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RESEARCH ARTICLE

Dietary intake of *Spirulina platensis* alters *HSP70* gene expression profiles in the brain of rats in an experimental model of mixed stress

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Abstract. *Spirulina platensis* has gradually gained more attention for its therapeutic, antioxidant, and anti-inflammatory potential worldwide. However, the current molecular knowledge about the effects of spirulina on stress-related genes is rather limited. The effects of dietary intake of spirulina on the *HSP70* gene expression were assessed in a controlled *in vivo* experimental design. Moreover, alterations in serum corticosterone levels, IL-2, IL-4, IFN-γ, triglyceride, ALT, AST, relative gene expression values, and the correlations between them were evaluated. A total of 36 rats were divided into four groups: control group, stress-only group, spirulina group, and spirulina+stress group. To control the dose administration, *S. platensis* was applied by a gastric gavage in stress groups. Crowded environment stress and hosting alone stress were applied to the stress-only group and spirulina + stress group. RNA was extracted from brain samples using TRIpure and the relative gene expression assessment was performed using Roche-LightCycler-480-II real-time PCR-System. Gene expression values were remarkably different among the four experimental groups. The differences between stress-only and the spirulina groups were statistically significant ($P<0.05$). The correlation between the *HSP70* gene expression and the IFN-γ was found to be statistically significant ($P<0.05$; $r=0.50$). Results indicate a novel effect of spirulina on the *HSP70* expression related to the stress-response. Data presented in this study may be useful for further studies to define not only the molecular genetic aspects through dietary *S. platensis* but also the effects of spirulina on stress-response and animal welfare.

Keywords. gene expression; *HSP70* gene; real-time polymerase chain reaction; *Spirulina* (*Arthrospira*) *platensis*; stress.

Introduction

Genomic data obtained as a result of the fact that environmental factors and nutrition can cause genetic changes and the widespread research of epigenetic mechanisms have led to a new era of genetics. Nutrigenomic studies have shown that various dietary components can contribute to altered gene expressions which ineluctably affect metabolism and health (Kozul *et al*. 2008; Zhang and Kutateladze 2018). On the other hand, the homeostatic balance of the organism is directly related to stress exposure because it is well-known that stress condition affects numerous biological processes (neuroendocrine, physiological, and behavioural responses) through the hypothalamus–pituitary–adrenal cortex axis (Do Yup Lee and Choi 2015; Seyidoglu *et al*. 2021). In this context, many nutritional sources or additives (natural or synthetic), and their specific components have been long studied *in vitro* and *in vivo* to determine their anti-stress, therapeutic, and immunomodulatory potential, such as *Spirulina platensis.*

S. (*Arthrospira*) *platensis* is a filamentous and multicellular Gram-negative cyanobacterium (blue-green algae) that is a member of the Phormidiaceae family (Seyidoglu *et al*. 2017; Zahran and Emam 2018). It is increasing in popularity through its high nutritional content (i.e., proteins, carbohydrates, lipids, minerals, vitamins and pigments) (Saranraj and Sivasakthi 2014; Seyidoglu *et al*. 2017) as well as bioactive components which conduce to its therapeutic

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potential (cancer, diabetes, arthritis, cardiovascular diseases, anaemia, allergic diseases) (Ramamoorthy and Premakumari 1996; Løbner *et al*. 2008; Juárez-Oropeza *et al*. 2009; Chen *et al*. 2012). *S. platensis* shows antioxidant and immunomodulatory properties through some biochemical constituents such as γ-linolenic acid (precursor of arachidonic acid), carotenes, tocopherol and minerals (e.g. Zn, Mn and Cu) (Juárez-Oropeza *et al*. 2009; Seyidoglu *et al*. 2017). Another significant antioxidant molecule derived from *S. platensis* is C-phycocyanin (Reddy *et al*. 2003). C-phycocyanin is a cyclooxygenase type 2 inhibitor which is 20% of all protein fractions of *S. platensis* (14000 mg/100 g). This protein-bound pigment has been shown to be a regulator of the immune system and it plays excellent roles in many important biological activities (e.g. antitumoral enhancement, hepatorenal protection, and anti-inflammation) to protect the organism from diseases (Bhat and Madyastha 2000; Reddy *et al.* 2003; Juárez-Oropeza *et al.* 2009; Seyidoglu *et al.* 2017).

Heat shock protein (HSP) 70 is one of the decisive and inducible HSPs in the mammalian brain and is highly conserved among eukaryotic cells (Kiang and Tsokos 1998; Ammon-Treiber *et al*. 2004). Increased HSP70 levels have been shown to be a protection mechanism against harmful conditions. However, HSP expression consumes much cellular energy and competes with the organisms' metabolism that leads to reduced cell growth rates and a reduction in productivity (Karl *et al*. 2009). In this respect, various stress factors transiently elevate the production of this protein which is genetically controlled by the same gene name, the *HSP70* (Kiang and Tsokos 1998). This gene is transcriptionally regulated by heat shock factors (HSFs) and heat shock rapidly stimulates the binding of these factors to the promoter of the *HSP70* gene. A genomic region involving five nGAAn sequences, namely heat shock elements (HSEs), is responsible for the HSF–HSE combination (Kiang and Tsokos 1998; Ammon-Treiber *et al*. 2004). Under normal unstressed conditions, the HSP90 is the predominant protein in several cell types and the HSF-HSP90 binding suppresses the *HSP70* gene expression (Izumoto and Herbert 1993; Sharp *et al*. 1999; Ammon-Treiber *et al*. 2004). The alterations in *HSP* genes expression levels and the synthesis of related proteins are important indicators of environmental stress exposure and the health status of the organism. Further, the elevation of *HSP70* mRNA levels or decrease in suppressor proteins (or vice versa) has been reported to be a remarkable genetic mechanism by *in vitro* and *in vivo* studies in response to the biological processes under various environmental stress conditions (Kiang and Tsokos 1998; Sorensen *et al*. 2007).

