Hepatoprotective Effects of Selenium-Enriched probiotics supplementation on heat-stressed Wistar rat through Anti-Inflammatory and Antioxidant Effects

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Abstract

The purpose of this study was to elucidate the effects of selenium-enriched probiotics on the liver of heat-stressed Wistar rats. Ten-week-old male rats were assigned to four groups: control (Con); high temperature (HT); high temperature plus probiotics (HT+P: 10^{11} CFU/mL *Lactobacillus acidophilus* and 10^9 CFU/mL *Saccharomyces cerevisiae*); or high temperature plus selenium-enriched probiotics (HT+SeP: 0.3 mg/kg Se, 10^{11} CFU/mL *L. acidophilus* and 10^9 CFU/mL *S. cerevisiae*). The HT, HT+P, and HT+SeP groups were maintained at higher ambient temperature (40-42 °C), while the control group were kept at room temperature (25 °C). After 42 days of thermal exposure, blood and liver tissues were collected and analyzed for morphological and molecular markers of liver physiology. The body weight of rats in the HT group decreased but liver weight and live index were increased. Histological examination showed dilation of liver sinusoids and congestion of interstitial veins in HT group. Moreover, the histomorphology of the liver in HT+P and HT+SeP groups was restored, and the serum AST, ALT, ALP, LDH and hepatic MDA level decreased significantly, but the serum total protein level and the liver SOD, T-AOC, and GSH-PX activities were increased significantly relative to the HT group. In addition, the mRNA level of Gpx1, SOD1, Nrf2, and Bcl-2 was significantly increased, while the expression level of Bax, IL-6, TNF- α , COX-2, NF- κ B, α -SMA, TGF β 1, Collagen I, HSP70, and HSP90 was significantly decreased in liver tissues after SeP supplementation. We concluded that SeP can protect Wistar rats from oxidative stress, inflammation, apoptosis, and liver fibrosis induced by heat stress.

Keywords: Liver; Selenium-Enriched probiotics; High ambient temperature; Inflammation; Oxidative stress, Apoptosis; Wistar rats

Introduction

Long-term heat stress in summer can affect domestic animals and ultimately lead to reduced productivity by affecting certain physiological mechanisms [1]. Exposure of aged rat to acute heat stress (40-42°C for 24-48 hours) can lead to liver damage, increased reactive oxygen species levels, and altered intracellular signal transduction [2]. It was also found that acute thermal stress increased oxidative stress and ROS levels in superoxide dismutase 1 (SOD1) gene knock-out mice, even after exposure to 42 °C conditions for just 15 minutes, and lead to impaired spermatogenesis [1]. Another study found that the levels of reactive oxygen species (ROS) and lipid peroxidation were increased in the small intestine of heat stress-exposed rats, while the activities of SOD and glutathione peroxidase (GSH-Px) were decreased [3].

Selenium (Se), zinc (Zn), and vitamin C and E are the most important antioxidants. Zn and Se are essential components of the antioxidant enzymes SOD1 and GSH-Px, respectively [4,5]. Se is an essential trace element and plays a fundamental role in several cellular processes, one of its most important functions is the antioxidant defense system [6]. It has previously been observed that increased body temperature (heat stress conditions) affects numerous aspects of animal physiology, and Se supplementation can reduce some of these heat stress effects. An effective model is that since selenium is added to the diet of heat-stressed animals, its antioxidant capacity is higher, which can increase the activity of certain stress marker enzymes [7]. Notably, considerable evidence suggests that selenium reduces liver chronic diseases [8].

Probiotics have also been shown to be potentially useful dietary supplements for the treatment of liver diseases caused by heavy drinking, viral infection, and metabolic disorders [9-11]. Moreover, probiotic organisms such as *Saccharomyces cerevisiae* and *Lactobacillus* spp. can help reduce liver damage caused by oxidative stress [12]. The Institute for Nutrition and Metabolic Disorders of domestic animal and poultry has developed a new selenium-enriched probiotics (SeP) product, produced under an appropriate microenvironment by adding sodium selenite into the culture medium in which *L. acidophilus* and *S. cerevisiae* are grown. Both strains have the ability to convert sodium selenite into organic selenium

[12]. Earlier studies have shown that addition of SeP into the diet of poultry, livestock, and mice results in a combined effect, synergizing a benefit of both selenium and probiotics [13-15].

