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Saccharomyces crevices and *Bacillus* spp. effectively enhance health tolerance of Nile tilapia under transportation stress

Nantaporn Sutthi[a], Hien Van Doan[b,c,*]

[a] Department of Agricultural Technology, Faculty of Technology, Mahasarakham University, Maha Sarakham 44150, Thailand
[b] Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University, 50200, Thailand
[c] Science and Technology Research Institute, Chiang Mai University, 239 Huay Keaw Rd., Suthep, Muang, Chiang Mai 50200, Thailand

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ABSTRACT

The effects of added probiotics on growth performance, blood chemical, and hematological responses; as well as immune-related gene expression in Nile tilapia (*Oreochromis niloticus*) under transportation stress were evaluated. Fish were treated over a period of 120 days in three groups: T_1, the control group (no probiotic), T_2, 5.6×10^8 cfu g^{-1} of *Saccharomyces cerevisiae*, and T_3, 1×10^6 cfu g^{-1} of *Bacillus* spp. The fish were then exposed to the transportation stress experiment for four hours (h). The resulted revealed that there were no significant differences in survival rate during and after transportation in all treatments. However, the survival rate of the control group significant decreased ($p < .05$) after 24 h post transportation stress, and significantly higher rate of cortisol, glucose, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were observed in the T_1 group, compared to the T_2 and T_3 groups. However, significantly lower malondialdehyde (MDA) was found in T_3 compared to those of T_1 and T_2 after 24 to 72 h post-transportation. The expression of heat shock proteins (*Hsp*70) and tumor necrosis factor-alpha (*TNF*-α) levels in the control group were significantly higher ($p < .05$) than those of T_2 and T_3 groups, especially at 0 h post-transportation stress. No significant differences in lysozyme activity and hematological profiles were detected between the control (T_1) and probiotic groups (T_2 and T_3). In conclusion, Nile tilapia reared for 120 days in aqueous probiotic showed significantly improved health tolerance under transportation stress.

1. Introduction

Fish and shrimp transportations, a commonplace activity in aquaculture practice, is known to cause mechanical stress and water quality deterioration (Herrera et al., 2019a; Hoseini et al., 2019). The transport phase consists of multiple procedures, both pre-transport and during-transport processes, which are stressful to fish (Dhanasiri et al., 2013; Pakhira et al., 2015a). The stress caused by transport also leads to a suppression of fish production, increased mortality (Tacchi et al., 2015), susceptibility to diseases (Cardoso et al., 2017), and increased culture costs (Refaey and Li, 2018). Consequently, transport procedures must be planned to reduce tension (Harmon, 2009). Various fish species also react to transportation pressures by eliminating their amounts of circulation catecholamines and corticosteroids, such as cortisol hormone, which are considered primary stress response bio-markers (Wendelaar Bonga, 1997). An increase in cortisol level is accompanied by several secondary responses, such as increased blood glucose content, and altered homeostasis of the electrolytes (Barton and Iwama, 1991; Magnoni et al., 2019). Metabolic changes, such as hyperglycaemia, hyperlactaemia, and hypercholesterolaemia also occur (Jerez-Cepa et al., 2019; Parodi et al., 2014; Zeppenfeld et al., 2014).

In aquaculture, methods of mitigating pressure throughout transporting are therefore of concern to aquaculturists (Refaey and Li, 2018), in which various zootechnical schemes and variables are adapted to obtain maximum animal welfare without influencing their productive yield (Herrera et al., 2019a). In addition to the technical and infrastructural improvements, the use of modern feeding methods is a realistic and straightforward way of enhancing fish welfare. The concept of functional food has emerged as a novel approach to enhance their overall health and welfare (Pérez-Sánchez et al., 2014). One such approach is the application of probiotics, which are live, favourable microorganisms administrated into either feed or water; to enhance water quality, digestion, and immune functions (Tarnecki et al., 2019). Probiotics occupy larval fish, promoting health through competitive exclusion and production of antimicrobial compounds against pathogenic and opportunistic bacteria (Pérez-Sánchez et al., 2014). Stimulation of

* Corresponding author at: Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand.
E-mail address: hien.d@cmu.ac.th (H.V. Doan).

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the larval fish's innate immune system through probiotics offers a fast triggering of antigens, which increases survival rates and pathogen resistance (Magnadóttir, 2006). The up-regulation of certain enzymes that enable the organism to cope with cell stress is critical to the enhancement of innate immunity (Tarnecki et al., 2019). Several probiotics, such as *Bacillus*, stimulate the production of these crucial enzymes. *Bacillus* probiotics are promising for commercial production, because they can form spores, allowing for greater shelf life (Balcázar et al., 2006). Many *Bacillus* species regulate the growth of opportunistic pathogens (Xu et al., 2014) and produce anti-viral compounds (Arena et al., 2006). Moreover, the supplementation of probiotics via live feeds, such as rotifers, Artemia, and copepods; has led to enhance fish health through increasing survival, growth rates, and enzymatic immune responses (Avella et al., 2010; Sun et al., 2013).

Tilapia, one of the world's most commonly cultivated fish (Delphino et al., 2019; Machimbirike et al., 2019), is cultured in over 100 countries, due to its rapid growth, adaptability, and high market value (Prabu et al., 2019). However, the intensification and expansion of tilapia cultivation have led to stresses on the quality of cultivated water, and have increased the susceptibility of infectious diseases, especially bacterial diseases. (Assefa and Abunna, 2018; Chen et al., 2019). This results in a high mortality rate for farmed fish and severe economic damages (Chen et al., 2019). *Aeromonas* spp. and *Streptococcus* spp. rank among the most frequent offenders causing significant financial losses in tilapia production (AlYahya et al., 2018; Fawzy et al., 2014; Neamat-Allah et al., 2019; Zahran et al., 2019). Antibiotics and chemotherapeutics have been implemented worldwide to inhibit and treat these diseases (Okocha et al., 2018; Santos and Ramos, 2018; Watts et al., 2017). Nevertheless, extensive use of such compounds has led to the establishment of antimicrobial-resistant bacteria, antimicrobial residues in fishery products, environmental hazards, and changes in the structure and diversity of aquaculture ecosystems (Done et al., 2015; Hong et al., 2018). Having determined that an ecologically friendly solution to the control of diseases in aquaculture is therefore needed, the present study was conducted to evaluate the effects of probiotics on survival, blood chemical, hematological, and gene expression of Nile tilapia, *Oreochromis niloticus* under transportation stress.