In the literature, many studies are focussing on the therapeutic and immunomodulatory potential of *S. platensis* (Ramamoorthy and Premakumari 1996; Løbner *et al*. 2008; Juárez-Oropeza *et al*. 2009). However, the capability of *S. platensis* on the genetic regulation of stress mechanisms has not been satisfactorily investigated in detail. On the other hand, many variables that can influence gene expression levels, including housing, sampling, handling, diurnal cycles, euthanasia, and necropsy procedures are often not controlled for *in vivo* experimental designs (Kozul *et al*. 2008). In this study, we focussed on the effects of *S. platensis* on the *HSP70* gene expression profiles by conducting a controlled experimental design using uniform Sprague-Dawley rat groups under stress conditions.

Materials and methods

Animals

Thirty-six male and healthy Sprague-Dawley rats (∼11 weeks old) were used in this study. All animals were housed under standard conditions in the laboratory situated in Bursa Uludag University Experimental Animal Breeding and Research Unit, Turkey. The rats had free access to tap water and standard commercial rodent chow (Korkutelim Yem Gida Sanayi, Turkey) containing 13.6% protein, 7.1% minerals, 4.1% fat, and 8.6% cellulose. They were kept in clear, meshed, and plastic cages of sizes 42×21×20 cm with wood shavings as bedding. The provided temperature gradient was 22±1 °C and humidity 55±10%. Each cage was furnished with stainless steel food hoppers and a watering nipple. The research has complied with all the relevant international regulations and the Turkish National Institute of Health Guidelines for the Care and Use of Laboratory Animals. Ethical approval for this study was granted by the Bursa Uludag University Local Research Ethics Committee (approval number: 2018-06/08).

S. platensis (Egert, Izmir, Turkey, AGHKN167) was applied by a gastric gavage and the doses were administered based on the previously published papers (Hoor *et al*. 1980; Yang *et al*. 2006). The content analysed and pure commercial product of *S. platensis* used in this study contains 65% proteins, 18% carbohydrates, 7% oils (especially linolenic, linoleic and arachidonic acid), and minerals.

Experimental design

The rats were allocated into four groups (nine rats each) with uniform conditions. The experimental process was initiated after two weeks of adaptation. The total experiment protocol was maintained for 28 days. Considering that rats could adapt to any mild stressor within about three days, we used a combination of acute and chronic stress, to simulate biological processes as accurately as possible. When a daily supplement is tested for its stress-relieving properties or its long-term impact, a model consisting of multiple stress exposures has shown to be ideal (Garcia *et al*. 2000; Smith 2012). The standard lightning procedure for laboratory animals, which was on a 12 h light/dark cycle with the lights off at 07:00–19:00 was applied during the first two weeks of the

study. Concerning the last two weeks of the study, rats were exposed to a light/dark cycle which is 18 h light / 6 h dark at 07:00–01:00 as suggested by relevant studies based on the murine biological rhythm (Hoor *et al*. 1980; Gancarczyk *et al*. 2004; Park *et al*. 2015).

Experimental rats were classified as follows: (i) control group (C), fed standard chow and no stress was applied throughout the study. (ii) Stress-only group (S): fed standard chow and the mixed stress protocol was applied throughout the study. (iii) *S. platensis* group (Sp): fed standard chow, same doses of *S. platensis* were administered by gastric gavage, and no stress was applied throughout the study. (iv) *S. platensis* + stress group (SpS): fed standard chow, same doses of *S. platensis* were administered by gastric gavage, and the mixed stress protocol was applied throughout the study.

S. platensis was applied by gastric gavage (1500 mg/kg/day) to Sp and SpS groups while 1 cc of tap water was applied to C and S groups for 28 days. Tap water was given to the rats with the same dose and duration as *S. platensis* administration to eliminate the stress factor that originated from the gastric gavage application. The gastric gavage application was performed at the same time each day during the light period. In this study, two stress conditions were set, including hosting alone stress and crowded environment stress. Stress protocols were applied at the last two weeks of the experimental process. Hosting alone stress was applied to S and SpS groups. Each rat from the corresponding groups was hosted in a separate cage with four sides and a white ground for 30 min on Monday, Wednesday, Friday and Sunday. Neither food nor water was given to the rats during this stress application. Crowded environment stress was applied (S and SpS groups) by leaving six rats in the same size cage for three rats (50% cramped) on Tuesday, Thursday, and Saturday for 30 min. Neither food nor water was given to the rats during this stress application.

