CHAPTER III

TRANSCRIPTOME ANALYSIS OF JEJUNAL MUCOSAL TISSUE IN BREEDER HENS EXPOSED TO ACUTE HEAT STRESS

3.1 Abstract

Heat stress (HS) severely compromises intestinal barrier function in poultry, resulting in significant production losses. This study aimed to explore the molecular response of the small intestine to acute HS in breeder hens. Fifty 28-week-old heat-sensitive breeder hens were raised individually in a cage and randomly assigned to control and heat-treated groups (25 hens each). Control group hens were maintained at thermoneutral conditions (23°C), and heat-treated group hens were subjected to acute HS (36°C for a 6-hour). The heart rate and cloacal temperature were measured in all hens. The jejunal mucosa tissues were collected from 12 randomly selected hens per group for transcriptomic analysis. The acute HS induced significant physiological alterations, with a marked increase in the heart rate and cloacal temperature in hens ($P = 0.001$). Transcriptome analysis revealed 138 genes with altered expression patterns under acute HS conditions. Of these, 75 genes, including heat shock proteins (HSPs), showed upregulated expression, while 63 genes, including a key bile acid transport molecule (SLC10A2), exhibited downregulated expression. Functional analysis through gene ontology classification, pathway mapping via the Kyoto encyclopedia of genes and genomes, and protein interaction networks identified several important regulatory genes in thermal response (HSPA8 and HSPA2), energy homeostasis and fat metabolism (PDK4, PPARA, and CD36), glucose transport (SLC2A5), and cholesterol synthesis pathway (SQLE, CYP51A1, and HSD17B7). The following genes were identified as candidate biomarker genes in the jejunal mucosa for HS response: HSPA2, HSPB9, IL-18BP, and CD36. Moreover, for the antioxidant trial, one hundred 33-week-old HS hens were randomly assigned to thermoneutral (TN; 23 °C); or heat stress (36 °C, 4 h/d from week 38 to 52) rooms, with four groups (25 hens each): T1) basal diet in TN zone; T2) basal diet under heat stress; T3) basal diet supplemented with synthetic antioxidants

under heat stress; and T4) basal diet supplemented with phytogenic antioxidants under heat stress. Results showed that either synthetic or phytogenic antioxidant supplementation increased the expression of CD36 while decreasing HSPB9, HSPA2, and IL18BP in the jejunum of HS hens compared to the heat stress group without supplementation ($P < 0.05$). This study provides insights into the molecular mechanisms of heat stress on intestinal function and identifies candidate genes that can be targeted by antioxidants to alleviate the effects of heat stress in poultry.

Keywords: Breeder hen, Acute heat stress, Jejunal mucosa, Transcriptome, Dietary antioxidant.

3.2 Introduction

Heat stress (HS) has emerged as an important issue in the poultry production industry (Gregory, 2010). HS can be categorized into acute and chronic HS, depending on its duration and severity (Saeed et al., 2019). HS adversely affects poultry health and performance by impairing development, feed efficiency, reproduction, gut health, and immune function, often resulting in high mortality (Kumar et al., 2012; Kim et al., 2023). The intestine is sensitive to various types of stress, including HS (Tellez Jr et al., 2017). Chronic HS disrupts the morphology and integrity of the small intestine, resulting in reduced jejunal weight, length, and villus height (Garriga et al., 2006), and causes systemic inflammation and infection (Elnesr and Abdel-Azim, 2023). Chronic HS can induce changes in the intestinal mucosal barrier, increase intestinal permeability, and reduce the activity of digestive enzymes such as lipase and trypsin, thereby impairing digestion and absorption (Song et al., 2018; Al-Zghoul et al., 2019). These changes were also observed in the jejunum of heat-stressed chickens following acute HS. However, the molecular mechanisms underlying the jejunal mucosal damage caused by acute HS remain unclear.

