Egypt. J. Chem. **Vol. 67**, No. 2 pp. 371 - 382 (2024)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Effective Microorganisms Amelioration against Copper, Lead and Cadmium Content on Nile tilapia, *Oreochromis niloticus* (L.)

Ragab G. Abdel-Salam[1, 2], Samah M. Bassem[3], Mahmoud Abdel-Latif[1], Eman Salah Abdel- Reheim [1], Giulia Guerriero[4], Fagr Kh Abdel-Gawad[3, 5]*

[1] *Department of Zoology, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt*
[2] *Egyptian Environment Affairs Agency, Fayoum, Egypt.*
[3] *Department of Water Pollution Research, CEAS,, Centre of Excellence for Research and Applied Studies on Climate Change and Sustainable Development, National Research Centre (NRC), Dokki, Cairo, Egypt. 33 El Bohouth st. (Former El Tahrir st.), Dokki, Giza, Egypt, P. O.12622.*
[4] *Department of Biology, University of Naples, Federico II, Naples, Italy.*
[5] *National Biotechnology Network of Expertise, Academy of Scientific Research Expertise and Technology, Cairo, Egypt.*

Abstract

The main goal of this study is to use effective microorganisms as a probiotic to improve the ability of tilapia fish to cope with temperature stress and heavy metal exposure. The experiment design was split block, incorporating three temperature levels (24 °C, 28 °C, and 32 °C) and three different heavy metals ($CuSO_4$, $CdCl_2$, and Pb $(NO_3)_2$), and the duration of the experiment was two weeks. All stages groups included EM groups in comparison with treatment groups. Quantitative real-time PCR (qRT-PCR) was used to follow the expression profiles of heat shock proteins (HSP70, HSP27, and HSP90) genes in Nile tilapia fish. Moreover, the activities of antioxidant enzymes catalase (CAT) and glutathione-S-transferase (GST) were examined in fish liver. Expression levels in HSP27 and HSP 90 genes were increased significantly ($p< 0.05$) in fish groups treated with $CuSO_4$ at all temperature levels while expression levels of HSP70 gene increased significantly ($p< 0.05$) in the fish group treated with $CuSO_4$ at 28 °C. At the same time, results varied in $CdCl_2$ treatment with variations in temperature. But in case of $Pb(NO_3)_2$ stage, expression levels in HSP27 gene increased significantly ($p< 0.05$) in fish groups subjected to (24°C and 28 °C), while increased in the HSP70 gene significantly ($p< 0.05$) in fish groups subjected to (24°C and 32°C). Results of antioxidants enzymes revealed that, the decrease was more detectable in the groups exposed to $0.1LC_{50}$ of (Cu, Cd, and Pb) at the most temperatures levels. Results revealed the positive impact of effective microorganisms on tilapia fish immunity and adaptation to climate change in aquaculture. As a recommendation EM could be used in fish farms to enhance fish productivity and reduce the toxic effects of pollutants.

Keywords: Oreochromis niloticus, climate change, heat shock proteins, effective microorganisms, antioxidant enzymes

1. Introduction

Since people have become more aware of the nutritional and medicinal benefits of fish, there has been a rise in fish consumption worldwide. Fish is an excellent source of protein, as well as being abundant in vitamins, unsaturated fatty acids, and crucial minerals. Fish, which is low in cholesterol having all nine essential amino acids, is thought to supply about 60% of the world's protein needs, where 60% of developing nations getting more than 30% of their needed protein from fish [1, 2]. Nile tilapia (*Oreochromis niloticus*) is a widely consumed fish species with high nutritional and economic value [3]. It has high productivity, adaptability, and high tolerance against different stressors which suggests Nile tilapia is a successful candidate for aquaculture[4]. In recent decades, there has been a