Sampling and ELISA analysis

At the end of the study, rats were euthanized with 5% isoflurane and the blood samples were obtained by puncturing the heart. Serum and plasma were immediately separated from blood cells and stored at −80°C until analysed. When the animals were sacrificed, craniotomy removal of brain tissue was performed and sectioned. Cerebellum was separated from the brains that were cut in the midline between the two cerebral hemispheres. Genetic analysis was carried out using whole cerebrum samples to determine the transcriptional regulation of the *HSP70* along the axis. The brain samples were immediately stored in RNAlater stabilization solution (Ambion, Austin, USA) at –80°C for the genetic analyses. All surgical procedures and blood sampling were completed on the same day by the same researchers to prevent unreliable results. Serum corticosterone (Elabscience, Houston, USA), IL-2, IL-4, and IFN-γ (ThermoFisher Scientific, Massachusetts, USA) were measured by enzyme-linked immune sorbent assay (ELISA) technique using commercially available rat-specific kits (Catalog numbers: BMS634, BMS628, and BMS621 for IL-2, IL-4, and IFN-γ, respectively). ELISA procedures were performed based on the instructions provided by the manufacturer companies and read in a microplate reader (Biotek Epoch, Winooski, USA). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by standard enzymatic methods. Serum triglyceride (mg/dL) was determined using a commercial kit (Sigma, Triglyceride Reagent #T2449 and Free Glycerol Reagent #F6428).

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

The brain samples were cut into ∼1×1 cm sections and were homogenized using a Magnalyser instrument and homogenization tubes containing ceramic beads (Roche MagNA Lyser Benchtop Homoginizaton System). Total RNA was extracted from frozen tissue samples using TRIpure® Isolation Reagent (Roche Life Science, Penzberg, Germany) based on the instructions with some modifications. Briefly, tissues were incubated with 1 mL TRIpure at room temperature for 5 min vortexed for 45 s. The mixture was incubated on ice for 2 min and then incubated for 5 min at room temperature. Chloroform 200 μL was added to the mixture and then incubated at room temperature for 5 min and subjected to centrifugation at 12,000 rpm for 20 min at 4°C. The supernatant of the mixture was then transferred to a sterile tube and incubated with 500 μL of isopropanol at –20°C overnight. After another centrifugation at 12,000 rpm for 10 min at 4°C, 1 mL 75% ethanol (EtOH) was added to the precipitation and subjected to centrifugation at 7500×g for 5 min at 4°C. EtOH was evaporated at 57°C and the precipitate was dissolved in 50–100 μL of RNase-free molecular biology grade double distilled H_2O to obtain final RNA samples. The concentration range (ng/μL) and the purity (260/280 ratio) of the RNA samples were measured with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, USA). The RNA was revers-transcribed using iScript cDNA Synthesis kit (BioRad, Laboratories, USA) according to the manufacturer's instructions. Real-time PCR was performed using the LightCycler 480 II Real-

Table 1. Primer sequences used in this study.

Gene name	Primer	Sequence
*ACTB**	Forward	ACCACCATGTACCCAGGCATT
	Reverse	CCACACAGAGTACTTGCGCTCA
HSP70	Forward	TTCGAGTTGAGCGGCATC
	Reverse	TGTCGTTGGTGATGGTGATC

ACTB, beta-actin (β-actin); *HSP70*, heat shock protein.
*Housekeeping gene.

Time PCR System (Roche Diagnostics, Indianapolis, USA) and SYBR Green I Master chemistry (Roche). For each PCR amplification (total volume: 10 μL), 2.5 μL of cDNA, 0.3 μL of forward and reverse primers (each), 5 μL of SYBR Green I Master, and 1.9 μL of nuclease-free water (Thermo Scientific) was used. Specific rat primer sequences (table 1) were obtained from Invitrogen (Invitrogen, Paisley, UK) and were verified for rat genome specificity by conducting BLAST searches of the NCBI Gen-Bank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The thermal profile consisted of preincubation at 95°C 5 min, followed by 45 cycles of denaturation for 10 s at 95°C, an annealing for 15 s at 57°C, an extension step of 10 s at 72°C, and melt-curve analysis. LightCycler 480 Relative Quantification Software (Roche) was used for quantification. The expression profile of *HSP70* was normalized to beta-actin (*ACTB*) expression (internal control), and the fold change was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). In this respect, the cycle threshold (*Ct*) value of each sample was estimated by subtracting the average *Ct* value of the reference gene (housekeeping gene, *ACTB*) from the average *Ct* value of the target gene. For each group, the ΔCt value was determined by subtracting the group average from this value. The target gene expression level relative to the sample was then estimated as $2^{-\Delta\Delta Ct}$. The gene-expression results were confirmed by the Roche Applied Science E-Method (Roche Diagnostics) using the crossing point (Cp) values of the target and the reference gene. The calculation (and the details) of the ratio of the regulated target gene (*HSP70*) to an unregulated housekeeping gene (*ACTB*) in the same sample was performed based on the study by Tellmann (2006). All analyses were performed in triplicate and the average of the values was accepted as the final value.

Statistical analysis

GraphPad Prism 9 was used for statistical analysis (GraphPad Software, La Jolla, USA). The Anderson–Darling test was performed to evaluate the normality of the data. To determine the differences among groups, Kruskal–Wallis with Dunn's posthoc multiple comparison tests were performed with a *P*-value <0.05 deemed statistically significant. Pearson correlation coefficient (*r*) was used for assessing the relationship between the *HSP70* gene expression, serum corticosterone, IL-2, IL-4, IFN-γ, triglyceride, ALT, and AST levels. Heatmap was generated based on the *r* values of the Pearson correlation.