2. Materials and methods

2.1. Fish preparation

Four hundred and fifty Nile tilapia (*Oreochromis niloticus*) fingerlings were used in this study, conducted at Khon Kaen Inland Fisheries Research and Development Center, Khon Kaen, Thailand. Fish were randomly distributed at a rate of 50 fish per tank into nine fiber glass tanks (volume 1000 L). A completely randomized design (CRD) with three treatments and three replications was applied. Three treatments of varied aqueous probiotic supplements were performed over a 120-day period, as follows: T_1, the control group (no probiotic); T_2, with the addition of 5.6×10^8 CFU g^{-1} of *Saccharomyces cerevisiae*; and T_3, 1×10^6 CFU g^{-1} of mixed *Bacillus* spp. (*B. subtilis*, *B. megaterium*, and *B. licheniformis*). Aqueous probiotic supplements were administered according to the method described by Sutthi et al. (2018). Briefly, 1 g of Baker's yeast (Perfect®, Thailand) at a concentration of 5.6×10^8 cfu g^{-1} mixed with 5 g of sugar and 1.5 g of glutinous rice flour and then added to 1 L of deionized water, mixed, and incubated at room temperature for 72 h. The *Bacillus* spp. (*B. subtilis*, *B. megaterium*, and *B. licheniformis*) was obtained from the Coastal Aquaculture Research and Development Regional Center 2 (Samut Sakhon, Thailand). The preparation consisted of adding 1 g of *Bacillus* spp. at a concentration of 1×10^6 cfu g^{-1}, mixed with 5 g of brown sugar and 5 g of commercial fish feed in 2.5 L of deionized water. The mixture was then incubated at room temperature for 36 h. Lastly, the aqueous probiotic was added to the different treatments at 0.5 mL L^{-1} of water in fiberglass tanks. The probiotic treatments were added each week after the removal of the water. Fish were fed a commercial diet, 3% of body weight, containing 30% protein, twice daily, at 08.30 and 16.30. Water qualities were maintained throughout the experiment; at a temperature of 23 ± 1.80 °C, pH of 7.15 ± 1.53, dissolved oxygen at 6.15 ± 1.3 mg L^{-1}, and total ammonia nitrogen (TAN) at 1.10 ± 1.02 mg L^{-1} (0.25–1.50 mg L^{-1}).

2.2. Transportation stress

One hundred and eighty healthy Nile tilapia, with an average weight of 70.00 ± 4.54 g and an average length of 12.20 ± 0.20 cm were selected for handling transportation stress experiment, 120 days post-culturing. The fish were starved for 24 h before transportation and then placed in 0.3 mm polyethylene containers, 100 L capacity, containing 60 L of water, at a density of 67.84 ± 7.25 g L^{-1}. Fish were placed in an aerated open-transport system for 4 h, with the following water conditions: pH, 7.58 ± 0.08; temperature, 20.53 ± 0.15 °C; and dissolved oxygen, 8.96 ± 0.19 mg L^{-1}; and TAN, 0.25 ± 0.01 mg L^{-1}. After transportation (12.00), the water's pH, temperature, dissolved oxygen, and TAN were recorded at 6.92 ± 0.1, 24 ± 0.28 °C, 8.06 ± 0.31 mg L^{-1}, and 0.31 ± 0.09 mg L^{-1}, respectively. The fish, separated by treatment, were immediately transferred to fiberglass tanks with a density of 20 fish/400 L of water. Survival rates were monitored, and blood samples were collected, five days post-transportation. Water qualities were 23 ± 1.28 °C for temperature, 7.12 ± 0.3 for pH, 7.56 ± 0.48 mg L^{-1} for dissolved oxygen, and 1.0 ± 0.52 mg L^{-1} for TAN.

2.3. Blood sampling

Blood samples were taken from six fish per treatment at 0, 24, 72, and 120 h post-transportation. Fish were anaesthetized with clove oil (100 mg L^{-1}) in water to inhibit stress. Afterward, 1 mL of blood was collected from the caudal vein and placed into anticoagulant tubes for collected plasma and hematological analysis. A second blood sample (1 mL) was transferred into sterile Eppendorf tubes without anticoagulant for serum collection and allowed to clot at room temperature for one hour and at 4 °C for four hours to collect serum. They were then centrifuged at 5,000 *g* for ten minutes at 4 °C. For plasma, whole blood with anticoagulant was separated by centrifugation at 1000*g* for ten minutes at 4 °C. All samples were stored in Eppendorf tubes at minus 20 °C until needed. The serum samples were used to determine blood cortisol, glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT); whereas plasma was used to detect malondialdehyde (MDA) levels, within a week after collection.

2.4. Blood chemical analysis

Serum glucose, AST, and ALT were determined using a TC6060L fully automated Chemistry Analyzer (Tecom Science Co. Ltd., China). Cortisol levels were measured via a cortisol radioimmunoassay (RIA) test kit and displayed with a WIZARD® 2470 Automatic Gamma Counter (Wallac, PerkinElmer, Waltham, MA). Plasma MDA concentration was determined by measuring thiobarbituric acid reactive substances (TBARS) following the method suggested by Aengwanich et al. (2011) with brief modifications, and described in detail by Sutthi et al. (2018).

2.5. Hematological parameters

The erythrocyte (RBC) and leukocyte (WBC) number were counted using a Neubaeur hemocytometer (Blaxhall, 1972). Lymphocytes were counted through WBC blood cell smears in combination with Giemsa/May-Grunwald (Davis et al., 2008). Hematocrit was validated using heparinized micro-hematocrit capillary tubes after centrifugation (12,000 rpm for five minutes) and reported as percentages (Zhao et al.,

2018). Hemoglobin concentrations were measured by the formation of cyanhemoglobin using spectrophotometry with a wavelength of 540 nm following method by Zhao et al. (2018).

2.6. Lysozyme activity

Lysozyme activity was detected turbidimetrically, based on the lysis of the lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus* (Sigma, USA) following the method of Demers and Bayne (1997); with some modifications. Brief, 25 μL of each serum sample was placed into wells of a 96-well plate in triplicate. Next, 125 μL of *M. lysodeikticus* suspension (75 mg mL^{-1} in 0.05 M phosphate buffer, pH 6.0) was added to each well. After rapid mixing, the absorbance was measured at 450 nm using a microplate reader at intervals of 30 s, over five minutes, at room temperature. The results were expressed as μg mL^{-1}, computed as one unit of lysozyme activity, and defined as the enzyme amount producing 0.001 unit decrease in absorbance per min in 1 mL serum.