Heat stress is one of the main harmful factors that affect liver tissues and cause liver damage [16]. Long-term acute heat stress can lead to chronic liver injury, which is also a recognized model of liver fibrosis [2]. So far, there is no report on the effects of selenium-enriched probiotics on the physiological functions of Wistar rat in high ambient temperature. The purpose of present study was to investigate the protective effects of supplemental selenium-enriched probiotics on heat stress-induced liver damage by studying the anti-inflammatory and antioxidant properties of Wistar rats inflated at high ambient temperature.

Materials and Methods

Reagents

Total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) activity and malondialdehyde (MDA) content kits were received from Wuhan Service Biotechnology Co., Ltd. (Wuhan, China). Selenium was obtained from Sigma (Shanghai, China). The RT and qRT-PCR chemical reagents were obtained from TakaRa (Dalian, China).

Animals and Experimental Design

A total of 48 male, 10-week old Wistar rats (mean weight 200 ± 20 g) were received from the Animal Experimental Center of Yangzhou University of China. The rats were placed in clean polypropylene cages (4 rats/cage) under controlled environmental conditions in a 12 h light/dark program. The temperature and relative humidity were 24 ± 2 °C and 60-70 %, respectively [17]. Drinking water and basal diet were used during the experiment without restriction. The research procedure was agreed by the Animal Protection and Utilization Committee of Nanjing Agricultural University (Certification No.: SYXK (Su) 2011- 0036) and the basic ingredients and nutritional composition of basal diet fed to Wister rats were formulated according to the basic requirements of the National Research Council [18], and are

reported in details in our previous study [19]. After a week of adaptation, the 48 Wistar rats were randomly allocated into four groups (12 rats in each group), with 3 replicates, and each replicate consisting of 4 rats: Control group (Con, n=12); high temperature group (HT, n=12); high temperature + probiotics group (HT+P, n=12); and high temperature + selenium-enriched probiotics group (HT+SeP, n=12). The probiotic (P) and Selenium-enriched probiotics (SeP) diets were fed (1 mL per day) through the stomach tube to each rat during the daytime for 42 days. The experiments lasted for 6 weeks, and all rats were fed with the basal diet for all 42 consecutive days (July 1 to August 12).

Sample Collection and Preparation

The liver was removed, and transverse or longitudinal sections were taken and fixed in 10% neutral-buffer formalin. The remaining liver tissue was washed in ice-cold phosphate-buffer saline, and stored at -80 °C for further experimental work. The mortality rate was 0% during the experiment. Wistar rats fed a basal diet were changed on daily basis, and fresh water was always available during the experiments. Rat weight was measured on the first and last days of the experiment. All parameters such as final body weight, liver weight and liver index (%) were calculated for each rat and group. At the beginning of the experiment, the environmental temperature for rats in the three treatment groups (HT, HT+P and HT+SeP) was 36-39°C, and in the last 20 days was 39-42°C (Fig1), while rats in the control group remained for 42 days at normal temperature (24-25 °C) and 60-70 % relative humidity to avoid the influence of high ambient temperature, as reported by Wang, et al [16].

Fig 1. Daily average of Normal and High temperature from 12:00 a.m. to 08:00 p.m. in all 42-days of the experiment period.

Probiotics and Selenium-Enriched Probiotics

These two products, probiotics (P) and Se-enriched probiotics (SeP), was composed of *L. acidophilus* (10^{11} CFU/mL) and *S. cerevisiae* (10^9 CFU/mL). In addition, according to the procedure described by reference [20] , the total Se content in SeP were determined to be 0.3 mg/L [17,20], and a selenium containing probiotics fermentation medium was prepared to transform inorganic form into an organic form.

Biochemical Analysis of Serum

Measurements of serum alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, lactate dehydrogenase (LDH) and total protein (TP) were determined using commercial kits according to the manufacturer's instructions (Wuhan Service Biotechnology Co., Ltd. Wuhan, China).

Determination of Antioxidant Activity in Liver Tissues

Ice fixed liver tissues (500 mg) were homogenized in 5 mL cold homogeneous buffer (0.32 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) at 12500 rpm for 30 seconds on ice pack and then centrifuged for 10 minutes at 3000 rpm at 4 °C. The activity of antioxidant enzymes such as T-AOC, GSH-Px, SOD and MDA was determined from the supernatant of liver tissues using commercial kits according to the manufacturer's instructions (Wuhan Service Biotechnology Co., Ltd. Wuhan, China).