Results

Relative quantification data (the ratio of a regulated target gene to an unregulated housekeeping gene) was successfully generated and the amplification curves obtained from the LightCycler 480 System were presented for the *ACTB* (figure 1) and the *HSP70* (figure 2) genes. Results revealed that dietary intake of *S. platensis* and the stress applications altered the gene expression profile of the murine *HSP70*. With reference to figure 3, relative gene expression values were remarkably different among the four experimental groups. As expected, the highest *HSP70* gene expression was observed in the stress-applied group (S). The lowest expression was estimated to be 0.810 and it belonged to group Sp. The *HSP70* expression values of both Sp and SpS groups were lower than the control group (1.198) but this was not substantiated in Dunn's posthoc multiple comparison test (*P*>0.05). The mean of the *HSP70* expression was 0.881 in the SpS group. Conspicuously, the differences between S and the *S. platensis* groups (S vs Sp and S vs SpS) were statistically significant (*P*<0.05) in multiple comparisons (figure 3). The result was also confirmed in the evaluation of fold change alterations based on the control group gene expression in the Roche E method (figure 1 in electronic supplementary material at https://www.ias.ac.in/jgenet/). This may indicate a novel potential effect of *S. platensis* on the *HSP70* gene expression related to the stress response. The relative gene expression results of each sample are presented in figure 2 in electronic supplementary material.

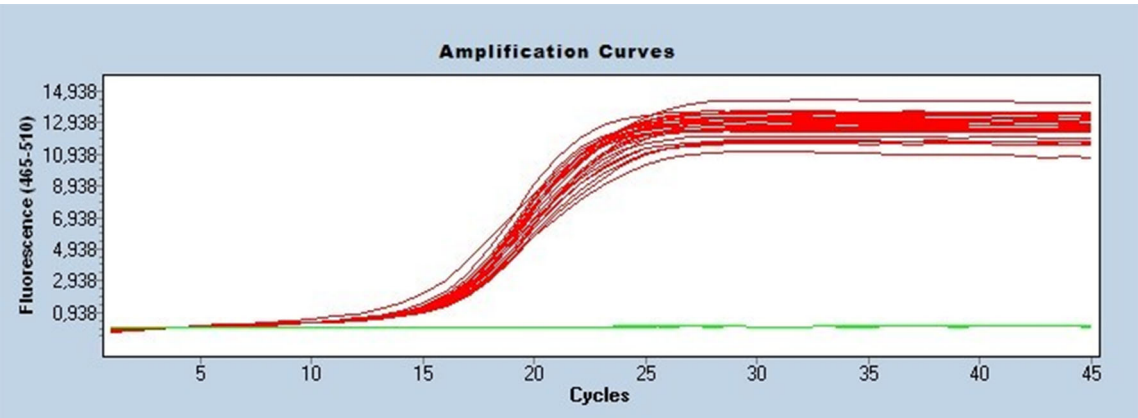

Figure 1. Amplification curves in the qRT-PCR analysis for β-actin (*ACTB*).

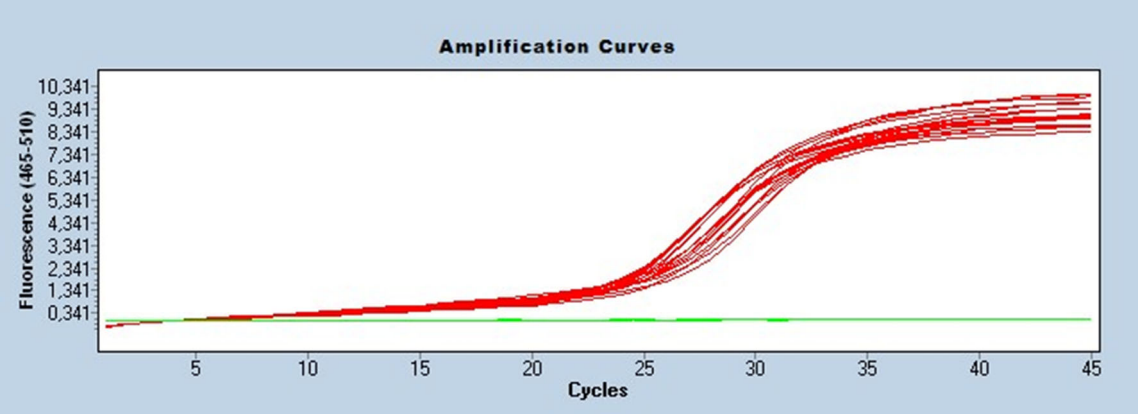

Figure 2. Amplification curves in the qRT-PCR analysis for the *HSP70*.

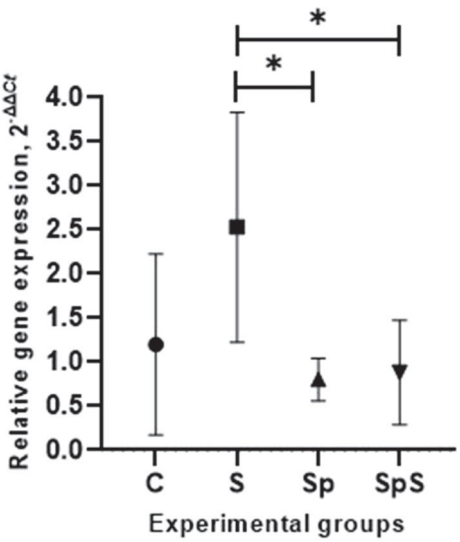

Figure 3. Gene expression of the *HSP70* in control rats and the three experimental rat groups. Data are expressed as mean $2^{-\Delta\Delta Ct}$ ± SD. C, control; S, stress-only group; Sp, *S. platensis* group; SpS, *S. platensis* + stress group. *P<0.05.