*Corresponding author e-mail: fagrabdlgawad@gmail.com; (Fagr Kh. Abdel-Gawad)
Received date 12 July 2023; revised date 01 August 2023; accepted date 07 August 2023
DOI: 10.21608/EJCHEM.2023.222604.8251

growing concern regarding the contamination of aquatic resources by various pollutants [5, 6]. The Nile tilapia, *O. niloticus*, has been utilized as a bio-indicator for a variety of contaminants, including heavy metals (zinc, cadmium, and mercury) [7] and environmental stresses [8, 9]. When cells experience stress from external stimuli, they produce proteins called heat shock proteins (HSPs) that protect the cells [10, 11]. HSPs are essential for maintaining cellular homeostasis because they promote proper protein folding and reduce misfolded proteins in cells that have been under stress in a specific way [12, 13]. HSPs are generally grouped into 5 families, (HSP100, HSP90, HSP70, HSP60, and small HSPs) based on their molecular weight, the homology of their amino acid sequence, and also their functions [14]. Heat shock proteins (HSPs), genes associated with oxidative stress, and immune system regulators, particularly cytokines, are among the genes whose expression is influenced by temperature. It has **been** discovered that HSPs can repair and stop cellular stress brought on by protein denaturation at high **and** low temperatures [15, 16]. However, HSPs expression is fluctuating at both stresses according to fish species [16, 17]. Because cells produce a range of protective responses in response to oxidative stress, which can be easily detected as altered enzymatic or genetic expression, oxidative stress is a convenient criterion to quantify toxicity and ecotoxicity [18, 19]. Catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST) and glutathione peroxidase (GPx), which are referred to as biomarkers to oxidative stress, make up the most powerful antioxidative physiological defense systems [20,21]. Using functional feed additives in aquaculture is one promising means for the reduction of environmental stress [22], as application of such additives works to mitigate growth retardation, immunosuppression, and oxidative damage in fish species [23]. Numerous microbial species have been tested as possible probiotics for aquaculture such as: yeast *Saccharomyces spp., Lactobacillus spp.*, which are used primarily as feed or water additives [24, 25, 26, and 27]. Recently, thermal stress has become a serious global concern. It could disturb cellular homeostasis and adversely impact aquatic species' susceptibility to toxins [28]. In the aquatic environment, the temperature varies dramatically from season to season, and living organisms experience altered metabolism in addition to other pathophysiological problems [29]. As protective mechanisms against the decreased dissolved oxygen that results from heat stress, the respiratory and metabolic rates are increased. This increased rate is followed by an increase in the amount of water that travels over fish gills carrying dissolved compounds (such as trace metals) and other dissolved particles, which increases both the bioavailability and bioaccumulation of the aquatic pollutants in fish organs [30, 31]. The present study aims to evaluate the antioxidative state and the gene expression of heat stress protein genes HSP27, HSP70, and HSP90 to highlight the effects of various heavy metals' toxicity on Nile tilapia (*O. niloticus*) during thermal stress.

2. Materials and Methods

2.1. Fish samples:

About 576 adult tilapia fish (*O. niloticus)*, with a weight ranges between 90 – 100 g, were collected from the National Research Centre farm (Nubaria, Egypt). Tilapia fish were brought to the Biotechnology and Biodiversity Conservation Lab, National Research Centre, in plastic water containers containing aerators as an oxygen source with de-chlorinated tap water. Fish were supplied with commercial fish diet (35% protein) all over the experiment**.**

2.2. Determination of lead, copper and cadmium toxicities 96 hr. LC50

A lethal concentration test (96-hr. LC_{50}) was applied for *O. niloticus* after the 96 hr. treatments of ($CuSO_4 \cdot 5H_2O$), ($CdCl_2.H_2O$) and $Pb(NO_3)_2$ as described by [32], LC_{50} was estimated for ($CuSO_4 \cdot 5H_2O$) according to [33] and [34], for ($CdCl_2.H_2O$) referring to [35], and for Pb $(NO_3)_2$ according to [36].

2.3. Experimental design

After acclimation, the fishes were separated to eight groups, with three different temperature levels for each group and then placed in glass aquaria (150 liters) in three replicate groups (8 fish/group). The experimental design (Fig. 1) included three stages: First stage; treatment with $CuSO_4$, Second stage; treatment with $CdCl_2$ and the third stage; treatment with Pb $(NO_3)_2$.