2.7. Gene expression

The collected samples were sent to the Animals Molecular Diagnostic Services (AMDS) Limited Company (19/2 Sanambin Kao Rd., Suthep, Muang, Chiang Mai 50,200, Thailand) for gene expression analysis. Briefly, total RNA extraction from the head kidney was followed via a PureLink RNA Mini Kit (Ambion, USA) according to the manufacturer's instructions. The quality and quantity of extracted RNA were determined by spectrophotometry with the 260:280 ratio of 1.8–2.0 and confirmed using agarose gel (1%). DNase I (ThermoScientific, USA) was used to ensure no DNA contamination. Afterward, complementary DNA (cDNA) was synthesized with a Tetro™ cDNA Synthesis Kit (Bioline, UK). Three transcripts, including heat shock proteins 70 (*Hsp*70), interleukin-1 beta (*IL*-1β), and tumor necrosis factor-alpha (*TNF*-α) were quantified in head kidney using beta-actin (β-actin) as an endogenous control. cDNA at 30 ng/μl was amplified with PerfeCTa SYBR® Green FastMix (Quantabio, USA) using specific primer pairs, as shown in Table 1. The amplification was operated via PCRmax Eco 48 Real-Time qPCR System (PCRmax, UK) at the following protocol: 95 °C for 10 min; 40 cycles of denaturation for 15 s at 95 °C; annealing for 15 s at 58 °C; and extension at 72 °C for ten seconds. The threshold cycle (C_T), obtained from fluorescent data, measured after each extension step; calculated the relative expression, which normalized the endogenous control (β-actin) via the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.8. Statistical analysis

Before statistical analysis, data were tested for normality using the Kolmogorov-Smirnov test and tested for homogeneity of variance using Levene's test. Data were performed using a one-way analysis of variance (ANOVA), followed by Duncan's multiple range test to determine the means at a significance level ($p < .05$). Results are expressed throughout as mean ± standard deviation (SD).

Table 1
Sequences of primer using in quantitative real-time PCR.

Genes	Primer sequence (5′ → 3′)	Accession no.
β-actin	Fw: GCTACTCCTTCACCACCACAG Rw: CGTCAGGCAGCTCGTAACTC	JF957365
*Hsp*70	Fw: TGCCTTTGTCCAGACCGTAG Rw: GTGTCCAACGCTGTCATCAC	JF957370
IL-1β	Fw: TGCACTGTCACTGACAGCCAA Rw: ATGTTCAGGTGCACTTTGCGG	JF957374
TNF-α	Fw: CCAGAAGCACTAAAGGCGAAGA Rw: CCTTGGCTTTGCTGCTGATC	AY428948.1

3. Results

3.1. Survival rate after transportation stress

The survival rates of the three fish groups treated with probiotics; *S. cerevisiae*, *Bacillus* spp., and the control group (no probiotic), show no significant differences ($p > .05$), displayed in Table 2. However, the survival rate significantly decreased (93.33 ± 2.89%, $p < .05$) in the control group at 72 and 120 post-transportation (Table 2).

3.2. Blood chemical profiles

The blood chemical profiles of fish after 4 h post-transportation are shown in Fig. 1. The results showed that the cortisol level of the control group (no probiotic; T_1) significantly increased in comparison to the probiotic groups (T_2 and T_3) over 1–120 h post-transportation, with the highest cortisol level observed in the control group at 0 h after transportation. The cortisol values of treated groups dropped at 120 h after transportation to pre-transportation values. However, the cortisol level of the control group at 120 h after transportation was higher than that of the pre-transportation value ($p < .05$).

Glucose levels of T_1 and T_2 were significantly higher than that of the T_3 group at 0 h after transportation ($p < .05$). However, the glucose values decreased in all treatments at 72 h post-transportation, and returned to basal levels similar to that of pre-transportation (Fig. 1b).

AST levels of tilapia at both pre- and post-transportation are presented in Fig. 1c. The control group showed a significantly higher AST level ($p < .05$) than those of the probiotics supplemented groups (T_2 and T_3) and was highest at 24 h post-transportation (Fig. 1d). Moreover, the highest ALT level was also found in the control group at 24 h post-transportation. In contrast, AST and ALT values were lower in the probiotic groups, especially T_3, and remained unchanged at both pre- and post-transportation periods. However, the AST and ALT levels in all treatment groups decreased after 72 h and dropped to pre-transportation levels at 120 h post-transportation.

3.3. Malondialdehyde and lysozyme activity

There were no significant differences in MDA levels in the control (T_1) and supplemented groups (T_2 and T_3) before transportation or at 0 h of post-transportation (Fig. 2a). However, the MDA concentrations of T_1 and T_2 were significantly higher than T_3 group ($p < .05$) at 24 h post-transportation. The highest levels were recorded at 72 h post-transportation, and each group dropped to be the same pre-transportation levels at 120 h post-transportation. No significant differences in lysozyme activity among all treatments were observed at either pre- or and post-transportations (Fig. 2b). However, the lowest concentrations of lysozyme were found in the T_1 and T_2 groups at 24 h post-transportation.

3.4. Hematological analysis

The results of the hematological analysis of Nile tilapia exposed to 4 h of transportation stress, given in Fig. 3, showed that the lymphocyte percentage (Fig. 3e) in all treatment groups significantly decreased at 24 h after transportation ($p < .05$), compared to the other times (pre, 0, 72, and 120 h post-transportation). However, the WBC, RBC, hematocrit percentage, and hemoglobin numbers were not affected at 4 h of post-transportation stress.

3.5. Gene expression

Transportation up-regulated the expression of the *Hsp*70 gene. The highest *Hsp*70 expression was found in the control group (T_1) compared to the probiotics-supplemented groups at both 0 and 24 h post-transportation ($p < .05$) (Fig. 4). The *Hsp*70 expression was significantly

Table 2
Survival rate (%) of Nile tilapia under transportation stress (4 h).

Time	Treatment		
	Control; T_1	*S. cerevisiae*; T_2	*Bacillus* spp.; T_3
0 h after transportation	$100.00 \pm 0.00^{a,A}$	$100.00 \pm 0.00^{a,A}$	$100.00 \pm 0.00^{a,A}$
24 h after transportation	$96.67 \pm 2.89^{a,AB}$	$98.33 \pm 2.89^{a,A}$	$98.33 \pm 2.89^{a,A}$
72 h after transportation	$93.33 \pm 2.89^{a,B}$	$96.67 \pm 2.89^{a,A}$	$96.67 \pm 5.77^{a,A}$
120 h after transportation	$93.33 \pm 2.89^{a,B}$	$96.67 \pm 2.89^{a,A}$	$96.67 \pm 5.77^{a,A}$
Mean	96.67 ± 3.61^{a}	98.33 ± 2.43^{a}	98.33 ± 3.61^{a}

Data are presented as mean ± SD. Different lowercase letters indicate significant differences between groups at the same time ($p < .05$). Uppercase letters indicate significant differences over time for the same group ($p < .05$).