Differences in serum corticosterone levels among groups are presented in table 2. Results revealed that the serum corticosterone levels were statistically different among the experimental rats and the highest value was observed in the stress-applied group (Kruskal–Wallis with Dunn's posthoc; P<0.05) which is a prerequisite for our study's main hypothesis. Figure 4 shows the correlation among serum corticosterone, IL-2, IL-4, IFN-γ, triglyceride, ALT, AST levels, and relative gene expression values. The correlation between the *HSP70* relative gene expression and the IFN-γ was found to be statistically significant (P<0.05; r=0.50). No significant correlation was found between the *HSP70* expression and the rest of the biochemical parameters. It is also worth noting that this study focusses on the alterations in the *HSP70* gene profile through the influence of dietary *S. platensis* on the stress response, and hence, the correlations between commonly studied serum biochemical parameters (e.g. ALT vs AST) will not be discussed further (nor the differences among groups), even if they are statistically significant.

Discussion

S. platensis has prominent effects on growth and immunomodulation and has been widely studied by researchers from various fields. Thus, it has been defined as a 'superfood' (Seyidoglu *et al*. 2017). *S. platensis* enhances the antioxidant defense system by increasing glutathione, superoxide dismutase activity, and the synthesis of various antioxidant enzymes (Park *et al*. 2018; Hajati *et al*. 2021). Although many molecules that are effective in different biological pathways have been studied, it is clearly seen from the literature that the studies related to *S. platensis* are mostly at the enzyme and/or protein level. The present study was designed to evaluate the potential stress-preventive effects of *S. platensis* on the *HSP70* gene expression profile in an *in vivo* experimental rat model. It is well known that corticosterone is an indicator of the presence of a stress or arousal reaction and is thus often used as a biomarker for stress. Moreover, corticosterone is the main glucocorticoid involved in the regulation of stress responses in rodents (Gong *et al*. 2015). Hence, alteration in corticosterone levels is one of the main indicators that experimental stress was adequately exposed to rats in the stress group of our study. In this context, the serum corticosterone significantly increased in the stress-exposed group (S) compared to other experimental groups (table 2). It is worth noting that this is an important detail in the experimental process and these results indicate that stress was induced successfully. Although control and the other groups fed with *S. platensis* were not significantly different, the lowest level of serum corticosterone was observed in the Sp (45.09±2.44). Another important point is that corticosterone decreased by 19.45% in the SpS compared to stress-exposed rats. In fact, several studies have demonstrated decreased corticosterone in animals fed with *S. platensis* under stress conditions. For

Table 2. Serum corticosterone levels in the studied rat groups. The results were expressed as means with their corresponding standard errors.

	Experimental groups			
	C	S	Sp	SpS
Serum corticosterone, ng/mL	46.02±1.72[b]	57.03±1.84[a]	45.09±2.44[b]	45.94±2.32[b]

C, control; S, stress-only group; Sp, *S. platensis* group; SpS, *S. platensis* + stress group.
[a;b]Different superscripts are significantly different at the 0.01 level.

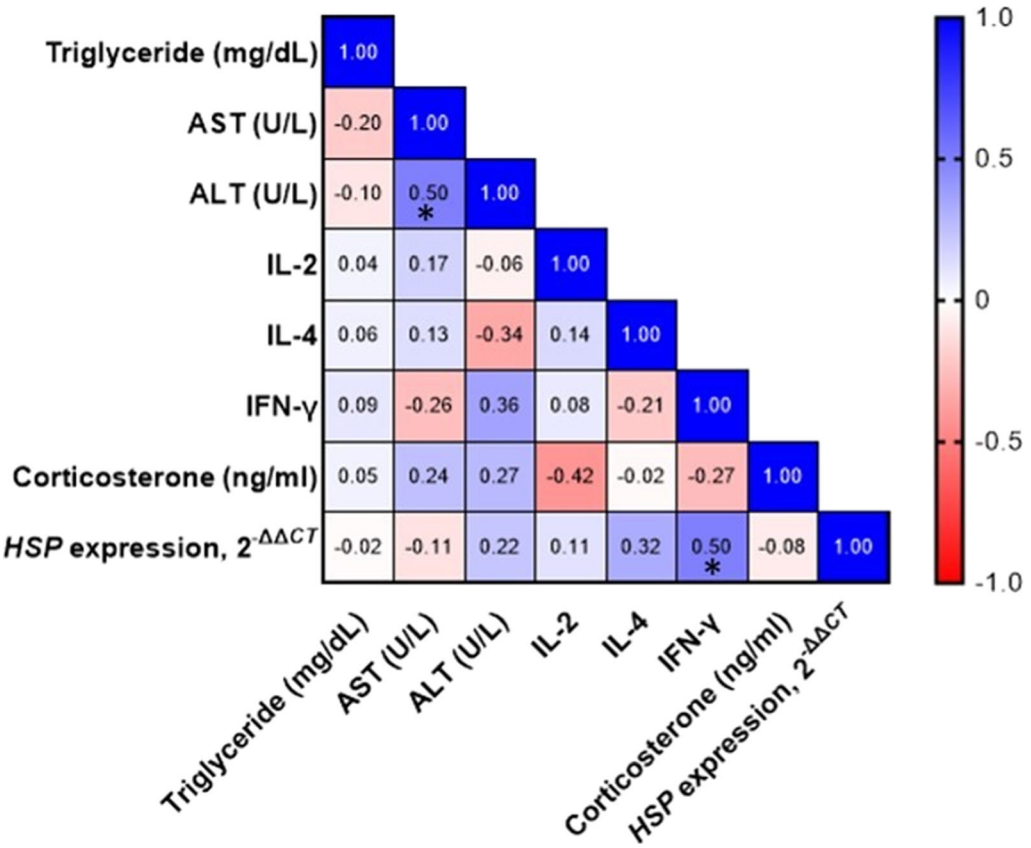

Figure 4. The heatmap of the correlation among serum corticosterone, IL-2, IL-4, IFN-γ, triglyceride, ALT, AST levels, and relative gene expression values of the *HSP70*. The heatmap was generated using Pearson correlation coefficients (*r*) values. *$P<0.05$.