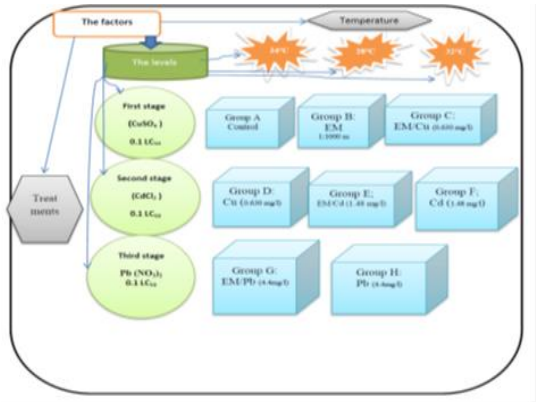

Fig.1. the experimental layout.

2.4. *Measurement of antioxidant enzymes activities.*

CAT activity was determined, for isolated samples from fish liver, using a colorimetric method, as reported in a commercial assay kit was used for this assay according to, Bio diagnostic Company in Cairo, Egypt [37]. The enzyme levels were measured at 510 nm and catalase activity was measured as $mmol^{-1}$ $H_2O_2/min^{-1}/mg^{-1}$ protein. The activity of Glutathione-S-transferase activity (GST) enzyme was examined in fish liver by a spectrophotometric process, as reported in a commercial assay kit was used for this assay according to [38]. The conjugation leads to an increase in absorbance at 340 nm.

2.5. *Gene expression of heat shock proteins*

Fish RNA was isolated from fish liver by using TRIzol® Reagent (Invitrogen, Germany). Then a unit of RQ1 RNAse-free DNAse (Invitrogen, Germany) was added to RNA to digest DNA precipitates, then DEPC water was added and measured photospectrometrically at 260 nm and stored at -80°C. Aliquots were used in reverse transcription (RT) reactions. RNA was converted to cDNA (20 µl total volume) by RevertAidTM cDNA Synthesis Kit (Fermentas, Germany). RT reactions were applied for 10 min at 25 °C, then 1 hr. at 42°C, ended by a denaturation step for 5 min at 99°C [39]. Step one Real-Time PCR system (Applied Biosystems, USA) was used to determine cDNA copy number of fish liver. Each reaction included 0.5 µL 0.2 µM sense primer, 0.5 µL 0.2 µM antisense primer, 12.5 µL 1× SYBR® Premix Ex Taq TM (TaKaRa, Biotech. Co. Ltd.), 6.5 µL dH2O, and 5 µL of cDNA template to a final 25 µL. The used primers are listed in Table (1). A melting curve analysis was done at 95.0°C at the end of each qPCR to detect the primers quality [40].

Table 1. The HSPs primers used in the current study

Gene	**Sequence (5'–3')a**	**GenBank number (accession number, NCBI)**
Hsp27	F: CCCAGAACTAATGACACCGCA R: GTGCTCGATGGCTGGTTTGA	KC 192887.1
Hsp70	F: CGGGAGTTGTAGCGATGAGA R: CTTCCTAAATAGCACTGAGCCATAA	GQ 386813.1
Hsp90	F: ATGCCTGAAGAAATGCGCCAAGAGGAG R: CCAATGGGCTCACCGTTGTCGACTCTG	GR 599873.1
β-Actin	F: TGGGGCAGTATGGCTTGTATG R: CTCTGGCACCCTAATCACCTCT	EF 206801.1

2.6. Statistical analyses

The study results were represented as mean ± SE. Data were statistically analyzed utilizing analysis of variance (F-test) and Duncan's multiple range test to determine differences in means as expressed by different case letters in the descending order A, B, C and D at P<0.05 using SAS (Statistical Analysis System) version 9.1[41] and [42].

3. Results

3.1. Antioxidant enzymes activities

The CAT activity in fish liver increased significantly (P < 0.05) in EM treated group than other groups exposed to 24°C. While increasing temperature to 28°C, the levels were highly significant in EM/Cu and control groups. At 32°C, the enzyme levels were highly significant in control followed by EM/Cu fish groups as shown in (Table 2). The GST activity levels showed different patterns than CAT (Table 2). At temperature levels of 24°C and 28°C, Enzyme levels increased significantly (P < 0.05) in EM group when compared to other groups. While at higher temperatures, 32 °C the highest GST level was detected in Cu group.