Hematoxylin and Eosin Staining (Histological Examinations)

To determine the histological changes, liver samples were fixed in 10% formalin, then dehydrated with different levels of ethanol, washed and cleaned with xylene, embedded in paraffin, sliced at a thickness of 4 μ m, and dyed with hematoxylin and eosin (H & E). The histological changes, such as dilatation in the hepatic sinusoids and an interstitial venous congestion, were observed under optical microscope (Tokyo, Japan) and were detected through a computer image analysis system (Image-Pro Plus version 6.0; Media Cybernetics, MD, U.S.A.) in four random areas of the slide.

Total RNA Extraction and Quantitative Real-time PCR assay (qRT-PCR)

qRT-PCR was used to detect the relative expression level of mRNAs encoding Hsp70, Hsp90, GPX1, SOD1, TNF- α , IL-6, COX-2, NF κ B, Collagen I, TGF- β 1, α -SMA, Nrf2, Bax and Bcl-2. NCBI's online primer design tool was used to design primers, as shown in Table 1. Trizol (Invitrogen) was used to extract total RNA from 50 mg of liver tissues, as described previously [17], and was reverse transcribed to cDNA using Prime-ScriptTM RT Master Mix kit using the manufacturer's protocol (Takara, Dalian, China). The purity of samples was determined by Nanodrop and the samples selected for further work showed a ratio between the A260 and A280 of between 1.8 and 2. Triplicate qPCR assays were performed using AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China) in 20 μ L PCR reaction mixture. The reaction mixture was composed of 10 μ L AceQ SYBR Green qPCR Master Mix (Vazyme, China), 0.4 μ L (10 μ M) forward primer, 0.4 μ L (10 μ M) reverse primer, 0.4 μ L ROX Reference Dye, 2

μL (10 ng/μL) of diluted cDNA; The reaction tube was supplemented 6.8 μL of RNase free dH₂O to a total volume of 20 μL. ABI prism 7300 software was used to detect fluorescent signal, and for PCR amplification, β -actin was used as an internal control to normalize the expression, and fold changes were determined by comparative threshold cycle and were calculated using the $2^{-\Delta \Delta CT}$ method.

Table 1. Primer pairs used for real-time PCR.

Target genes	Forward primer (5'→ 3')	Reverse primer (5'→ 3')
β-actin	CACGGCATTGTCACCAACTG	AACACAGCCTGGATGGCTAC
Bcl-2	ACTCTTCAGGGATGGGGTGA	TGACATCTCCCTGTTGACGC
Bax	AGGACGCATCCACCAAGAAG	CAGTTGAAGTTGCCGTCTGC
A-SMA	ACCATCGGGAATGAACGCTT	CTGTCAGCAATGCCTGGGTA
TGF-β1	AGGGCTACCATGCCAACTTC	CCACGTAGTAGACGATGGGC
Collagen I	GTACATCAGCCAAACCCCA	CAGGATCGGAACCTTCGCTT
Nrf2	GCACATCCAGACAGACACCA	CTCTCAACGTGGCTGGGAAT
GPX1	GCTCACCCGCTCTTTACCTT	GATGTCGATGGTGCGAAAGC
SOD1	AGGGCGTCATTCACTTCGAG	CCTCTCTTCATCCGCTGGAC
NF-kB	CATACGCTGACCCTAGCCTG	TCACTGAGCTCCCGATCAGA
COX-2	GATGACGAGCGACTGTTCCA	TGGTAACCGCTCAGGTGTTG
IL-6	AGAGACTTCCAGCCAGTTGC	AGTCTCCTCTCCGGACTTGT
TNF-α	CTGTGCCTCAGCCTCTTCTC	ACTGATGAGAGGGAGCCCAT
HSP70	GTACAGCAGCTCAAGGAGTTC	GGGGGAAGACACAAGCCTATT
HSP90	GAAAGGACGGTGTGGCCAAT	TCGTGGACCACCATAGTACG

Statistical Analysis

The collected data was analyzed by the SPSS 19.0 software. Significant differences among groups were investigated through the application of one-way Analysis of Variance (ANOVA) followed by Duncan's multiple comparison test. The significance level was set at 0.01 and 0.05 for different variables. The analyzed data was reported as mean $\pm$ Standard Error of Mean (SEM).