instance, de Mattos *et al*. (2019) reported that dietary inclusion of *S. platensis* reduced serum corticosterone and glucose levels and also improved haematological and serum biochemical parameters in the rainbow trout (*Oncorhynchus mykiss*). These authors suggested that the reason for these changes may be related to the reduced expression levels of some stress-related genes. Similarly, Yeganeh *et al*. (2015) showed that plasma cortisol of *S. platensis* treatments was 1.3–2.2 fold lower than the control group and they suggested that rainbow trout fed with *S. platensis* supplemented diet may be more resistant to the stress conditions. Although many other studies have emphasized the presence of bioactive compounds of *S. platensis* and their immunostimulant ability which enables organisms to better fight against stress, related genetic studies on the subject are rather insufficient. In this study, rats fed with *S. platensis* significantly lowered the *HSP70* gene expression ($P<0.05$) after a challenge with two types of stress exposure (hosting alone and crowded environment stress), which was consistent with serum corticosterone results. As expected, the highest *HSP70* expression was observed in the stress-only group. Although close results were obtained for the Sp and SpS groups, the lowest level was found in the Sp group (figure 3). A significant difference was expectedly observed between the control and S groups regarding the *HSP70* expression in nonparametric ANOVA ($P<0.05$), but this result could not be corroborated in multiple comparisons (Dunn's multiple comparisons, $P>0.05$). One possible

explanation is the individual differences observed in the control and S groups (figure 2 in electronic supplementary material), which is a common consequence of *in vivo* experimental studies. Another explanation for this situation may be the limited sample size per experimental group. In accordance with our recently reported interpretations for *S. platensis,* Al-Deriny *et al*. (2020) demonstrated that the transcription of *HSP70* was decreased by feeding spirulina compared to the control group ($P < 0.05$) in Nile tilapia (*Oreochromis niloticus*). In a Parkinson's disease model of *Drosophila melanogaster*, supplementation of *S. platensis* reduced the cellular stress via deregulating the expression of the *HSP70* gene (Kumar *et al*. 2017). Moreover, in a recent paper by Hajati *et al*. (2021), *in ovo* injection of *S. platensis*/egg (25 mg) decreased *HSP70* gene expression in newly hatched broiler chicks without any adverse effect on their health status after hatching. On the contrary, Hajati *et al*. (2020) found that there was no statistically significant relationship between the *HSP70* gene expression in the liver/-heart and supplementation with *S. platensis* in quails under heat stress. Although the molecular basis of these effects of spirulina has not been fully elucidated, several hypotheses have been established. First, *S. platensis* contains important antioxidant phytochemicals including β-carotene, phycocyanin, phycobiliproteins, allophycocyanin, and phenolic compounds (e.g. salicylic, chlorogenic, quinic, and caffeic acids), etc. (Hajati *et al*. 2021). Secondly, spirulina includes mediators of immune and cardiovascular systems such as gamma-linolenic acid and linolenic acid (precursor effects of prostaglandins and leukotrienes), highly digestible carbohydrates (especially, a specific polysaccharide molecule, called 'immulina'), and many minerals (Seyidoglu *et al*. 2017). The animal cells exhibit high levels of HSP70 associated with an increase in protein integrity and a decrease in apoptosis status under stress conditions. The relation between *S. platensis* and the dynamics of the immune response through corresponding protein expressions has been previously reported in various species. Nevertheless, recent genetic knowledge is insufficient, especially for mammals. The present study is the first of its kind in mammal species that established the significant relationship between *HSP70* gene expression profiles with the functionality of *S. platensis* supplementation, concerning stress conditions and related biological regulations. Such knowledge is critical because this study could also provide important data for livestock where crowd stress is a particular problem and also for animal welfare. Although the present results indicate a novel effect of *S. platensis*, further genetic studies are needed to support our findings and to provide more detailed data about the subject, especially epigenetic analyses.