At the molecular level, cells coordinate several mechanisms to protect themselves from the harmful effects of HS. Heat shock proteins (HSPs) are induced in response to HS to protect cells and cellular proteins (Archana et al., 2017). The HSP family consists of molecules ranging in size from 10 kDa to more than 100 kDa (Jee,

2016). HSP110 has an immune and protein-folding function (Chen et al., 2018). HSP90 is a family of highly conserved molecular chaperones that play critical functions in signal transduction, protein folding, degradation, and morphological evolution (Wegele et al., 2004). HSP70 is activated and removes the denatured or abnormal proteins in the cell, which could improve cell viability and its resistance to HS (Bhat et al., 2016). HSP60 forms a hetero-oligomeric complex that assists in protein assembly (Zuo et al., 2016). HSP40 is derived from the DnaJ protein family and works as an HSP70 cochaperone (Hageman et al., 2010). Small heat shock proteins, i.e., HSP25 and HSP27, are ATP-independent chaperones that bind unfolded proteins and require HSP70 for complete refolding during large-scale unfolding (Hwang et al., 2016; Mackei et al., 2021). Among them, HSP70 and HSP90 have been well-studied and are known for their roles in protecting and repairing cells and tissues (Gu et al., 2012; Arnal and Lalles, 2016). Similar to HSPs, the expression of genes responsible for nutrient transport and lipid metabolism is also affected by HS (Goel et al., 2021). For instance, chronic cyclic HS has been shown to downregulate glucose transporter (GLUT) 2, 10, 11, and 12 in the jejunum of modern broilers while increasing GLUT1, 5, 10, and 11 expressions in wild jungle fowls (Abdelli et al., 2021). Previous studies have also demonstrated the decreased expression of GLUT2, the cluster of differentiation 36 (CD36), and fatty acid-binding protein 1, which are crucial for glucose and lipid transport in the jejunum of broiler chickens under periodic heat exposure (Sun et al., 2015). However, to the best of our knowledge, no study has examined the changes and interactions of these genes using genome-wide transcripts in breeder hens exposed to acute HS.

Various nutritional strategies have been explored to mitigate heat stress effects on chickens (Saeed et al., 2019). Vitamins C and E, selenium (Se), L-carnitine, and phytogenic help reduce cellular damage and against heat stress (Surai et al., 2018). The combined supplementation of Se and vitamin E in broilers exposed to high temperatures enhances jejunal tissue accumulation of Se and vitamin E, which in turn reduces the expression of HSP90, HSP70, and HSP60 mRNA (Kumbhar et al., 2018). In addition, L-carnitine demonstrated antioxidant and anti-inflammatory effects and enhanced intestinal histology (Agarwal et al., 2018). Camellia sinensis (green tea) with its primary antioxidant catechins and Syzygium aromatum (clove) rich in eugenol inhibits the activation of nuclear factor-kB in response to various inflammatory stimuli,

which suppresses various pro-inflammatory cytokines expressions (Liu et al., 2020; Pasri et al., 2023; Saracila et al., 2023). Our previous study revealed that both synthetic antioxidants (a combination of vitamin E, vitamin C, Se, and L-carnitine) and phytogenic antioxidants (a combination of clove, green tea pomace, and Vietnamese coriander) downregulated HSP70 and HSP90 mRNA expressions in the liver of breeder hens under heat stress (Pasri et al., 2024). However, the role of these antioxidants in gut health and production, along with the underlying mechanism in heat-stressed chickens, is not completely explored.

Transcriptome sequencing technology (RNA-seq) can accurately and efficiently obtain almost all transcripts of specific tissues and reveal subtle changes in the differential expression of each gene in the tissue (Haas and Zody, 2010), which allows the identification of key genes and molecular regulatory mechanisms. Using RNA-seq, a previous study found that genes related to immune responses, glutathione metabolism, defense systems, and xenobiotic detoxification were differentially expressed in the jejunal mucosa of chronic heat-stressed broilers (Kim et al., 2022). Therefore, the current study used RNA-seq to analyze the transcriptome of the jejunal mucosa of breeder hens subjected to acute HS, and tracking expression changes of selected candidate genes in heat-sensitive breeder hens supplemented with dietary antioxidants under HS conditions. The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis highlighted several significant pathways, including steroid biosynthesis, steroid hormone biosynthesis, protein processing in endoplasmic reticulum, the peroxisome proliferator-activated receptor (PPAR) signaling pathway, and the adipocytokine signaling pathway. Protein-protein interaction network analysis involves two large networks: one containing several upregulated HSPs and genes related to energy homeostasis and fat metabolism (pyruvate dehydrogenase kinase 4 [PDK4], peroxisome proliferator-activated receptor alpha [PPARA], and CD36) and solute carrier family 2 member 5 (SLC2A5), known as a glucose transporter, and the other containing downregulated genes related to cholesterol biosynthesis. These findings provide a scientific basis for understanding the potential molecular mechanisms by which acute HS affects intestinal health. These insights offer valuable clues for developing strategies to mitigate HS in chickens.