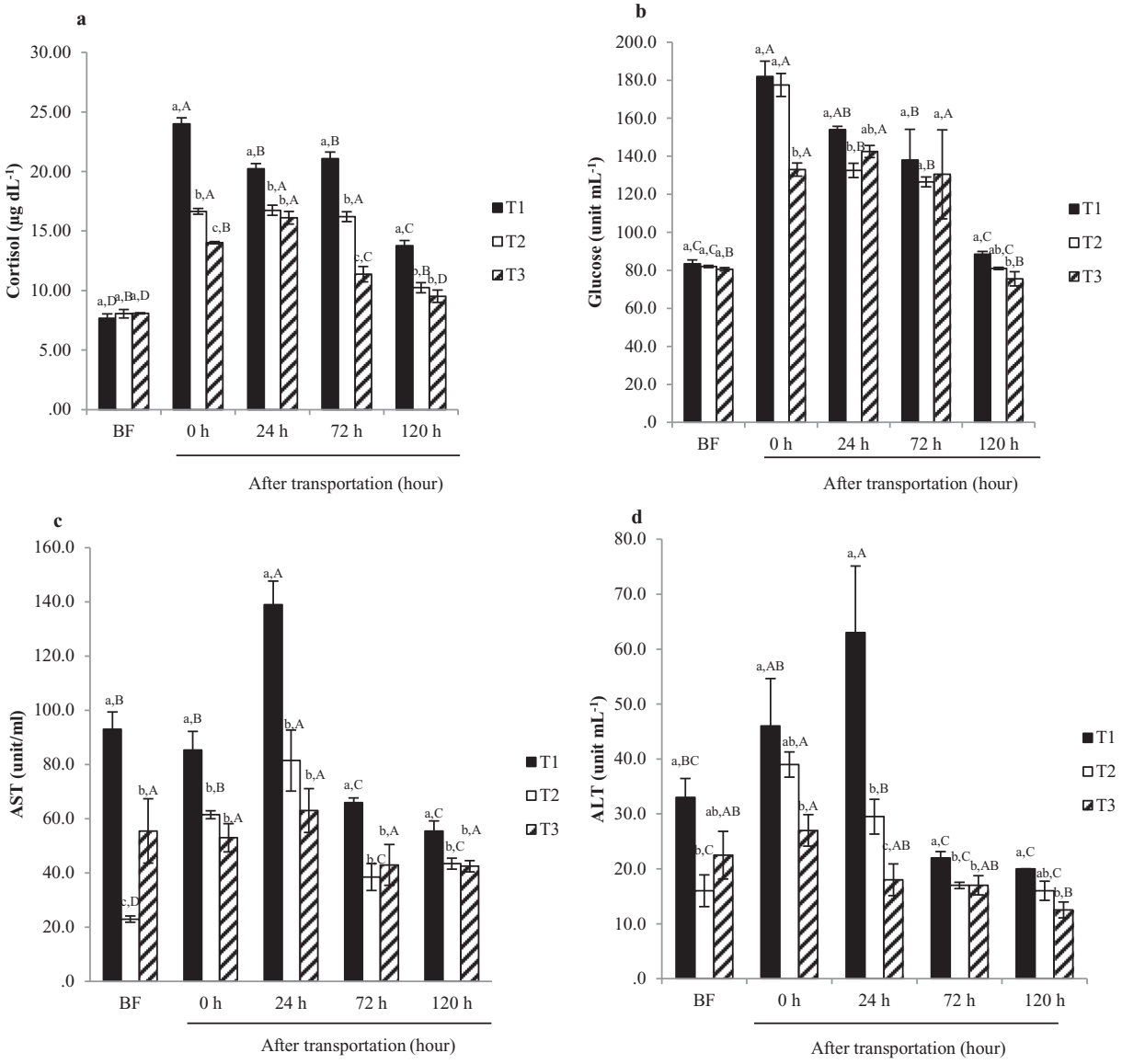

Fig. 1. Effect of water supplementation of probiotic on serum cortisol level (**a**), serum glucose level (**b**), serum AST level (c) and serum ALT level (**d**) in Nile tilapia during 4 h of transportation stress. T_1: control group (no probiotic); T_2: fish group reared in water treated with *S. cerevisiae*; T_3: fish group reared in water treated with *Bacillus* spp. Data are given as mean ± SD. Different lowercase letters indicate significant differences between groups for the same time period ($p < .05$). Different uppercase letters indicate significant differences over time within the same group ($p < .05$).

higher in all treatments at 0 h compared to those at 24 and 72 h post-transportation ($p < .05$). Moreover, the *Hsp*70 expression level recovered to the basal level (before transportation) after 120 h post-transportation (Fig. 4a).

The *TNF*-α expression level in the T_1 group was significantly higher at 0 h after transportation compared with T_2 and T_3 groups ($p < .05$). The expression of *TNF*-α significantly increased in the control group at 0 and 24 h after transportation ($p < .05$) and recovered to basal level after 72 h post-transportation. However, no significant differences in *TNF*-α were observed in the T_2 and T_3 probiotic-supplemented groups.