Table 2. Effect of treatment by CuSO4 on CAT and GST activity in thermal stressed Tilapia fish

Experimental groups	CAT (u/g)			GST (u/g)		
	24 °C	28 °C	32°C	24 °C	28 °C	32°C
Control	**0.291 ±0.001 C**	**0.324 ± 0.005 A**	**0.317 ± 0.002 A**	**12.405 ± 0.13 B**	**11.303 ± 0.03 B**	**12.127 ± 0.07 B**
E.M	**0.316 ±0.002 A**	**0.305 ± 0.003 B**	**0.310 ± 0.002 B**	**17.320 ± 0.01 A**	**12.767 ± 0.02 A**	**9.04 ± 0.01 C**
E.M. and Cu	**0.302±0.003 B**	**0.318 ±0.003 A**	**0.314 ± 0.001 AB**	**9.73 ± 0.08 C**	**11.500 ± 0.09 B**	**5.11 ± 0.30 D**
Cu	**0.298 ± .003 BC**	**0.296 ± 0.002 B**	**0.299 ± 0.002 C**	**12.65 ± 0.72 B**	**7.70 ± 0.17 C**	**18.077 ± 0.11 A**

- Data are represented as means of eight samples ± S.E.
- Statistical significant differences (*P*<0.05) are shown with different capital letters in the same column.

Results of CAT activity in liver of tilapia fish increased significantly (P < 0.05) in EM and control groups compared to other groups subjected to 28°C and 32°C. While the highest enzyme values were detected in EM fish group exposed to 24°C as shown in (Table 3). The GST activity levels in fish liver increased significantly (P < 0.05) in EM group more than other groups subjected to both temperatures; 24 °C and 28 °C. While at 32 °C the maximum enzyme level was detected in EM/Cd group (Table 3). Results of CAT activity in liver of tilapia fish increased significantly (P < 0.05) in EM fish group at both temperature levels of 24°C and 32°C. The maximum levels were detected in both control and EM fish groups subjected to 28°C. Concerning the GST activity, the levels increased significantly (P < 0.05) in EM group exposed to all temperature levels of 24°C, 28°C and 32 °C (Table 4).

Table 3. Effect of treatment by $CdCl_2$ on CAT and GST activity in thermal stressed tilapia fish

Experimental groups	CAT (u/g)			GST (u/g)		
	24°C	28°C	32°C	24°C	28 °C	32 °C
Control	0.284 ± 0.003 B	0.318 ±0.005 A	0.314 ±0.003 A	12.158±0.16 B	11.51 ± 0.19 B	12.36±0.077 B
E.M	0.319 ± 0.002 A	0.304 ± 0.004 A	0.307 ±0.001 A	17.63 ± 0.38 A	12.94±0.35 A	9.11 ± 0.10 C
E.M. and Cd	0.252 ± 0.002 C	0.281 ± 0.005 B	0.286 ±0.005 B	9.15 ± 0.17 C	8.74±0.17 C	13.74 ± 0.32 A
Cd	0.237 ± 0.002 D	0.265 ± 0.003 B	0.268±0.004 C	7.63 ± 0.24 D	5.83 ± 0.20 D	4.54 ± 0.18 D

- Data are represented as means of eight samples ± S.E.
- Statistical significant differences (P<0.05) are shown with different capital letters in the same column.

Table. 4. Effect of treatment by Pb $(NO_3)_2$ on CAT and GST activity in thermal stressed tilapia fish

Experimental groups	CAT (u/g)			GST (u/g)		
	24°C	28°C	32°C	24°C	28°C	32°C
Control	0.284 ± 0.002 B	0.303± 0.002 A	0.309 ±0.001 B	9.13± 0.20 B	8.21± 0.16 B	7.83 ±0.17 B
E.M	0.318 ± 0.001 A	0.306 ±0.002 A	0.315± 0.001 A	11.13±0.27 A	9.85± 0.18 A	8.98± 0.04 A
E.M. and Pb	0.261± 0.001 C	0.256± 0.003 B	0.261 ±0.001 C	2.27 ± 0.03 C	2.62± 0.11 C	2.48 ± 0.01 C
Pb	0.228 ± 0.001 D	0.241± 0.001 C	0.248 ±0.001 D	1.49± 0.01 D	0.50 ±0.07 D	1.0 ± 0.03 D

- Data are represented as means of eight samples ± S.E.
- Statistical significant differences (P<0.05) are shown with different capital letters in the same column.