3.3 Materials and methods

3.3.1 Ethics statement

The experiments were carried out at the Suranaree University of Technology (SUT) farm according to the approved protocol by the Animal Care and Use Committee of SUT, Thailand (document no. SUT-IACUC-012/2020).

3.3.2 Housing, birds, and sample collection

This study consists of two interconnected experimental trials, the first focusing on transcriptomic analysis to examine the gene expression responses to HS in heat-sensitive breeder hens, and the second investigating the effects of antioxidant supplementation on modulating gene expression alterations induced by HS in heat-sensitive breeder hens.

Trial 1: Transcriptomic study, a total of fifty 22-week-old SUT breeder hens, a synthesized line developed for producing Thai indigenous crossbred chickens, raised at the SUT farm, were used. Prior to the start of the experiment, the hens were individually housed in wire cages measuring 45 × 40 × 40 cm^3 (width × length × height) and were adapted to a controlled temperature of 23 ± 1°C for 6 weeks. All hens were provided with a daily feed allowance of 140 g, formulated following the guidelines of the National Research Council (1994) and Aviagen (2016) (containing 2,800 kcal of metabolizable energy/kg and 15% crude protein), and were maintained on a 16-hour light cycle daily, with water available ad libitum. At 28 weeks of age, the hens were randomly divided into control and heat-treated groups, each consisting of 25 hens, using a completely randomized design. In the control group, the hens were raised at 23 ± 1°C with 40–70% relative humidity in an air-conditioned room ([TN] condition), while in the heat-treated group, the hens were exposed to HS (i.e., 36 ± 1°C with 67% relative humidity) for 6 hours (HS condition). The HS conditions were determined using the temperature–humidity index (Duangjinda et al., 2017), and the heat-treated groups experienced the HS condition only once. After the hens were exposed to HS for 6 hours, the cloacal temperature and heart rate were measured in both groups. Subsequently, 12 hens in CS and HS conditions were selected and euthanized by severing the vein in the neck and dissecting them to collect jejunal mucosa tissues. These tissues were collected into RNA protect tissue tubes (Qiagen, Duesseldorf, Germany), which were snap-frozen in liquid nitrogen and stored at −80°C until RNA

extraction. The results of the transcriptomic analysis in trial 1 will inform the subsequent antioxidant study, exploring how dietary antioxidants may modulate these marker gene expression changes in heat-sensitive hens.

Trial 2: Antioxidant study, 100 SUT female breeder hens (33 weeks of age) were housed individually in cages and randomly assigned to four treatment groups, each consisting of 25 hens, and acclimated for 5 weeks in a TN (23±1°C) room. Group 1 was maintained in a TN room, while groups 2, 3, and 4 were exposed to an HS room for 4 hours daily. The experimental diets were as follows: T1) basal diet under TN, T2) basal diet under HS, T3) basal diet supplemented with combined synthetic antioxidants (200 mg of vitamin C/kg, 150 mg of vitamin E/kg, 0.30 mg of Se yeast/kg, and 150 mg of carnitine/kg) under HS and T4) basal diet supplemented with 1% phytogenic antioxidants (a mixture of clove, green tea pomace, and Vietnamese coriander powder) under HS. All hens were provided 140 g of feed (15% crude protein, 2800 kcal metabolizable energy/kg), as outlined in Table 3.1, with 16 hours of light per day and ad libitum access to water throughout the experimental period (38–52 weeks of age). At the end of the experiments, all breeder hens were euthanized after exposure to 36°C heat for 4 hours. Jejunal mucosal tissue was immediately collected in liquid nitrogen and stored at −80°C for subsequent gene expression analysis. This connection between the results of the transcriptomic study and the antioxidant study allows us to investigate the potential of antioxidant interventions in improving HS tolerance by targeting key genes involved in heat-induced cellular damage, gut health, and nutrient absorption. Thus, the antioxidant supplementation in trial 2 serves as a strategic intervention to address the molecular disruptions identified in trial 1.

Table 3.1 Ingredients and chemical composition of the basal diet for trial 2.