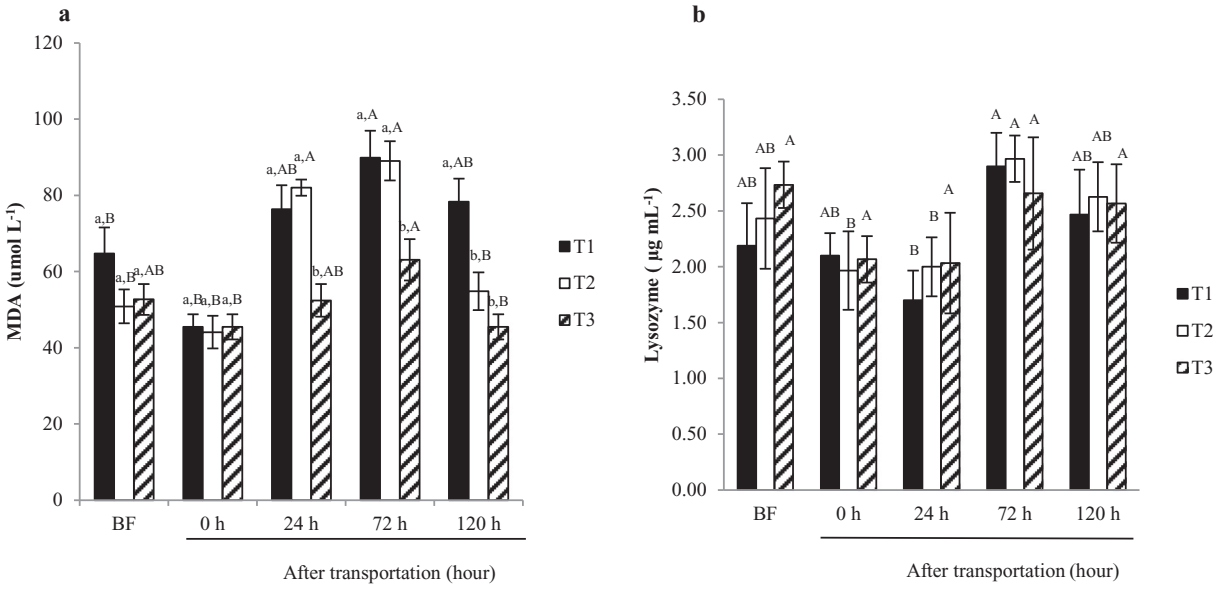

Fig. 2. Effect of water supplementation of probiotic on plasma MDA level (**a**) and serum lysozyme activity (**b**) of Nile tilapia during 4 h of transportation stress. T_1: control group (no probiotic); T_2: fish group reared in water treated with *S. cerevisiae*; T_3: fish group reared in water treated with *Bacillus* spp. Data are given as mean values ± SD. Different lowercase letters indicate significant differences between groups for the same time period ($p < .05$). Different uppercase letters indicate significant differences over time within the same group ($p < .05$).

Similarly, no significant differences in *IL*-1β expression were observed in any of the treatments.

4. Discussion

Probiotics are widely used in aquatic animal production to boost growth rate, disease tolerant, and immunity (He et al., 2015; Hoseinifar et al., 2018; Ringø, 2020; Tan et al., 2019; Zhang et al., 2019). They can be applied to feed or introduced directly in cultivated water to enhance the water quality and development of Nile tilapia under various aquaculture systems (Abarike et al., 2018; Adeoye et al., 2016; Chen and Chen, 2001; Jahangiri and Esteban, 2018; Padmavathi et al., 2012; Sunitha and Padmavathi, 2013; Xia et al., 2020). In the present study, Nile tilapia were treated with probiotics that were added to the water for 120 days and then tested under transportation stress for four hours. The results displayed no significant discrepancies ($p < .05$) among groups during transportation (4 h) or after transportation ($0-120h$). However, survival rates of the control group significantly decreased ($p < .05$) during the 24–120 h after transportation. This finding concurred with previous result of Gomes et al. (2008), who found no differences in the survival rates of marbled hatchet fish, *Carnegiella strigata* between groups treated with probiotic (Efinol®L) and the control (3–12 h after transportation). However, our control group showed a significantly decreased survival rate ($p < .05$) 24-120 h after transportation. These results agreed with Raj (2008), who reported that the fry of Indian major carp (*Catla catla*) not treated with probiotic (Efinol®L) demonstrated low cumulative survival compared to that of the probiotic treated group in the five-day post-transportation trials. Moreover, after the transportation trials, > 50% of fish showed clinical signs of tail and fin rot, especially in T_1. The reasons for this may be due to: (1) capture and transport stress, which can induce high mortality and reduce performance (Gomes et al., 2003); and (2) seasonal temperature, as most fish diseases occurred during the winter season (Hasan et al., 2013). The elevated survival rate may be attributable to the probiotic administration. Montalban-Arques et al. (2015) reported that nutritional inputs generate a positive loop in maintaining host health, and are essential in shaping the composition of the commensal gastro-intestinal microflora communities. Probiotics, which are live exogenous microorganisms, selectively provided to the host, are a promising concept for manipulating the microbiota, and thus for increasing the host health status Montalban-Arques et al., 2015. Furthermore, the supplementation of yeast has been demonstrated to modulate gut microflora, growth, and biochemical parameters of grass carp (Liu et al., 2018) and zebrafish (Siriyappagouder et al., 2018).

Transport stress can promote physiological changes in cortisol and glucose levels in fish (Balasch and Tort, 2019), which are frequently viewed as stress indicators (Morgan and Iwama, 2011). Cortisol level is a primary feature and a good target indicator for stress studies in fish (Aerts et al., 2015). We found, herein, significantly increased cortisol levels in the control group (T_1) versus the fish groups treated with probiotic (T_1 and T_2) 1–120 h post-transportation ($p < .05$). Fish treated with probiotics showed slightly decreased cortisol levels after 72 h, and levels returned to basal values 120 h after transportation. However, the control group showed a basal level higher than that of the pre-transportation level. Milla et al. (2010) determined that fish presented the highest cortisol levels one hour after stress, and returned to basal levels after six hours. In another study of transportation stress, Bolasina (2011) recorded the highest cortisol levels in juvenile flounders (*P. orbignyanus*) at one hour post-transportation, which dropped to the basal level after one day. In the channel catfish (*Ictalurus punctatus*), cortisol levels during transport stress also returned to the basal level after one day (Refaey and Li, 2018). Information regarding the effects of cortisol levels in Nile tilapia under transportation stress is lacking; however, our results determined that the cortisol level in Nile tilapia was highest at one hour after stress, but returned to the basal levels at 120 h. Similarly, the cortisol concentration of Eurasian perch (*Perca fluviatilis*, L.) was highest on the 2nd day after transport and recovered to basal levels between the 7th and 14th day (Acerete et al., 2004). In salmonids, cortisol levels under transportation stress may take from a few days to up to a week to return to basal levels (Barton, 2000; Maule et al., 1988; Sundh et al., 2010; Waagbø et al., 2017), as cortisol level recovery depends on the type of stress and varies between species (Balasch and Tort, 2019; Barton and Iwama, 1991; Herrera et al., 2019b; Pickering and Pottinger, 1989). The variations in these results may due to the different species of fish, the extent of tissue damage, or environmental factors (Wendelaar Bonga, 1997). Our results agreed with this suggestion in terms of tissue damage. We observed that Nile tilapia post-transportation showed greater clinical signs of tail and fin