Results

Effects of Probiotics and Selenium-Enriched Probiotics on Body Weight and Liver Index

The difference between the body weight, liver weight and liver index of the control and experimental groups are reported in Table 2. According to the results, the initial body weight of almost all rats in all groups was the same. The final body weight of rats exposed to high temperature (HT) was significantly lower than that of the control group ($p < 0.01$), while there was a slight decrease in the final body weight of the HT + P and HT + SeP groups as compared to control group but were not significant ($p > 0.05$). However there was a significant increase in the final body weight of the HT + P and HT + SeP groups compared to HT group ($p < 0.01$). Moreover, the liver weight was significantly increased in the HT group in comparison with the control, HT+P and HT+SeP groups ($p < 0.01$), but there were no significant differences between control, HT+P and HT+SeP groups ($p > 0.05$). In addition, the liver index was significantly increased in the HT group ($p < 0.05$) as compared to control group. The liver index of the HT + P and HT + SeP groups was slightly higher compared to control group, however there was no significant difference between HT + P, HT + SeP, and control groups, but was significantly lower ($p < 0.01$) as compared to HT group.

Table 2. Effects of HT+P and HT+SeP on body weight, liver weight and liver index (liver weight/body weight × 100) of Wistar rat.

	Con	HT	HT+P	HT+SeP
Initial BW (g)	220±4.1 ^{*a}	221±3.28 ^a	222±3.21 ^a	220±2 ^a
Final BW (g)	310±4.18 ^a	232±6.56 ^b	280±6.98 ^a	295±6.35 ^a
Liver weight (g)	6.5±0.7 ^b	9.84±0.31 ^a	7.03±0.16 ^b	7.21±0.31 ^b
Liver index (%)	2.1±0.1 ^b	4.2±0.8 ^a	2.5±0.21 ^b	2.4±0.32 ^b

* Data is presented as mean ± SEM (n = 12).

^{a-b} Means followed by different superscript letter in the same row are significantly different ($p < 0.05$).

Effects of Selenium-Enriched Probiotics on Serum Enzyme Activity in Heat-Treated Wistar rats

The data pertaining to the activity of AST, ALT, ALP and LDH, and the total protein present in animal serum are depicted in Fig 2. The enzyme activity of AST, ALT, ALP, and LDH was significantly higher in the HT group in comparison with the control group ($p < 0.01$). Moreover, the activity of AST, ALT, ALP and LDH in both HT+P and HT+SeP groups were significantly decreased compared to HT group ($p < 0.05$), demonstrating a beneficial effects of probiotics treatment. Importantly, the effects of HT + SeP group on serum enzyme activity of AST, ALP, LDH and ALT ($p < 0.01$) was more prominent as compared to HT + P group, proving the beneficial effects of selenium-enriched probiotics treatment. It is worth noting that the HT + SeP group significantly reduced the serum enzyme level to almost the control level, and there was no significant difference between control and the HT + SeP group.

Fig 2. The effects of HT, P and SeP supplementation on liver marker enzymes and total protein in serum. Bar graphs show the mean of groups (n=8) and error bars depict the standard error of the mean. **(A)** Serum aspartate aminotransferase activity (AST); **(B)** serum alanine aminotransferase activity (ALT); **(C)** serum alkaline phosphatase activity (ALP); **(D)** serum lactate dehydrogenase activity (LDH); and **(E)** serum total protein content. ^{a-c} Bar graphs for each variable are followed by different lowercase letters to demonstrate which are significantly different (a represents $p < 0.01$; b represents $p < 0.05$; c represents $p > 0.05$). The results showed significant differences between control and treatment groups in terms of enzyme activity of ALT, AST, ALP and LDH, and total protein.

Effects of Selenium-Enriched Probiotics on Total Serum Protein in Heat-treated Rats

The results also showed a significant decrease in total serum protein in the HT group in comparison with control group ($p < 0.05$) (Fig. 2E). Interestingly, this measure was significantly increased by both probiotics treatments, and the protein level detected in the HT + P and HT + SeP groups was higher compared to the HT group ($p < 0.05$). Among all treatment groups, the HT + SeP group obtained high

significant value for total serum proteins ($p < 0.05$). On the contrary, as shown in Fig. 2E, there was no significant difference between the total serum proteins of control and the HT + SeP group ($p > 0.05$).

Selenium-Enriched Probiotics Activate Liver Antioxidant Enzymes in Heat-Treated Rats

The data pertaining to the level of T-AOC, GSH-Px, and SOD activity in liver tissues are shown in Fig 3. The HT group showed significantly decreased antioxidant activity of T-AOC, GSH-Px and SOD compared to the control group ($p < 0.01$). However, both probiotic treatments improved antioxidant activity of T-AOC, GSH-Px, and SOD and were significantly increased in the HT + P and HT + SeP groups compared with the HT group ($p < 0.05$), whereas there was no significant difference in the level of antioxidant activity of T-AOC, GSH-Px and SOD between control and HT+SeP groups ($p > 0.05$). Moreover, the antioxidant activity of T-AOC, GSH-Px and SOD in HT + SeP group was significantly higher ($p < 0.05$) compared to the HT group and were more prominent than HT + P group, however it was at the range of control group and there was no significant difference between the HT + SeP and control group ($p > 0.05$).