	25-50 weeks of age	**After 50 weeks of age**
Ingredients (%)		
Corn	64.60	63.50
Soybean meal, 44 %CP	18.20	16.52
Full-fat soybean meal	6.70	9.00
Calcium carbonate	8.50	8.90
Monocalcium phosphate	0.94	1.00
Salt	0.41	0.44
DL-methionine	0.135	0.134
L-lysine	-	-
L-threonine	-	-
[1]Premix	0.521	0.521
Calculated compositions (%)		
Metabolizable energy (kcal/kg)	2,800	2,800
Calcium	3.51	3.71
Total Phosphorus	0.53	0.54
Available phosphorus	0.31	0.32
Digestible lysine	0.70	0.70
Digestible methionine	0.35	0.35
Digestible methionine + Cystine	0.57	0.57
Digestible threonine	0.50	0.50
Analyzed compositions (%)		
Dry matter	93.06	93.10
Crude protein	16.02	16.20
Crude fiber	3.06	3.04
Ash	11.08	11.66
Ether extract	3.35	4.49

[1]Premix for breeder hens (0.52%) provided the following (per kg of diet) by withdrawing vitamin E and Se; vitamin A, 15,000 IU; vitamin D3, 3,750 IU; vitamin K3, 5 mg; vitamin B1, 2 mg; vitamin B2, 9.8 mg; vitamin B6, 4 mg; vitamin B12, 25 mg; pantothenic acid, 11.04 mg; nicotinic acid, 35 mg; folic acid, 1 mg; biotin, 15.5 µg; choline chloride, 250 mg; Cu, 2.1 mg; Mn, 84 mg; Zn, 66.5 mg; Fe, 80 mg; I, 1.2 mg.

3.3.3 Extraction of total RNA for transcriptome analysis

Total RNA was isolated from the jejunal mucosal tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and purified using a QIAamp spin column (Qiagen), according to the manufacturer's instructions. The purified RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and the quality was checked using 1% agarose gel electrophoresis with 0.5×TAE as a buffer and an electric current of 100 V for 25 min. Three RNA pools were generated for each condition group from 12 hens, with each pool consisting of four individual jejunal mucosa samples. The pooled samples were utilized to create an RNA-seq library. The capillary electrophoresis with a QIAxcel Connect (Qiagen) was used to evaluate RNA integrity number (RIN), and RNA samples with a RIN ≥ 7 were used in RNA library constructions.

Total RNA was extracted from 8 jejunal mucosa tissue samples of heat-sensitive breeder hens from each T1, T2, T3, and T4 by using NucleoSpin® RNA Midi kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and purified using a QIAamp spin column (Qiagen, Hilden, Germany). The extracted RNA from 2 individual jejunal mucosa samples was pooled, and 4 replications were generated in each treatment and the purity and quantification of RNA were measured, as previously described.

3.3.4 Library construction and data processing

Construction of the cDNA library and RNA-seq were performed by BGI Co., Ltd. (BGI, Shenzhen, China). Six libraries were tested on the DNBSEQ platform. Sequencing data were filtered using SOAPnuke Version v1.5.6 (Cock et al., 2010). Reads containing adapters, reads with unknown base N content greater than 5%, and low-quality reads (reads with a base quality value less than 15, accounting for more than 20% of the total base number of the reads) were removed to obtain clean reads. Subsequently, we used HISAT2 v2.1.0 (Kim et al., 2015) to align the clean reads to the chicken reference genome (GCF_000002315.6_GRCg6a) and then used RSEM Version v1.3.1 (Li and Dewey, 2011) to align the clean reads to the reference gene set.

3.3.5 Differential gene expression and functional enrichment analyses

Differential gene expression analysis was performed using DESeq2 (v1.4.5) (Love et al., 2014). The differentially expressed genes (DEGs) were identified with fold-change (FC) of ≥ 1 and adjusted values of $P < 0.05$. Gene ontology (GO) and KEGG

enrichment analyses on DEGs were performed to explore the gene functions. GO terms and KEGG pathways with $P < 0.05$ were defined as significantly enriched. STRING (Szklarczyk et al., 2018) analysis was performed using DIAMOND (v0.8.31) (Buchfink et al., 2015) to obtain the interactions between DEGs encoding proteins. Eight genes were selected for validation analysis based on the function in HS (heat shock protein family B (small) member 9 [HSPB9], (heat shock protein family H (Hsp110) member 1 [HSPH1], heat shock 70 kDa protein 2 [HSPA2], and DnaJ heat shock protein family (Hsp40) member A4 [DNAJA4]), energy homeostasis metabolism (PDK4), signal transduction (calcium/calmodulin-dependent protein kinase IG [CAMK1G]), and immunity (guanosine triphosphatase-binding protein [GBP7] and interleukin18 binding protein [IL18BP]).