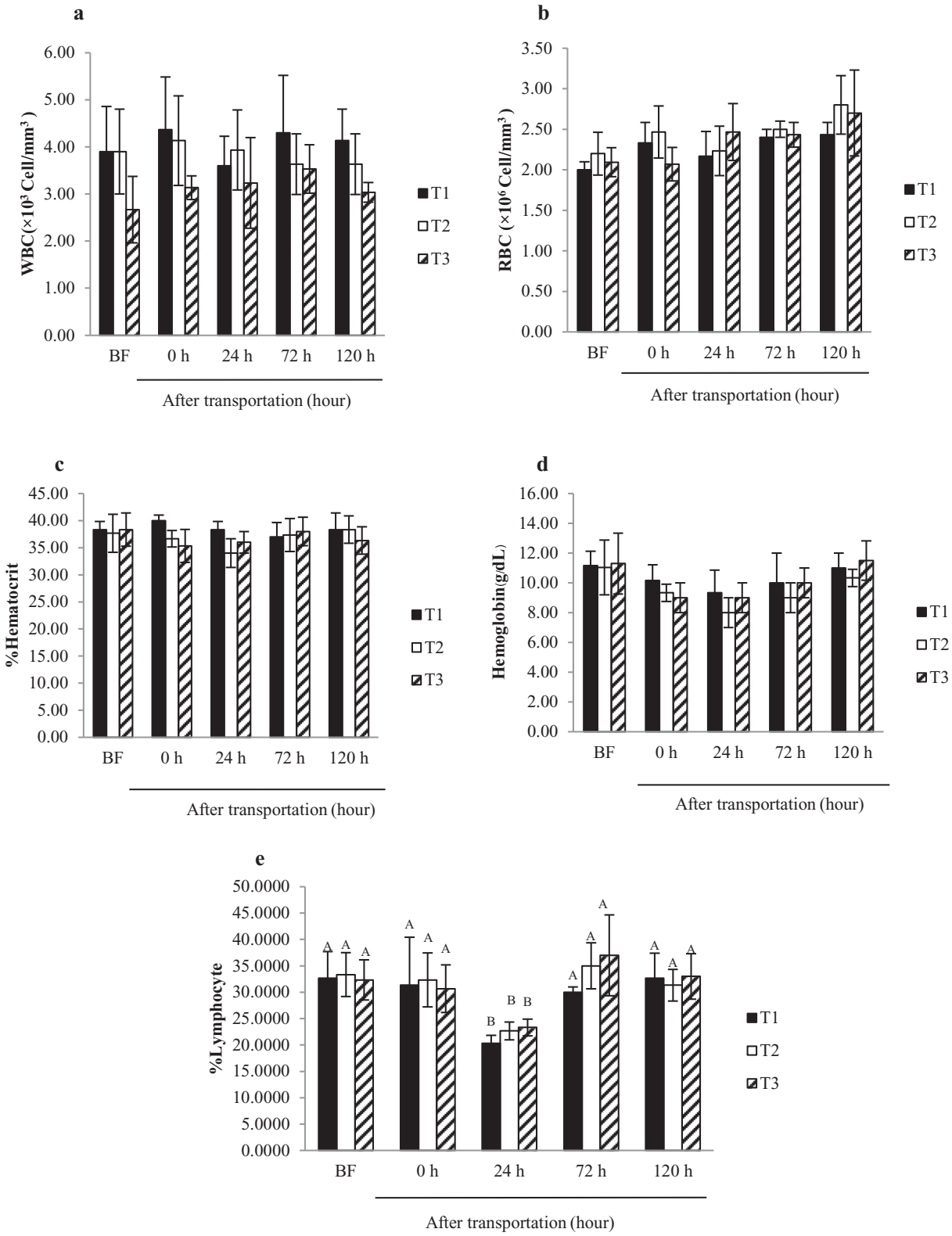

Fig. 3. Effect of water supplementation of probiotic on WCB (**a**), RCB (**b**), hematocrit (**c**), hemoglobin (**d**) and lymphocyte (**e**) of Nile tilapia during 4 h of transportation stress. T_1: control group (no probiotic); T_2: fish group reared in water treated with *S. cerevisiae*; T_3: fish group reared in water treated with *Bacillus* spp. Data are given as mean values ± SD. Different uppercase letters indicate significant differences over time within the same group ($p < .05$).

rot in T_1 than that of the treated groups. We may conclude that fish treated with probiotics (*S. cerevisiae* and *Bacillus* spp.) for 120 days showed improved transportation stress tolerance. While probiotics added into water for fish culture generally does not improve stress tolerance directly, improved water quality may reduce pathogens, as well as enhance growth, health, and survival rates (Dalmin et al., 2001; Martínez Cruz et al., 2012). Few studies have been made on fish reared in water with added probiotics to combat transportation stress. Raj

