in the study. All methods were performed following the relevant IACUC guidelines and regulations. The researchers handled and transported live fish from a private hatchery farm and chose fish for experimental use.

Consent for publication
Not Applicable.

Competing interests
The authors declare no competing interests.

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Received: 20 July 2024 / Accepted: 25 September 2024
Published online: 16 October 2024

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