Fig 3. Effects of heat stress, P and SeP supplementation on hepatic oxidative stress parameters. Bar graphs show the mean of the groups (n=8) and error bars depict the standard error of the mean. **(A)** Level of Total Antioxidant

Capacity (T-AOC); **(B)** activity of Glutathione peroxidase (GSH-Px); **(C)** activity of Superoxide dismutase (SOD); and **(D)** the level of malondialdehyde (MDA). ^{a-c} Bar graphs are shown for each group, and labeled with different lowercase letters to illustrate which are significantly different ($p < 0.05$). The T-AOC level, GSH-Px, and SOD activity in liver tissue differed significantly among control and treatment groups.

Effects of Probiotics and Selenium-Enriched Probiotics on Malondialdehyde (MDA)

The results from analysis of MDA levels in the liver tissues are shown in Fig. 3D, which revealed significant elevation in liver MDA level in the HT group compared to the control group ($p < 0.01$), but a significant decrease in both HT+P and HT+SeP groups compared to the HT group ($p < 0.01$). Furthermore, the hepatic MDA level was significantly decreased by SeP supplementation compared to solely P supplementation ($p < 0.05$), resulting in decrease level of MDA in the HT+SeP group showed no significant difference from the control group.

Selenium-Enriched Probiotics inhibit the Expression of Heat Shock-Related Genes in Heat-Treated Rats

To investigate the possible mechanism by which HT+SeP supplementation reduces the damage of activated Kupffer cells of the liver, we measured the mRNA expression levels of heat shock proteins (HSPs) HSP70 and HSP90 in liver tissues. The results are shown in Fig. 4. The expression of both the HSP70- and HSP90-encoding genes were significantly down-regulated in both HT+P and HT+SeP groups relative to the HT group ($p < 0.05$). Moreover, mRNA in the SeP-supplemented group was also significantly less than that in the HT+P group ($p < 0.05$), demonstrating a beneficial effect of SeP supplementation. The HSP expression in the HT+SeP group was not significantly different from that in the control group ($p > 0.05$).

Fig 4. The effects of heat stress, and heat stress combined with P or SeP supplementation, on heat shock protein-encoding gene expression (Heat shock protein 70 (HSP70), Heat shock protein 90 (Hsp90)). Bar graphs show the mean mRNA level of groups (n=8) detected by qRT-PCR, and error bars show the standard error of mean. ^{a-c} Bar graphs representing each group of animals are labeled with different lowercase letters to show significant differences ($p < 0.05$). The results showed that heat stress plus either P or SeP supplementation significantly down-regulated the expression of HSP70 and HSP90 in heat-stressed Wistar rats, with SeP supplementation resulting in expression equivalent to control rats.

Selenium-Enriched Probiotic Supplementation Activates the Expression of Antioxidant-Related Genes in Heat-treated Rats

To determine whether P and SeP supplementation in heat-treated rats can suppress HT-induced oxidative stress, we examined the mRNA expression level of antioxidant-related genes in liver tissues (Fig 5). The mRNA level of SOD1, GPX1 and Nrf2 was significantly reduced in the HT group relative to the control group ($p < 0.01$). On the contrary, the expression level of these genes were significantly elevated in HT+P and HT+SeP groups relative to the HT group ($p < 0.05$). However, there was no significant difference in the expression level of SOD1, GPX1 and Nrf2 mRNA between control and HT + SeP groups.

287

288 **Fig 5.** The effects of heat stress, P and SeP supplementations on expression of antioxidant-related genes
 289 (Glutathione peroxidase 1 (GPX1), Superoxide Dismutase 1 (SOD1), and Nuclear factor, erythroid 2 like 2 (Nrf2)
 290 measured by qRT-PCR. Bar graphs portray the means of the groups (n=8) and the error bars show the standard error
 291 of the mean. ^{a-c} Bar graphs in each variable are labeled with lowercase letters to illustrate which are significantly
 292 different ($p < 0.05$). The results showed that heat stress reduced expression of these genes, whereas P or SeP
 293 supplementation significantly increased the expression level of these antioxidant-related genes in liver tissues, with
 294 the HT + SeP group having expression equivalent to the control group.