3.1. Gene expression of heat shock protein genes

The obtained results of gene expression of HSP27, HSP70 and HSP90, in liver of O. niloticus, treated with $CuSO_4$, $CdCl_2$. and Pb $(NO_3)_2$ under different temperature levels are summarized in Figures (2-4). Results from the CuSO4 treatment revealed that HSP70 gene expression levels significantly increased only in the fish group at 28°C while in case of both HSP27 and HSP90 genes, the expression levels increased at all temperature levels (Fig. 2). In case of the fish groups treated with $CdCl_2$ at different temperature levels (Fig. 3), the expression levels of HSP27 increased significantly in control groups at 24°C, 28°C and in EM group at 32°C. However, expression levels of HSP 70 reached highest levels in EM/Cd groups at both 28°C and 32°C, but increased significantly in EM group after exposure to 24°C Concerning the third gene, HSP 90 expression levels increased significantly in EM and control groups at 24°C and 28°C respectively, while at 32°C the expression decreased only in Cd fish group. In the treatment with Pb $(NO_3)_2$ results are shown in Fig. (4). In case of HSP27, highest expression levels were seen only in control groups at all temperature levels, while for HSP70 the expression levels increased significantly in Pb groups at both 24°C and 32°C. Then expression levels of HSP90 increased significantly in EM group at all temperature levels. From the obtained results, the most affected metal by temperature changes is $CuSO_4$ followed by Pb $(NO_3)_2$

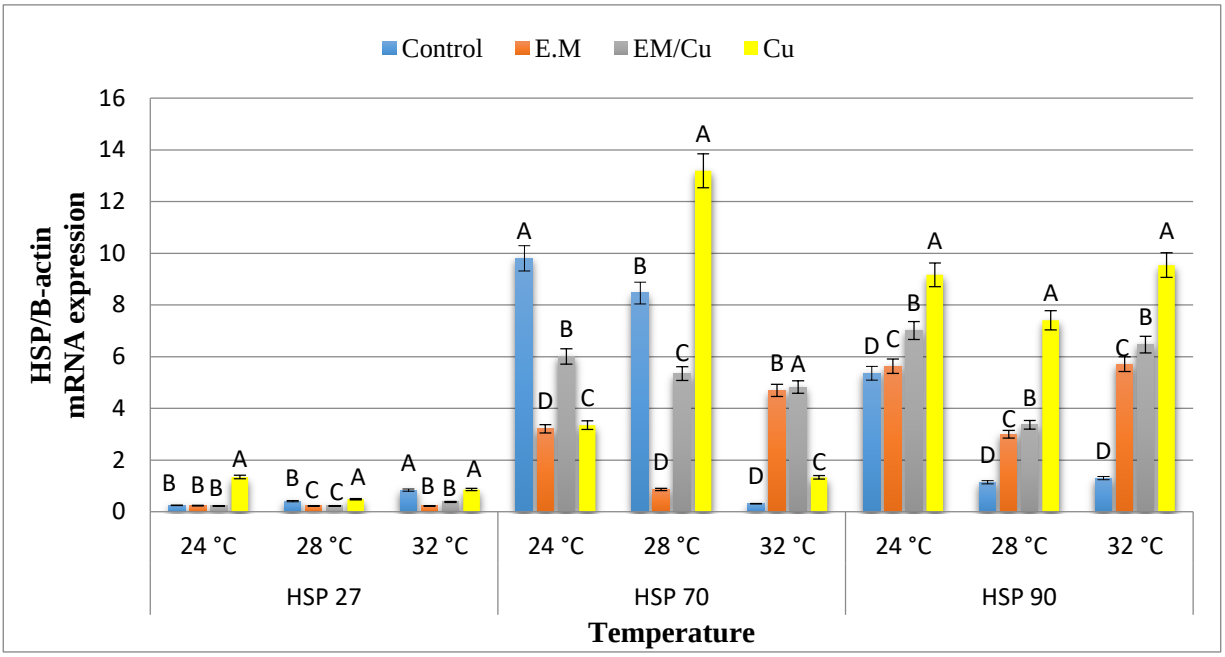

Fig.2. RTqPCR expression of HSP27, HSP70 and HSP90genes in liver tissues of tilapia fish treated by CuSO4 (Control, EM, EM/Cu and Cu) at 24 °C, 28 °C or 32 °C. Data are represented as mean ± SE (n = 8). The data showed effect of CuSO4 on HSP27, HSP70 and HSP90 genes (P<0.05).