HSP70 gene plays a major role in various cellular mechanisms owing to its protein product (HSP70) which displays chaperone activity. In this sense, HSP70 regulates newly synthesized proteins by numerous pathways including folding, degradation and transportation (Yu *et al*. 2021). This is a crucial point of this gene regulation because many stress types disrupt protein folding (Baird *et al*. 2006). Hence, the upregulation of the *HSP70* gene has been shown to be an indicator of stress in many organisms. HSF-related system is one of the most essential mechanisms in stress response. In normal conditions, HSF1 is monomeric but HSF1 trimers form and bind to HSEs in stress exposure which leads to *HSP70* transcription. The HSF1-dependent transactivation system is generally conserved among eukaryotes (Yu *et al*. 2021) and appears to provide protection against stress. Notably, *S. platensis* enhances the immune status, which is consistent with the upregulation of the genes related to stress and immune responses (de Mattos *et al*. 2019). In this study, Pearson correlation results revealed that there was a statistically significant relationship between the *HSP70* transcription and the expression of IFN-γ ($r = 0.50$; $P < 0.05$). Previously published papers have indicated that *S. platensis* supplementation influences the expression of IFN-γ. Hajati *et al*. (2021) reported that the expression of IFN-γ in the chicks consumed 1.5–3.5 mg *S. platensis*/egg was significantly higher than control groups ($P < 0.001$). Moreover, Mao *et al*. (2000) have shown that spirulina-induced secretion of IFN-γ was ∼13.6 times more than basal levels in human blood. The authors have also reported a 3.3-fold increase in IL-4 and concluded that the inclusion of *S. platensis* to *in vitro* peripheral blood mononuclear cells cultures can significantly enhance the production of IL-1β, IL-4, and IFN- γ. It is well known that IFN-y and IL4 have an antagonistic interaction and they differentially affect the immune response and counteract each other (Mao *et al*. 2000). However, these relations were not observed in this study, and moreover, IL-2, IL-4, and IFN-γ expressions showed statistically significant changes neither in the correlation analysis (figure 4) nor in the group-specific assessment (figure 3 in electronic supplementary material). It is worth noting that IFN-γ expression was the highest in the Sp group but this effect was not substantiated in the statistical analysis. *S. platensis* consists of nutrients that modulate the immune system which is directly related to stress conditions of the organism (Seyidoglu *et al*. 2017). However, systems developed against stress are quite complex biological pathways since their evolution (Yu *et al*. 2021). Accordingly, the alterations in cytokine expression levels and inconsistent results from different experimental designs are not surprising, especially for *in vivo* studies.

Hypolipidaemic activity and hepatoprotective effects of spirulina are still under investigation. Experimental studies conducted on rats have shown that *S. platensis* inhibits ileal bile acid reabsorption, jejunal cholesterol absorption (Nagaoka *et al*. 2005), and pancreatic lipase activity (Han *et al*. 2006). The active ingredients in spirulina responsible for these effects are proposed to be C-phycocyanin and γ-linolenic acid (Mazokopakis *et al*. 2014). However, in this study, there were no significant differences in triglyceride, AST, and ALT responses among the experimental groups. Although the triglyceride levels were remarkably higher in

Sp and SpS compared to C and S groups (figure 3 in electronic supplementary material), this relationship was not substantiated in the statistical analysis (Dunn's multiple comparisons, $P > 0.05$).

In general, stress can be defined as intrinsic (e.g. genetic factors) or extrinsic (environmental) conditions that affect homeostasis negatively or decrease the fitness of the biological system (Sørensen *et al*. 2003). The evolutionary process has allowed the development of various cellular mechanisms to overcome this situation. In this respect, one of the most conserved and widespread mechanisms is the regulation by *HSP* genes. The low variation in *HSP* genes and their universal presence (from bacteria to plants and animals) indicate evolutionary importance and a role in the protection of cells during or after stress (Hoffmann and Merila 1999; Sørensen *et al*. 2003). Among the superfamily of HSPs, HSP70 is the most abundant and sensitive protein and it protects the cell via its chaperone functions by transporting the damaged proteins to target organelles for repair or degradation (Li *et al*. 2017). Further, HSP70 can interfere with apoptosis at multiple levels (Kim and Yenari 2017). The responsible gene, *HSP70*, mediates the apoptosis-related mechanisms through recruitment of procaspase-9 into the apoptosome and antiapoptotic response by preventing the translocation of apoptosis-inducing factor (AIF) from mitochondria to the nucleus, inhibiting the mitochondrial release of the proapoptotic Bcl-2 family member Bax, and caspase-3. It also induces Bcl-2 to block the mitochondrial release of cytochrome c and AIF (Yenari *et al*. 2005; Giffard *et al*. 2008; Kim and Yenari 2017). On the other hand, the *HSP70* gene has crucial effects on the modulation of inflammation by interacting with various molecules including proinflammatory cytokines and inflammatory transcription factors (Giffard *et al*. 2008). As mentioned above, the overexpression of *HSP70* acts as the alert system for cellular responses under stress conditions, and the related molecular mechanisms have been well-documented. But one important question is whether the expression profiles are altered by extrinsic factors such as feed supplementation. Here, we demonstrated a potential novel effect of *S. platensis* on *HSP70* gene expression profiles in a controlled *in vivo* experimental design. Interestingly, spirulina supplementation significantly lowered the expression of the *HSP70* in stress-applied rats. From a broader perspective, although *HSP70* overexpression is known to have a cellular protective feature, *in vivo* responses may be much more complex. The effect of *S. platensis* here may be to contribute to the management of stress perception rather than prevent stress. The stress types applied in this study (hosting alone and crowded environment stress) also contribute to this hypothesis because these stress types also include psychological features. There is evidence that *HSP* genes may have pathophysiological importance for major mental disorders at the molecular level (Iwamoto *et al*. 2004). The key role in the relationship between *HSP* dynamics and mental health belongs to the mitochondria. Iwamoto *et al*. (2004) indicated that the altered expression of *HSP40* could be involved in the aberration of protein translocation systems into the mitochondria and/or endoplasmic reticulum, which in turn affects the functions of these organelles. Moreover, Bei *et al*. (2013) showed that bipolar disorder is accompanied by alterations in the glucocorticoid receptor (GR) signalling and HSP70 (HSP70-GR heterocomplex). These findings may lead to forming a hypothesis that alterations in stress-driven pathways and related gene expressions could result in changes through stress response and the management of stress perception of the individuals.