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296 **Selenium-Enriched Probiotics Reduce Expression of Proinflammatory Cytokines and NF- κ B in** 297 **Heat-Treated Rats**

298 To investigate whether P and SeP supplementation inhibit heat stress-induced inflammation, we examined
 299 the mRNA expression level of TNF- α , IL-6, COX-2 and NF- κ B genes in liver tissues (Fig 6). Compared
 300 to the control group, the expression level of IL-6, TNF- α , COX-2 and NF- κ B genes in the HT group were
 301 significantly increased ($p < 0.01$). However, the mRNA expression level of these genes were significantly
 302 decreased in HT+P and HT+SeP groups compared to HT group ($p < 0.05$). Crucially, the expression level
 303 of these genes in the HT+SeP group was significantly decreased in comparison with HT+P group ($p <$

0.05), resulting in no significant difference in their expression level between the control and the HT+SeP groups ($p > 0.05$).

Fig 6. The effects of heat stress, P and SeP supplementations on inflammation-related gene expression (Tumor necrosis factor alpha (TNF- α), Interleukin 6 (IL-6), Cytochrome c oxidase subunit II (COX-2), and Nuclear factor kappa B subunit 1 (NF- κ B), measured by qRT-PCR. Bar graphs reflect the means of the groups (n=8) and error bars portray the standard error of the mean. ^{a-c} Bar graphs in each variable are labeled with lowercase letters to demonstrate which are significantly different ($p < 0.05$). The results showed that heat stress increased, but P or SeP supplementations significantly decreased the expression level of inflammation related genes.

Selenium-Enriched Probiotics Reduce Expression of Fibrogenic Indicator Genes in Heat-Treated Rats

The results concerning the effects of heat stress and supplementation of P or SeP on Collagen I, α -SMA and TGF- β 1 mRNA expression level are portrayed in Fig 7. The results showed a significant increment in the expression level of Collagen I, α -SMA and TGF- β 1 genes in the HT group in comparison with the control group ($p < 0.01$). Conversely, in HT+P and HT+SeP groups, a significant decrement in the expression of these genes were observed compared to the HT group ($p < 0.05$). In addition, a decrement

was recorded for the HT+SeP group in comparison with the HT+P group ($p < 0.05$), suggesting an additional benefit of Se supplementation. Collagen I and TGF- β 1 mRNA expression was not significantly different between the control and the HT+SeP group. Furthermore, the HT+SeP group had lower levels of mRNA expression of α -SMA and TGF- β 1 ($p < 0.05$) than the HT P group.

Fig 7. The effects of heat stress, P and SeP supplementations on expression of fibrogenesis-related genes (Alpha-smooth muscle actin (α -SMA), Transforming growth factor beta 1 (TGF β 1), and Collagen I) as measured by qRT-PCR. Bar graphs reflect the mean of groups (n=8) and error bars portray the standard error of the mean. ^{a-c} Bar graphs for each variable are labeled with lowercase letters to show which are significantly different ($p < 0.05$). The results showed significant differences between control and treatment groups in the terms of expression of fibrogenesis-related genes.

Selenium-Enriched Probiotics Reduce Expression of Apoptosis-Related Genes in Heat-Treated Rats

In order to study the possible mechanisms of heat stress, and P and SeP supplement-induced apoptosis of activated hepatic stellate cells (HSC) to reduce liver injury, we examined Bcl-2 and Bax mRNA expression levels in liver tissues (Fig 8). The expression of Bcl-2 mRNA was significantly up-regulated, while Bax mRNA expression level was significantly down-regulated in the HT group in comparison with control group ($p < 0.05$). Moreover, the HT-induced increase in expression of Bcl-2 and the HT-induced decrease in expression of Bax mRNA was significantly inhibited in the HT+P and HT+SeP groups

($p < 0.05$). Furthermore, the expression of Bcl-2 mRNA and Bax mRNA was further up-regulated and down-regulated in the HT+SeP compared to the HT+P group, respectively ($p < 0.05$). Crucially, there were no significant differences in the expression levels of Bcl-2 and Bax between the control group and the HT + SeP group.

Fig 8. The effects of heat stress, P and SeP supplementations on expression of apoptosis-related genes (Bcl-2 Associated X Protein (Bax) and B cell leukemia/lymphoma 2 (Bcl-2), as measured by qRT-PCR. Bar graphs show the mean of groups (n=8) and error bars depict the standard error of the mean. ^{a-c} Bar graphs for each variable are labeled with lowercase letters to illustrate which are significantly different ($p < 0.05$). The results showed significant differences among control and treatment groups in the terms of apoptosis-related gene expression.