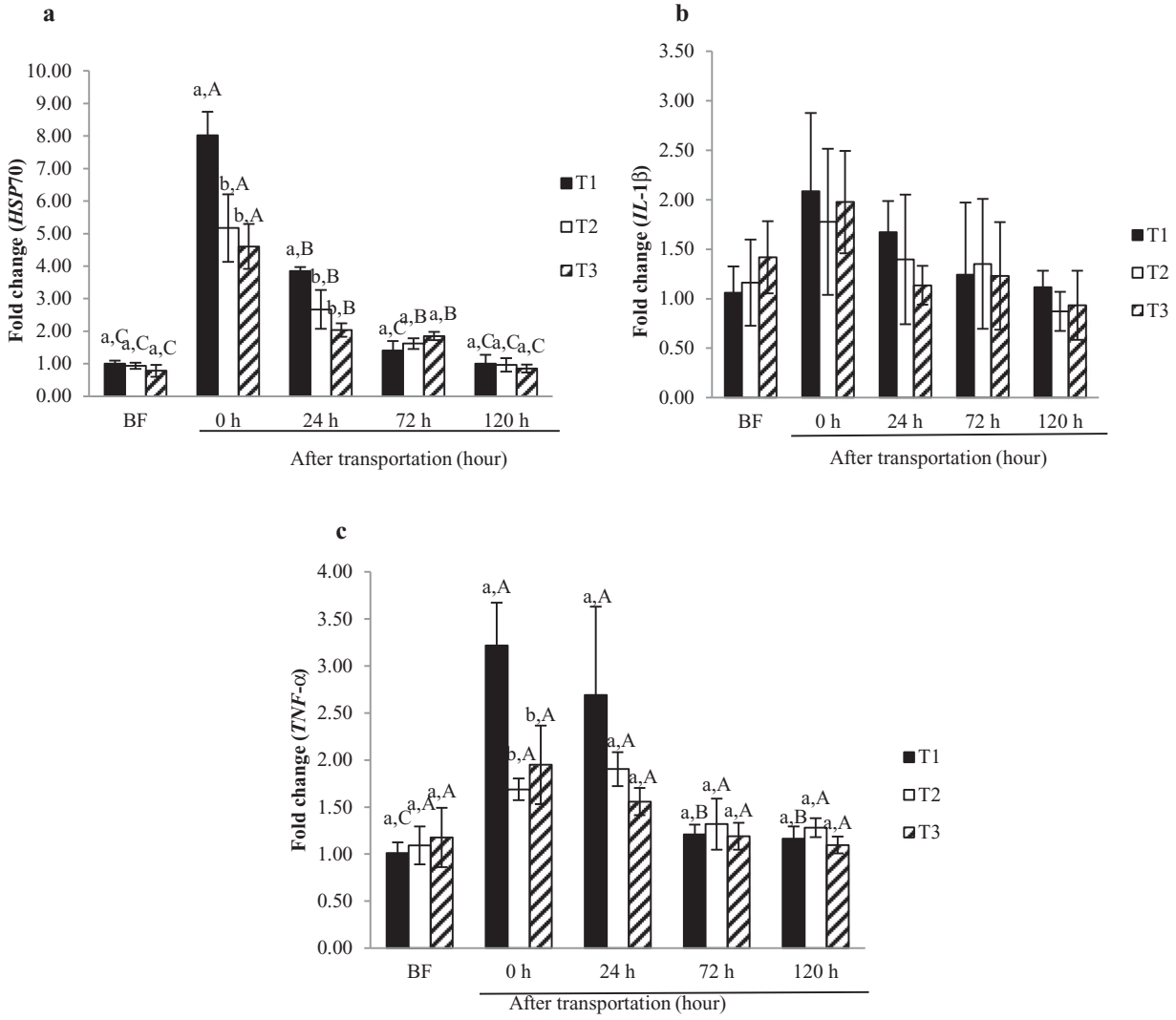

Fig. 4. Effect of water supplementation of probiotic on genes expression; *Hsp*70 (**a**), *IL*-1β (**b**) and *TNF*-α (**c**) of Nile tilapia during 4 h of transportation stress. T_1: control group (no probiotic); T_2: fish group reared in water treated with *S. cerevisiae*; T_3: fish group reared in water treated with *Bacillus* spp. Data are given as mean values ± SD. Different lowercase letters indicate significant differences between groups for the same time period ($p < .05$). Different uppercase letters indicate significant differences over time within the same group ($p < .05$).

(2008) reported on the survival rate of fry Indian major carp, *C. catla* treated with probiotic (Efinol®L). Gomes et al. (2009) also studied the effects of Efinol®L probiotic added to water during the transportation (24 h) of cardinal tetra, *Paracheirodon axelrodi*. They found that cortisol levels in the probiotic group were significantly lower than that of the control group.

Glucose level is generally the most evaluated indicator of secondary phase stress response in fish (Barton and Iwama, 1991; Fazio et al., 2015; Martínez-Porchas et al., 2009). Under stress conditions; catecholamine hormones, adrenaline, and noradrenaline are released into blood circulation. These compounds, in conjunction with cortisol, could elevate glucose production through glucogenesis and glycogenolysis pathways (Iwama et al., 1999) to cope with the energy demand, produced by the stressor. In the present study, the plasma glucose levels of T_1 (control group) and T_2 (*S. cerevisiae* group) were significantly ($p < .05$) higher than that of the T_3 (*Bacillus* spp. group) at 1 h post-transportation. The glucose levels in all treatments showed similar patterns, and decreased at 72 h after transportation, returning to basal levels at 120 h. These results were similar to the cortisol levels, suggesting that an increase in glucose level is positively correlated with the cortisol level. Previous studies have indicated that stressors increase the secretion of cortisol, which initially suppresses insulin secretion and subsequently increases plasma glucose (Iwama et al., 1999; Pakhira et al., 2015b). In general, the transportation stressor induces glucose level in fish; however, the expression level was dependent on the species. For example, in matrinxã, *Brycon amazonicus* stress was observed immediately after transport (Abreu et al., 2008); in salmonids, a few hours after transport (Barton, 2000); and after a few days in red drum, *Sciaenops ocellatus* (Robertson et al., 1987) and Eurasian perch, *Perca fluviatilis* (Acerete et al., 2004). There is a lack of information regarding glucose levels of Nile tilapia reared in probiotics before transportation stresses. In tambaqui, *C. macropomum*, Carvalho et al. (2009) reported that glucose levels in fish treated with the probiotic Efinol®L at 16 h of post-transportation presented similar glucose patterns to that of the control. Glucose levels significantly increased after transportation, and remained high for 24–48 h, then returned to basal levels at 96 h post-transportation. In contrast, our study showed that T_3 treated with *Bacillus* spp. resulted in lower glucose levels than that of the control group (T_1) at 120 h after transportation ($p < .05$). The glucose patterns, similar to the cortisol levels, suggest that transportation stress-induced cortisol and increased glucose level. Similarly, other stress conditions, such as hypoxia and overcrowding, induced increased glucose levels in Nile tilapia, higher than that of the control at 72-144 h (EL-Khaldi, 2010). Furthermore, stress response to glucose levels may affect liver

glycogen storage through fish activity factors; such as diet, life stage, environment, and season (Martínez-Porchas et al., 2009). Thus, *Bacillus* spp. probiotic added to water could indirectly promote fish health through the improvement of water quality and the reduction of pathogenic bacteria (Dalmin et al., 2001). It is well-documented that healthy fish can adapt more easily to physical changes and enhance their homeostasis under stressful, as the variation in values of plasma cortisol and glucose are caused by the changes in the immune system (Kucukgul and Sahan, 2008).

AST and ALT activities involved in aminotransferases were produced in the hepatocyte cells in the liver. Serum levels are low in healthy animals and high in sick animals. These enzymes leaked out of the bloodstream, causing liver damage or death (Pakhira et al., 2015a; Park et al., 2012). Stress conditions also induce higher levels of AST and ALT. For example, olive flounder (*Paralichthys olivaceus*) exposed to the stress of long-term starvation recorded a significant increase in AST and ALT levels compared to the probiotic fed group (Pakhira et al., 2015a). In rohu, *Labeo rohita* under pathogenic stress, AST and ALT levels were significantly higher than the probiotic fed group (Nandi et al., 2018). In rohu (*L. rohita* fry) AST and ALT levels increased at 12 to 36 h, and showed the highest levels of high stock density (Chatterjee et al., 2010). Fry catfish, *Pangasianodon hypophthalmus* fed with a dietary nucleotide for ten weeks showed no significant increase in AST and ALT levels in both handling and crowding stress (Yaghobi et al., 2015); however, the effect of probiotics added into the water on AST and ALT levels were not considered. Our results showed that the control group T_1 recorded significantly higher AST and ALT levels than fish treated with probiotics (T_2 and T_3), both before and after transportation stress (Fig. 1c-d). Similarly, Dobšíková et al. (2006) reported that AST and ALT levels of common carp, *C. carpio* were significantly influenced by transportation stress.