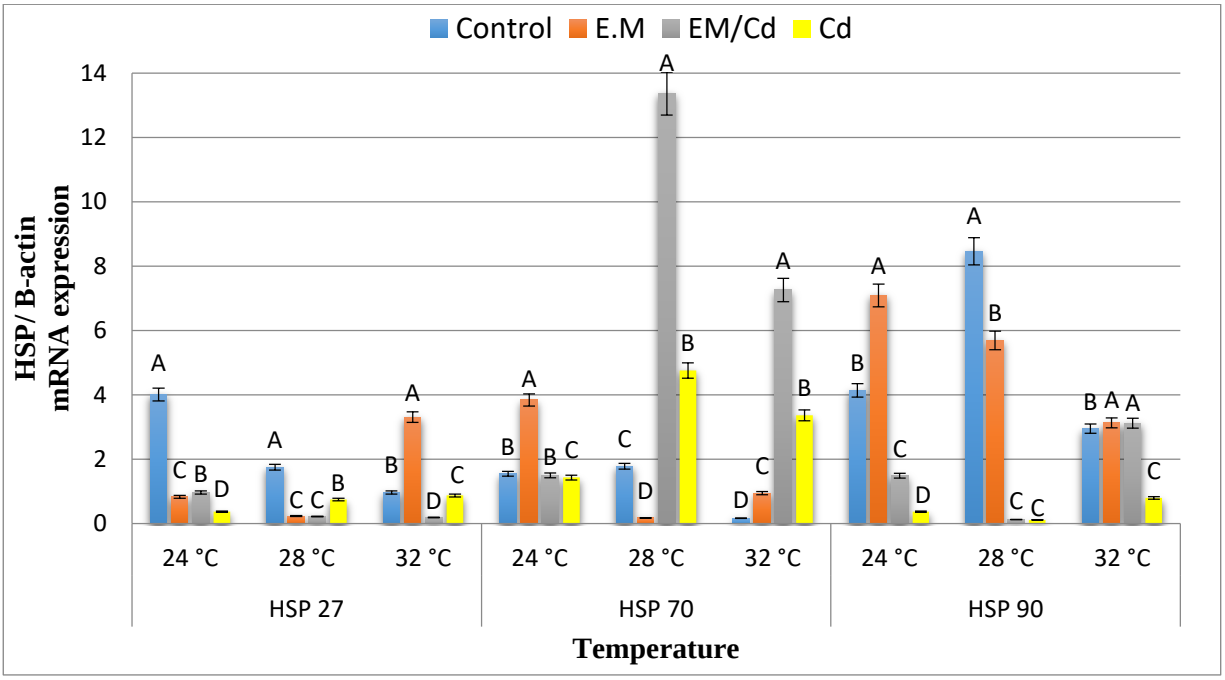

Fig.3. RTqPCR expression of HSP27, HSP70 and HSP90 genes in liver tissues of tilapia fish exposed to $CdCl_2$ (Control, EM, EM/Cd and Cd) with 24 °C, 28 °C or 32 °C. Data are represented as mean ± SE (n = 8). The data showed effect of EM on HSP27gene (P<0.05).