There are studies in different species that partially support the results of this paper. Remarkably, in a recent paper by Al-Deriny *et al*. (2020), spirulina supplementation was significantly associated ($P<0.05$) with the decreased transcription levels of *HSP70* in Nile tilapia (*Oreochromis niloticus*). More particularly, dietary supplementation of spirulina/C-phycocyanin has been shown to be effective in elevated levels of lifespan and improved health status in *Drosophila melanogaster* by boosting the antioxidant defense system and lowering the HSP70 signalling (Kumar *et al*. 2017). On other hand, from an evolutionary perspective, *HSP* expression results in higher cellular energy consumption and competes with the housekeeping metabolism, and thus, continuous or frequent exposure to stress may therefore reduce the expression of *HSP70* through evolution (Sørensen *et al*. 2001; Karl *et al*. 2009). As Karl *et al*. (2009) pointed out, the associated cellular energy costs regarding *HSP70* overexpression may outweigh its benefits. This also indicates that there may be alternative mechanistic *in vivo* responses that can be developed by the organism under different stress dynamics.

Spirulina has a rich source of protein that supports the development of the nervous system and brain normal physiological functions. Furthermore, spirulina compensates for nutritional deficiencies, improves immune responses and reduces adverse effects (Trotta *et al*. 2022). C-phycocyanine, which is the most important component of spirulina, can be broken down by proteolysis of peptides related to phycocyanobilin or phycocyanobilin that play a role in the pharmacological actions of biliproteins. Phycocyanobilin has a similar chemical structure as bilirubin, which is a NADPH oxidase inhibitor. This implies that phycocyanobilin can contribute to oxidative stress (McCarty 2007; Riss *et al*. 2007). McCarty (2015) noted that oxidative stress from NADPH oxidase may contribute to the damage caused by blood brain barrier and spirulina phycocyanobilin can enhance the cerebral small vessel disease course by suppressing the activity of NADPH oxidase. It has been reported that C-phycocyanin oral administration reduced microglia and astrocyte activation. The neuroprotective role of the effect of three different treatments with C-phycocyanin was studied in kainate-injured brains of rats and the incidence of neurobehavioral changes was significantly

lower in animals receiving C-phycocyanin (Rimbau *et al*. 1999; Piniella-Matamoros *et al*. 2021).

To the best of our knowledge, there are no controlled experimental results on how the impact of dietary spirulina supplementation creates an *in vivo* response with *HSP* gene regulation mechanisms under stress conditions, especially in mammalian species. This study may contribute to the understanding of the interaction of molecular genetic responses developed under stress conditions with dietary practices. It is worth noting that, however, related mechanistic dynamics may be much more complex than expected and hence more detailed genetic analysis supported by ethological aspects is needed to be performed to reveal the peculiar characteristics of *S. platensis,* the 'superfood'.

This study was mainly designed to evaluate the effects of *S. platensis* on the *HSP70* gene expression. Hence, the genetic variation of experimental animals is a crucial point that cannot be overlooked. To prevent erroneous results that may arise from individual genotypic differences, inbred Sprague–Dawley rats that were created following more than 20 generations of sibling or parent–offspring mating were used as animal material. Another important point is that gastric gavage was applied to ensure that all animals consume the same dose of spirulina. All conditions were kept equal for all groups throughout the entire study period, as even minor changes could alter the selected gene expressions. These variables are often not controlled for *in vivo* experimental designs and they can remarkably influence gene expression, confounding the effects of the experimental treatment (Kozul *et al*. 2008). Herein, we think that the present results can be reliable and explanatory about the spirulina effects on stress-response and related biological regulations. On the other hand, the main limitation of this study is the lack of Western blot analysis due to a limited research budget. Another limitation is that only *HSP70* expression of the *HSP* was evaluated. *HSP* superfamily consists of several closely related genes from major families including *HSP100*, *HSP90*, *HSP70*, *HSP60*, *HSP40* and the small HSPs (sHsp: product sizes below 30 kDa), and smaller cofactors. But, here, it should be noted that HSP70 is the most abundant, highly conserved, and sensitive protein of the super family of HSPs (Li *et al*. 2017). Moreover, it is considered to be the major HSP family consisting of solely inducible, constitutive and inducible, and solely constitutive proteins (heat shock cognates) in many organisms (Sørensen *et al*. 2003). Thus, our study provides important evidence for a novel molecular effect of *S. platensis* under stress conditions at the gene expression level. This knowledge may be useful for further genetic studies on the corresponding subject and the present results may also be indicative for genetic analyses about the novel stress-response-related feed additives in livestock, especially for avian species.

Coping with the exposure to stress is a complex biological process that includes many proteins and genes. Considering the fact that *S. platensis* has many beneficial effects on many biological mechanisms associated with stress response, it could be suggested as a food supplement for the prevention of stress-related damage not only in laboratory animals but also livestock species.

Conclusions

This paper focusses on the relationship of *S. platensis* supplementation with murine *HSP70* gene expression using an *in vivo* experimental design. Moreover, some cytokines including IL-2, IL-4, and IFN-γ, serum corticosterone levels, and some hepatotoxic parameters including triglyceride, ALT, and AST were considered. Considering the data of real-time PCR analysis, we suggest that *S. platensis* alters the *HSP70* gene expression profile with stress induction by crowded environment and hosting alone stress procedures. Our results showed that spirulina supplemented group had lower *HSP* gene expression compared to the stress-exposed group, which was consistent with the serum corticosterone levels. The correlation between the *HSP70* relative gene expression and the IFN-γ was found to be statistically significant. However, no significant difference was found in cytokines and hepatotoxic parameters among experimental groups. Based on these findings, *S. platensis* appear as promising dietary protein sources to promote homeostasis under stress conditions and to contribute to animal welfare.

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