Selenium-Enriched Probiotics Alleviate Liver Injury in Heat-Treated Rats as Examined by Histomorphology

The histopathological morphology of the liver of both control and experimental rats is presented in Fig 9. The histological examination of the liver sections of the control group showed normal hepatic lobular structure and central vein. Hepatocytes have abundant eosinophilic cytoplasm and round nuclei (Fig 9A). It was observed that the expansion of liver sinusoids and interstitial hemorrhage were the main changes in the histopathological morphology of the liver in heat-treated rats. However, heat-stressed rats supplemented with probiotics showed central venous congestion in the liver (Fig 9C). Moreover, SeP supplementation tempered most of the histological alterations that were observed due to heat stress. This

modulation resulted in the liver showing a normal, decongested central vein surrounded by healthy
 hepatic lobules (Fig 9D).

Fig 9. The effects of heat stress, P and SeP supplementation on liver histology of male rats. **A-** Control rat's liver section, illustrating normal hepatocytes, hepatic sinusoids and central vein (H&E, x100). **B** – Heat stressed rat's liver section, presenting dilatation of the hepatic sinusoids and interstitial hemorrhage (H&E, ×100). **C** – Liver section of the probiotics-supplemented group rats that were incubated under high ambient temperature for 42 days, exhibiting a dilated, decongested central vein with the little-vacuolated cytoplasm in the hepatocytes (H&E, ×100). **D-**Liver section of the SeP group rats that were inflated under high ambient temperature for 42 days, presenting a nearly normal central vein that is surrounded by normal hepatocytes (H&E, ×100). The results revealed that heat stress caused distinct changes in liver histology of Wistar rats, which are ameliorated by P and SeP supplementation.

Discussion

This study shows that dietary SeP supplement can reduce liver oxidative damage in rats after high ambient temperature treatment, and further indicates that SeP may have antioxidant, anti-inflammatory and anti-apoptosis effects through activated liver marker enzymes, hepatic antioxidant status and hepatic stress-related genes (e.g. antioxidant, inflammation, fibrogenesis, apoptosis and heat shock).

In summer, animals are more likely to be exposed to environmental thermal stress [1]. Previous studies have shown that heat stress in pigs and laying hens reduced growth performance, food intake and weight gain when reared under high ambient temperature [21,22], while in broilers, heat exposure can cause significantly reduced liver weight, which may be due to reduced feed intake [23,24]. In addition, studies of chronic mild heat stress in mice have shown that heat stress reduces liver weight by affecting liver cell physiology, a heat-sensitive organ [25,26]. Our results showed that HT reduced the final body weight gain but increased the liver weight, and liver index (%), which suggest that the liver's physiological functions are altered due to changes in temperature.

Numerous studies have reported that dietary supplementation with SeP is able to improve growth performance of pigs [27,28], and increases egg production and quality in laying hens [29]. Our study also showed that SeP products increased body weight, liver weight and liver index, which indicates that SeP products can improve the liver's physiological functions and reduced liver tissue alteration. Increased plasma specific enzyme activity is considered as an indicator for liver diseases, and these enzymes are released into the blood in response to the organ deteriorations [30]. Activity of liver marker enzymes in the serum, for example AST, ALT, ALP, and LDH activities, is regarded as a sensitive indicator of liver damage [31,16]. Similarly, prior studies have found that during heat exposure, Alkaline phosphatase (AKP) concentrations were reduced significantly, while AST and ALT were induced relative to negative controls [16]. Moreover, ALT, AST, and LDH activities in the plasma were released into the blood stream when the liver body suffers some type of injury [32]. Furthermore, animals studies have shown that these liver marker enzymes, such as AST, ALP, and ALT activities, increased in heat stressed animals [33,34],

and that thermal exposure of liver can induce oxidative stress damage, with the damage mainly related to elevated levels of ALT and AST, increased contents of hepatic MDA, and decreased liver SOD, CAT and GSH activity [35,36]. Our results demonstrated that thermal stress significantly increased ALT, AST, ALP, and LDH activities and increased MDA content of liver tissue, but significantly reduced serum TP level and reduced SOD, T-AOC, and GSH-Px activities. Collectively, these measures indicate significant liver damage in heat-stressed rats. However, our findings also demonstrated that supplementation with probiotics, and especially with the selenium-enriched probiotics, reduced all of these measures of heat stress-induced liver injury. It has been observed that oral administration of SeP to rat has helped in reducing liver injury [12]. In addition, our histological observations also showed that the liver of heat-stressed rats was undergoing hepatic sinusoids dilation and an interstitial hemorrhage, resulted in irregular liver morphology of the HT group. The present study showed that dietary SeP supplement not only improve these multiple molecular measures of liver damage, but also reduce liver histological irregularities caused by heat stress. The current findings are in agreement with those of previous reports regarding the antioxidative effect of selenium-enriched probiotics [27,12]. The above interpretation were further supported by analyses of gene expression of SOD1, GPx1, and Nrf2, which showed a marked increase in mRNA level when supplemented by SeP as compared to the heat-exposed group, the results that are in line with previously reported data [37]. The accumulation of free radicals during oxidative stress mainly destroy liver cells membranes via lipid peroxidation, which may increase synthesis of collagen and exacerbate liver cirrhosis and fibrosis [12,38]. Therefore, SeP group may be hypothesized to decrease lipid peroxidation due to its antioxidant capability.