3.3.6 Validation by real-time PCR

To verify the reproducibility and accuracy of gene expression data in RNA-Seq of breeder hen CS and HS conditions, quantitative PCR (qPCR) was performed using the same RNA samples. For cDNA synthesis, one microgram of RNA from each RNA pool was individually reverse-transcribed using the SuperScript III RNAse H-Reverse transcriptase kit (Toyobo, Osaka, Japan) with random primers (Promega, Madison, WI, USA), following the manufacturer's protocol. Primers were designed using Primer3 primer-design software (https://primer3.ut.ee/) and are shown in Table 3.2. The qPCR was performed using the QuantiNova SYBR Green PCR kit (Qiagen, Hilden, Germany), with the reaction conditions set as follows: initial heat activation at 94°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and final extension at 72°C for 30 s. Amplification reactions were performed in triplicate for each gene. The relative quantification of gene-specific expression was calculated using the $2^{-\Delta\Delta CT}$ method. The glyceraldehyde-3-phosphate dehydrogenase gene was used as an internal control.

3.3.7 Statistical Analysis

The differences in mean heart rate and cloacal temperature between treatment groups were assessed using a t-test in SPSS version 27.0 (SPSS Inc.) (Salcedo and McCormick, 2020). The gene expression data from T1, T2, T3, and T4 were analyzed using analysis of variance (ANOVA) in a completely randomized design (CRD) with SPSS version 27.0 (SPSS Inc.) (Salcedo and McCormick, 2020). Orthogonal contrasts were performed to compare the following conditions: 1) thermoneutral (T1) vs. heat stress (T2, T3, T4); 2) non-supplementation (T2) vs. supplementation (T3, T4); and 3) synthetic (T3) vs. phytogenic

(T4) antioxidants. Tukey's test was applied to determine significant differences among treatments. Statistical significance was considered at $P < 0.05$.

Table 3.2 Primer sequences used in real-time PCR.

Gene	Primer sequences	Accession No.
HSPB9	F-5'-CAAGTACGAGGTGCTGAAGCG-3'	NM_033194.3
	R-5'-TGACAGCTCCATCCTTGGCT-3'	
PDK4	F-5'-TCCTTCCCTCTCTCCAGTTGA-3'	NM_001199909.3
	R-5'-CATATCCAAAGCCAGCAAGAGG-3'	
DNAJA4	F-5'-AGTTGCTGCGCTGTCAGTAT-3'	NM_ 040680548.2
	R-5'-AGTTGGTTCTCAGCTGTGTGA-3'	
HSPH1	F-5'-CCCAGATGTCAAGAAAACAAGTGA-3'	NM_ 001159698.2
	R-5'-AGCTTCAATAGGCAGTTCCACA-3'	
HSPA2	F-5'-CCGTGGAGTTCCTCAGATCG-3'	NM_001006685.1
	R-5'-GCTAAGGCGACCCTTGTCAT-3'	
IL18BP	F-5'-CTTCTGCTGCCACTGCTCT-3'	XM_015280902.4
	R-5'-CTCACGTTGCTGCCCATCT-3'	
GBP7	F-5'-CCTGGAGAACCTGCACTACG-3'	NM_145545.4
	R-5'-CCACACGAAGGTTGGGAAGA-3'	
CAMK1G	F-5'-CCCACCCGATTATACAGGGC-3'	XM_040652982.2
	R-5'-CTGGTTGTCTGGCGATCCAT-3'	
GAPDH	F-5'-AGAACATCATCCCAGCGT-3'	K01458
	R-5'-AGCCTTCACTACCCTCTTG-3'	

Abbreviations: HSPB9, heat shock protein family B (small) member 9; PDK4, pyruvate dehydrogenase kinase 4; HSPA2, heat shock 70kDa protein 2; HSPH1, heat shock 110kDa protein 1; DNAJA4, heat shock 40kDa protein (HSP40); IL18BP, interleukin-18 (IL-18); GBP7, guanylate-binding protein 7; CAMK1G, calcium/calmodulin-dependent protein kinase 1G; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

3.4 Results

3.4.1 Heart rates and cloacal temperature of breeder hens

The heart rate and cloacal temperature in breeder hens subjected to acute HS treatment were significantly higher compared to those in hens raised under CS (P = 0.001) (Table 3.3). When breeder hens were challenged with 6 hours of heat exposure, the average heart rate and cloacal temperature were 236 times/min and 42.9°C for breeder hens under HS, and 198 times/min and 40.8°C for breeder hens under CS, respectively.