Malondialdehyde (MDA) is one of the most important biomarkers as a simply assayed end product of lipid peroxidation (Winston, 1991). It can increase during oxidative stress causing cell damage (Livingstone, 2001; Valavanidis et al., 2006), and pollution monitoring in fish Favari et al., 2002. In our study, MDA levels of tilapia under transportation stress both before and after transportation are presented in Fig. 3. The data indicated that fish groups treated with probiotics showed lower MDA levels than that of the control group. However, limited information exists concerning probiotics added into the water on the MDA level in Nile tilapia under transportation stress. The results were agreed with Zeppenfeld et al. (2014), who found that levels of MDA were significantly lower in silver catfish, *R. quelen* transported with essential oil of *Aloysia triphylla* compared to their control fish. Moreover, other stressors also responded to MDA level of tilapia fed with probiotic (*Lactobacillus plantarum* CCFM8610) reared in cadmium (Cd) water, in which MDA levels were lower than that of the control group (Zhai et al., 2017).

Lysozymes are essential humoral components in teleost fish (Smith et al., 2019). They possesses antibacterial actions (lytic enzyme), including bactericidal, hydrolyzing b-linked glycoside bonds of bacterial cell wall peptidoglycans (Fearon and Locksley, 1996). Moreover, they also serve as signaling molecules that promote the immune system during infection and stress (Saurabh and Sahoo, 2008). In the present study, the results showed that after four hours of transportation stress, lysozyme activity was reduced; yet returned to normal 72 h after transportation. Similarly, Möck and Peters (1990), found that 2 h post-transport and handling stress decreased lysozyme activity, while glucose level increased. Furthermore, acute stress, such as thermal stress, decreased lysozyme activity (Balta et al., 2017). However, in our study, the lysozyme activity of fish reared with probiotics was not significantly different than that of the control group. These results were similar to those of Zhou et al. (2010), who found that treatment with probiotics (*Bacillus subtilis* B10; *Bacillus coagulans* B16, and *Rhodopseudomonas palustris* G06) as water additives could affect lysozyme activity of tilapia. However, the plasma lysozyme activity of Japanese flounder (*Paralichthys olivaceus*) reared in probiotic-added water was significantly higher than that of the control group (Taoka et al., 2006). Such variations of lysozyme activity may be due to sampling time, environment, and nature stress (Hoseini et al., 2019).

Transportation and acute handling stress suppresses an animal's immunity (Blecha et al., 1984) and changes the hematological profile of fish (Ellsaesser and Clem, 1986). Stress can also increase morbidity and inhibit the immune system function (Salak-Johnson and McGlone, 2007). In our study, no significant differences were observed in WBC, RBC, hematocrit, and hemoglobin of all treatment groups under the transportation period. This was in agreement with Adeyemo et al. (2009), who found that the RBC, hematocrit, and hemoglobin of African catfish, *Clarias gariepinus* were not affected after acute handling and transport stress. In common carp (*Cyprinus carpio*), WBC significantly decreased in a high-density group under four hours of transportation stress (Hoseini et al., 2019). Lymphocytes play vital roles in immune regulation, inflammation, and protective immune response (Gerner et al., 2009). In the present study, the lymphocyte number in all treatment groups significantly decreased after 24 h post-transportation ($p < .05$) compared to the other transportation periods (BF, 0, 72, and 120 h). Ellsaesser and Clem (1986) also reported a reduction in the number of lymphocytes in channel catfish at 18 h after transport stress. Similarly, the lymphocyte number in porcine also decreased when subjected to transport stress (Dalin et al., 1993). Lymphocyte in African catfish also increased when induced shortly after acute handling and transport stress (Adeyemo et al., 2009). Variations in lymphocyte levels may be attributable to the time at which the samples were collected. Our results showed that lymphocyte numbers (%) decreased after transport stress, further indicating the immune status suppression of Nile tilapia.

Heat shock proteins (*HSPs*) represent a family of molecular chaperones, which play a vital role in the protection of stressed cells and organisms (Basu et al., 2002; Iwama et al., 1999) through the prevention of protein degradative pathways (Ran et al., 2016). *Hsps*, in particular the 70 kDa (*Hsp70*) family, are released at detectable levels after an acute stressor (Yamashita et al., 2004), which may further be used as a biomarker of stress in aquatic animals (Rollo et al., 2006). In the present study, the control group showed a higher *Hsp*70 expression level compared to the groups reared in water with probiotics (T_2 and T_3) after 0 and 24 h post-transportation. The results agreed with the previous study of Avella et al. (2011); where the level of *Hsp70* expression in common sole (*Solea solea*) larvae was significantly reduced in fish reared in water treated with *Enterococcus faecium* IMC 511. Ran et al. (2016); reported that Nile tilapia fed a live yeast supplement under crowding stress tolerance improved the expression of *Hsp70*, suggesting that live probiotics added into the water could enter the host through feed supplementation, as well as culture water and skin (Sayes et al., 2018).

Cytokines genes, including interleukin-1β (*IL*-1β) (Zou et al., 1999) and tumor necrosis factor-alpha (*TNF*-α) (Laing et al., 2001) are commonly used as biomarkers in regulating fish immune responses (Hossein et al., 2018). *IL*-1β and *TNF*-α genes were found to stimulate the major pro-inflammatory and inflammatory mediators of the host (Dinarello, 2002); including lymphocytes, macrophages, and natural killer cells (Dinarello, 2009; Selim and Reda, 2015). Our results showed that four hours post-transportation stress, cytokine gene expression was induced. Similarly, a previous study of common carp demonstrated that high transportation densities can cause an up-regulation of cytokine genes (*IL*-1β and *TNF*-α) (Hoseini et al., 2019). Cytokines may influence an immune homeostasis response under environmental challenges via bi-directional communication between endocrine-immune interactions in fish during stress (Castillo et al., 2009; Yildirim and Yurekli, 2010). We further noted that MDA levels significantly induced the cytokines gene. These results agreed with previous studies in both fish (Hoseini et al., 2019) and human (Cindrova-Davies et al., 2007). Lymphocyte levels were higher under transportation stress, owing to the activation of

cytokine genes (Dinarello, 2009; Selim and Reda, 2015). Probiotics can improve cytokine gene expression, influence the immune system of fish via feeding supplements (Panigrahi et al., 2007), and stimulate the cytokine secretion of *IL*-1β and *TNF*-α (Heumann et al., 1994; Tufano et al., 1991) via lymphocyte binding sites at the cell wall component (peptidoglycan) of lactic acid bacteria (Dziarski, 1991).

In conclusion, the addition of probiotics cultivated water before fish undergo transportation stress was shown to improve survival, blood chemical profiles, and immune-related genes expression in Nile tilapia. *Bacillus* spp. (T3) supplementation effectively enhanced health tolerance of Nile tilapia under transportation stress.

Ethical approval

Animal use protocol was followed the Institutional Animal Care and Use Committee, Mahasarakham University (IACUC-MSU), Thailand (IACUC-MSU-036/2019).

Declaration of Competing Interest

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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