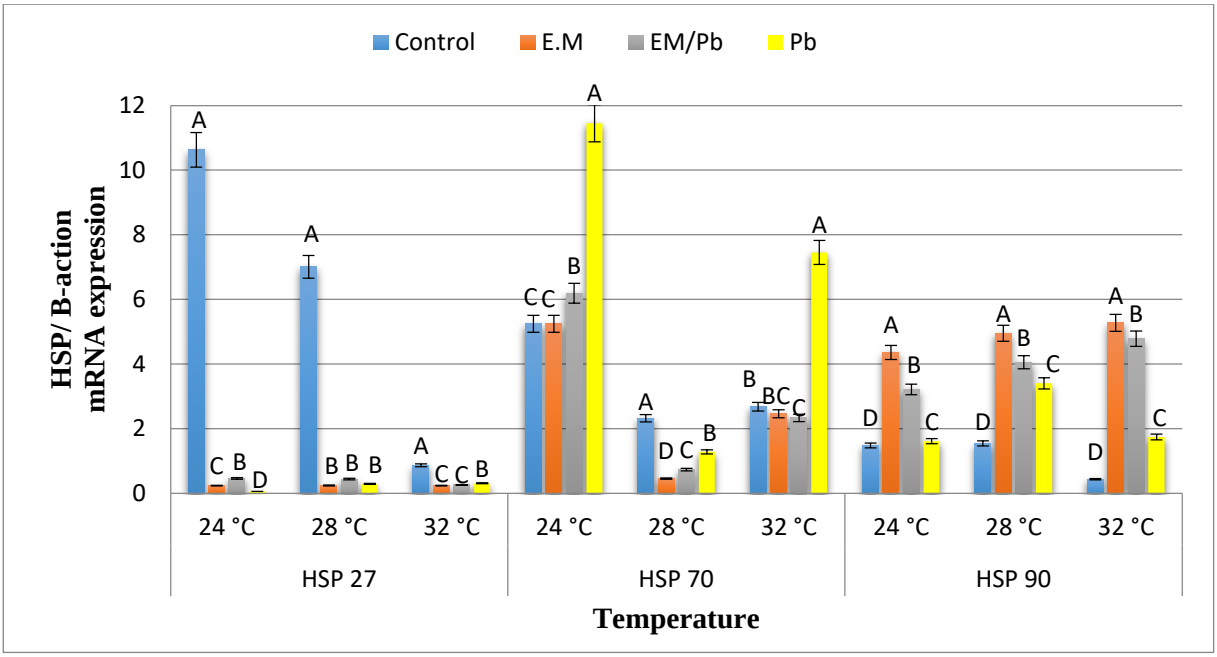

Fig.4. RTqPCR expression of HSP27, HSP70 and HSP90 genes in liver tissues of tilapia fish exposed to Pb (NO3)2 (Control, EM, EM/Pb and Pb) with 24 °C, 28 °C or 32°C. Data are represented as mean ± SE (n = 8). The data showed effect of EM on HSP27gene (P<0.05).

4. Discussion

Utilizing healthy microorganisms that have been isolated from the environment is one of the key technologies used to enhance the environment, prevent disease, maintain eco-equilibrium, minimize adverse effects, and boost immunity [43, 44]. In this study, we examined the impact of EM as a probiotic for remediation of some toxic metals and improving thermal stress adaptation in Nile tilapia. The data in this research reflected the impact of EM on fish immunity which appeared in the high significance of both antioxidants (CAT and GST) in EM fish groups subjected to combined stresses of heavy metals and temperature levels. Fish that have accumulated heavy metals may have toxicological consequences [45, 46] and cause oxidative deterioration to animal tissues defining cell function damage [47, 48]. Cu induces oxidative stress in fish (Gasterosteus aculeatus) [49]. Major antioxidant activities are inhibited by lead, generating oxidative stress and other disfunctions in proteins, lipids, and DNA [50]. In our investigation the levels of GST and CAT significantly decreased as a result of the metal contamination, which suggested damaged antioxidant defense mechanisms. These results agree with the recent findings of [51], which stated that the exposure to Cu and Zn enhanced DNA damage and significantly reduced transcription of SOD, CAT, and GST. In accordance with what has been suggested in a previous study by the authors [48], we thus agree that a variety of parameters, including the intensity and duration of chemical stress and the sensitivity of the species under study, might either increase or decrease the expression of antioxidant biomarkers. HSPs allow fish to cope with environmental pressures such as: temperature variations, osmotic stress and exposure to different xenobiotic parameters. Cross-protection is the capability of one stressor to elevate the resistance of any organism to a later heterologous pressure [52, 53]. Alteration in expression levels of HSP genes may be resulted from application of EM as in all stages of our research during treatment with: CuSO4, $CdCl_2$ and Pb $(NO_3)_2$. The results revealed that HSP genes were significantly increased and reached maximum levels in fish treated with Cu followed by Pb at varying temperature levels. According to our data, the expression levels of HSP70 in liver tissues considerably rose in the Pb group and control group at both 24°C and 32°C followed by the Pb group at 28°C. This might be explained by the beneficial effects of EM on fish at different temperatures and its ability to reduce the negative effects of some pollutants. In agreement with our results, it was found that fish secrete large quantities of HSP70 as a