Table 3.3 Heart rates and cloacal temperature of breeder hens under thermoneutral and heat stress conditions[1].

Conditions	Heart rate (times/min)	Cloaca temperature (°C)
Thermoneutral	198.3[b]	40.8[b]
Heat stress	236.0[a]	42.9[a]
Pooled SEM	5.8	0.1

[1]Values are means from 25 breeder hens (*n* = 25).

[a,b] Values within each column with different superscripts are significantly different (P < 0.05).

3.4.2 Summary of the raw RNA-seq reads

A comparative RNA-seq analysis of the jejunal mucosal transcriptomes from the CS and HS groups was conducted to investigate the global response of the jejunal mucosal transcriptome to acute HS in breeder hens. The RNA-seq results for the six jejunal mucosa samples are presented in Table 3.4. Raw data reads ranged from 40.39 million to 45.44 million, averaging 43.73 million reads per sample. After filtering out low-quality reads, contamination, and other artifacts from the raw data, clean reads totaled between 39.33 million and 44.23 million, averaging 42.53 million clean reads per sample. The Q20 score exceeded 97%, indicating high sequencing quality, and the GC content of the clean reads ranged from 47.01% to 47.51%. The total mapping rate ranged from 94.90% to 95.75%, with an average of 95.50%.

Table 3.4 RNA-sequencing reads and mapping rates in the jejunal mucosa in breeder hens.

Sample[1]	Raw Reads (million)	Clean Reads (million)	Clean Reads Q20[2] (%)	GC content (%)	Total Mapping (%)
CS1	45.44	44.05	97.84	47.41	95.75
CS2	45.44	44.23	97.85	47.51	94.90
CS3	45.44	44.08	97.72	47.29	95.68
HS1	40.39	39.33	97.91	47.01	95.49
HS2	40.78	39.76	97.84	47.13	95.55
HS3	44.90	43.70	97.91	47.24	95.65
Average	43.73	42.53	97.85	47.27	95.50

[1]Each sample consists of four individual jejunal mucosa of breeder hens under thermoneutral conditions (CS) (n = 12) and heat stress conditions (HS) (n = 12).

[2]Q20 indicates the percentage of bases with a Phred value ≥ 20.

3.4.3 DEGs analysis

The overall distribution of DEGs was visualized using a volcano plot (Figure 3.1). In total, 138 DEGs were identified, with 75 upregulated and 63 downregulated genes in hens exposed to acute HS. Detailed information concerning the DEGs is shown in Table S3.1. Among these, seven HSP genes, heat shock protein family B (small) member 9 (HSPB9), HSPA2, HSPH1, heat shock protein 90 alpha family class A member 1 (HSP90AA1), heat shock protein family A (Hsp70) member 8 (HSPA8), DNAJA4, and DnaJ heat shock protein family (Hsp40) member A1 (DNAJA1) were identified. Table 3.5 shows the top 20 upregulated and downregulated DEGs.