The main pro-inflammatory cytokines, such as TNF- α , IL-6, and COX-2, are produced by Kupffer cells, which are mainly related to the pathogenesis of infection. They also activate and stimulate hepatic stellate cells (HSCs), thus enhancing extra cellular matrix (ECM) deposition and aggravating fibrogenesis [39-41]. Moreover, the NF- κ B pathway is the main signaling pathway for inducing the inflammatory process [42]. Therefore, inflammatory response suppression might be helpful in preventing the hepatic damage and fibrosis caused by heat stress. Our findings showed that SeP reduced liver pro-

inflammatory cytokines and NF- κ B expression, compared to the heat stress group, resulting in expression that was comparable with control animals not exposed to heat stress. These findings are in line with previous reports that SeP and selenium have anti-inflammatory properties [12,43].

Hepatic stellate cells (HSC) have been shown to be the major cell type involved in extracellular matrix protein (ECM) deposition [44]. During liver damage, HSCs can be stimulated by conversion to myofibroblast-like cells expressing alpha smooth muscle actin (α -SMA) and desmin, and they produce inflammatory cytokines, including TGF- β 1, which leads to excessive production of ECM, fibrosis and liver inflammation [40,44,45]. Therefore, HSC activation causes liver fibrosis, and inhibition of fibrogenic markers may considerably decreases liver fibrosis in experimental rats. In this study, compared with HT rats, animals supplemented with SeP had significantly decreased Collagen I, α -SMA and TGF- β 1 expression in the liver, consistent with previous studies [12], indicating the liver fibrosis caused by an activated HSCs can be improved by SeP supplementation, potentially by inducing apoptosis. Our findings demonstrated that SeP considerably increased the expression of Bcl-2 and decreased the expression of Bax compared to the HT group. This is consistent with the earlier work [46], and provides evidence that cell survival is controlled by anti-apoptotic and pro-apoptotic family proteins of Bcl-2/Bad. In another study [12], supplementation of Selenium-enriched probiotics were proposed to decrease CCl₄-induced liver fibrosis through inducing apoptosis of activated HSCs in mouse. The present findings show that SeP supplementation can successfully decrease liver fibrosis by inducing activated HSCs apoptosis.

In rats, the expression of HSPs, such as HSP70 and HSP 90, in the liver, heart, and kidney tissues are increased by heat stress, but the expression levels of these genes were decreased by feeding of zinc-supplemented probiotics (ZnP) [17]. In addition, studies from pigs have reported that the expression level of HSPs were increased in the liver tissues under heat stress, presumably to protect from the harmful effects of this stress on liver tissues [15]. Our results show that the expression of HSP90 and HSP70 mRNA in liver was increased in heat stressed rats compared to the control group. Our findings also indicate that supplementation with Selenium-enriched probiotics can reduce the induction of HSPs caused

by thermal stress, thereby suggesting a potentially beneficial effect of SeP supplementation against the harmful effects of high ambient temperatures.

In conclusion, numerous measures of liver function and physiology demonstrated a clear effect of high ambient temperature on the liver in HT rats. Both P, and especially SeP supplementation showed a protective effect on liver damage and fibrosis caused by heat stress by inhibiting liver oxidative stress, inflammation, fibrogenesis, and inducing apoptosis and cell cycle arrest in hepatic stellate and Kupffer cells. Crucially, in all measures, there was a clear, additional benefit of SeP supplementation, relative to P supplementation. Our study suggests that SeP may be a potentially useful dietary agent to prevent liver fibrosis in animals exposed to high ambient temperatures. As our results indicate that SeP treatment can alleviate the damage caused by heat stress in the liver of rats. It is worth considering whether SeP should be used as an additive in livestock feed that are exposed to high ambient temperatures.

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Conflict of interest. The authors declare that they have no conflicts of interest.

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