warning signal to enhance protein integrity and reduce apoptosis in response to stress [54, 55]. Even though some studies claimed that fish express HSP70 at lower levels, several in vivo studies showed an opposite trend in Nile tilapia [56, 57, 58]. Application of probiotics reduced expression of HSP70 gene and improved growth and survival of fish [59]. Our findings are consistent with a prior work, which showed that HSP70 expression levels in Nile tilapia subjected to some heavy metals could be utilized as indicator to comprehend the fish reaction and adaptability to high concentrations of Pb and Cd [60]. Also agree with Jatoba et al. [61] who found that probiotics can improve growth, digestive physiology and the immune response of animals. Our findings agree with Acunzo et al. [62] who found that the most investigated member of the tiny HSP family, HSP27, is essential for several signaling pathways that are involved in treatment resistance and apoptosis inhibition. The expression level of the small heat shock protein 27 (HSP27) is increased in response to various stressors [63]. In agreement with previous studies, our results revealed that in the fish groups treated with $CuSO_4$, we found that expression levels increased significantly in Cu group at all temperature levels in comparison to other groups, while in the treatment with Pb $(NO_3)_2$ and $CdCl_2$ increased significantly in control group at all temperature levels except in case of CdCl2 subjected to 32°C. Which indicated the positive role of EM in reduction of some environmental pollutants and adaptive the fish with variation in temperature levels as an approach for adaptation to climate change on aquaculture and biodiversity. HSP90 is one of the genes that is known to be most highly expressed under thermal stress and protects the organism's cells by interacting with a few co-chaperones [64]. Additionally, it plays a part in protein folding of misfolded substrates as well as substrate discrimination. In the embryonic organisms of loggerhead turtles, HSP90 has been found to be a useful biomarker of temperature stress [65]. HSP90s initially recognized as proteins that respond to stress, HSP90 has now been linked to a number of homeostatic functions. Furthermore, the extracellular HSP90s are able to connect to the surface receptors and trigger cellular operations associated with immune reaction [66]. In accordance with our results, adding EM and other functional feed additives to fish meals significantly improved the defense against stressors, this may be linked to their beneficial effects. These effects included increasing food intake, fostering fish growth, and boosting immunity. Little immunological responses may be brought on by feeding EM supplements, as evidenced by their low EM concentration [67, 68]. As obtained in our research, tilapia fish was adapted to different stressors. Our study showed that heat stress can cause oxidative stress and inflammation, while EM supplementation significantly improves the defense against stress. This may be linked to the beneficial effects of EM on enhancing antioxidant capacity and immune functions of tilapia fish. Further research is intended on the application of EM on a large scale for longer periods to reduce various impacts of pollutants on fish.

5. Conclusion

Based on our results, oxidative stress was found to be significantly increased, and the levels of these proteins were found to be significantly higher in the groups that received heavy metal treatments. By reducing oxidative stress, these proteins function as a protective factor. Three new molecular biomarkers (HSP27, HSP90, and HSP70) and antioxidant enzymes (CAT and GST) for Pb, Cu, and Cd exposure in Nile tilapia with EM were successfully developed in this study. We recommend the using of probiotics as a technique for EM-induced tilapia fish immunity and facing multiple stressors (heavy metals - temperature variations). Our Further directions are intended on the application of EM as a probiotic for longer periods on large scale to minimize different effects of stressors on fish.

6. Funding information

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

7. Ethical statement

The Faculty of Science at Beni-Suef University's Institutional Animal Care and Use Committee (IACUC), where the research were conducted, approved all techniques used in this study(FS/2018/10) that involved using animals (fish), and they all complied with their ethical standards.

8. Conflict interest.

The author declares that there is no conflict of interest.

9. Acknowledgements.

This work was supported by the Biotechnology and Genetic Conservation group laboratory Center of Research and Applied Studies for Climate Change and Sustainable Development, Water Pollution Research Department, CEAS, National Research Centre (NRC), Egypt. A deep appreciation for the experimental participation at the Zoology Department, Faculty of Science, Beni-Suef University, Egypt. Deep appreciation for the experimental participation at the NationalResearch Centre (Dokki, Giza) of Dr. Giulia Guerriero, Visiting Professor in the framework of MoU between the National Research Centre of Giza and the Federico II University of Naples, Italy.

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