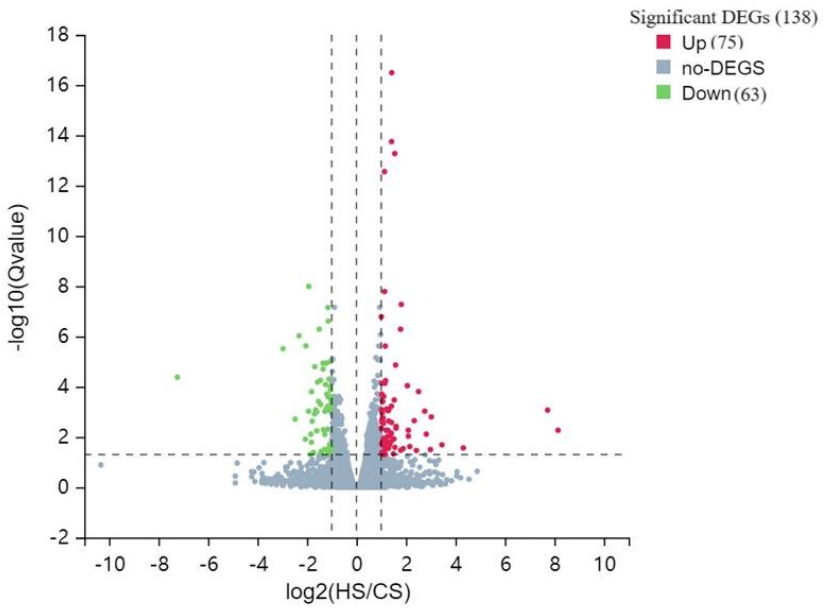

Figure 3.1 Volcano plot of differentially expressed genes in the jejunal mucosa in heat-stressed breeder hens. Genes meeting the conditions of adjusted P (q value) < 0.05 and |log2 FC| ≥ 1 are shown as significantly differentially expressed genes (DEGs), with red and green dots representing upregulated and downregulated genes, respectively. Gray dots represent insignificant DEGs. The x and y axes of the volcano plots show the log2 fold changes in breeder hens under heat stress conditions (HS) (n = 12) compared to thermoneutral conditions (CS) (n = 12) and –log10 q value, respectively.

Table 3.5 Top 20 upregulated and downregulated differentially expressed genes in the jejunal mucosa in heat-stressed breeder hens.

Gene ID	Gene Symbol	log2 fold change	Qvalue[1]	Regulated[2]
772158	-	8.1468	0.0054	Up
428310	*HSPB9*	7.7253	0.0009	Up
423504	*HSPA2*	4.3191	0.0272	Up
107051217	-	3.4531	0.0205	Up
425711	*C2H8ORF22*	3.0249	0.0016	Up
415360	*DNAJA4*	2.9905	0.0317	Up
420943	*ABCA13*	2.8216	0.0077	Up
420570	*PDK4*	2.7599	0.0009	Up
100857694	*FMO5*	2.5097	0.0002	Up
418917	*HSPH1*	2.4235	0.0344	Up
424391	*MYOC*	2.3367	0.0022	Up
423463	*HSP90AA1*	2.1537	0.0240	Up
396041	*SLC16A8*	2.0977	0.0093	Up
418800	*PCDH9*	2.0949	0.0054	Up
107054090	-	2.0581	0.0001	Up
112530324	-	1.8870	0.0291	Up
425431	-	1.8125	5.34E−08	Up
776543	*FANCD2OS*	1.7896	0.0337	Up
420321	*NDRG1*	1.7828	5.20E−07	Up
427907	*SSTR3*	1.6020	0.0038	Up

Table 3.5 (continued).

Gene ID	Gene Symbol	log2 fold change	Qvalue[1]	Regulated[2]
769715	*XRRA1*	−7.2383	4.18E−05	Down
421322	*DNAH14*	−2.9693	3.06E−06	Down
428018	*SLC10A2*	−2.4798	0.0020	Down
418544	-	−2.3208	9.43E−07	Down
770450	-	−2.0644	0.0123	Down
418167	*USP18*	−2.0429	2.38E−06	Down
107055301	*NR1D1*	−1.9357	9.45E−04	Down
429696	*GBP7*	−1.9264	1.02E−08	Down
107050574	-	−1.8605	0.0489	Down
112531455	-	−1.8422	0.0077	Down
419859	*CAMK1G*	−1.8188	0.0163	Down
770663	*SMPD2*	−1.8140	1.58E−04	Down
415409	*DYX1C1*	−1.7909	0.0024	Down
112531351	-	−1.7355	0.0411	Down
112533303	-	−1.6935	0.0012	Down
428187	*CA6*	−1.6820	1.60E−05	Down
418775	*CLYBL*	−1.6476	8.40E−04	Down
107052690	-	−1.6059	0.0057	Down
112531454	-	−1.6048	9.81E−04	Down
420459	*IDI1*	−1.5727	6.55E−05	Down

[1]Q-value is the corrected *P*-value.

[2]Upregulated and downregulated genes were detected in the jejunal mucosa of breeder hens under heat stress conditions (n = 12) compared to thermoneutral conditions (n = 12).

3.4.4 GO and KEGG pathway analysis of DEGs

Functions and pathways of the 138 DEGs were assessed using GO and KEGG pathway analyses. GO analysis categorized the DEGs into biological processes (BP), molecular functions (MF), and cellular components (CC), revealing enrichment in 352 GO terms, as presented in Table S3.2. A total of 213 GO terms were significantly enriched in the BP category. The top five significant GO terms within the BP category were responses to heat (P = 9.10E–08), cholesterol biosynthetic process (P = 3.77E–07), response to organonitrogen compounds (P = 5.47E–06), sterol biosynthetic process (P = 9.77E–06), and protein refolding (P = 6.37E–05). In the MF category, 105 GO terms were significantly enriched. Of these, the top five GO terms were unfolded protein binding (P = 1.38E–06), C3HC4-type RING finger domain binding (P = 2.19E–06), heat shock protein binding (P = 2.56E–06), oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen (P = 7.89E–06), and iron ion binding (P = 2.68E–04). In the CC category, 34 GO terms were significantly enriched. The top five GO terms were nucleosome (P = 0.0011), Prp19 complex (P = 0.0035), myelin sheath (P = 0.0105), receptor complex (P = 0.0118), and ribonucleoprotein complex (P = 0.0118). The top 20 most significant GO terms are shown in Figure 3.2. KEGG pathway analysis of the jejunal mucosa identified nine significant pathways: steroid biosynthesis (P = 1.36E–06), terpenoid backbone biosynthesis (P = 0.0013), steroid hormone biosynthesis (P = 0.0080), protein processing in endoplasmic reticulum (P = 0.0087), nitrogen metabolism (P = 0.0160), butanoate metabolism (P = 0.0308), PPAR signaling pathway (P = 0.0357), adipocytokine signaling pathway (P = 0.0372), and DNA replication (P = 0.0492) (Table 3.6).

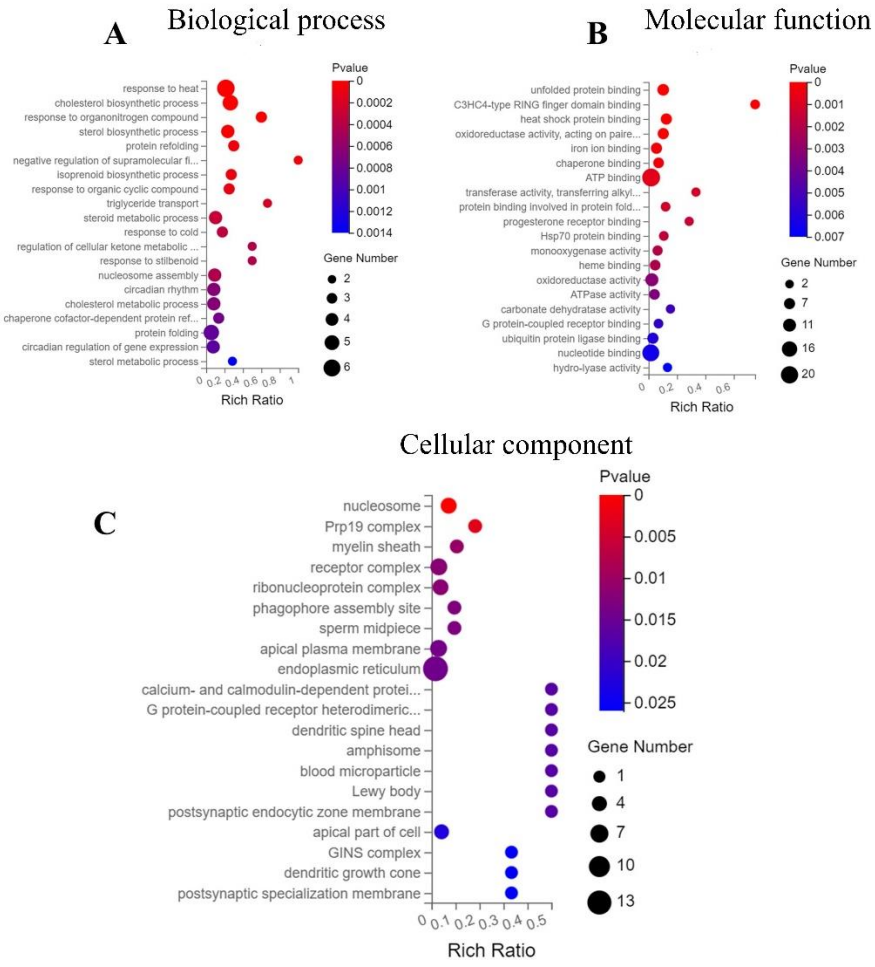

Figure 3.2 Top 20 enriched gene ontology terms of differentially expressed genes in jejunal mucosa in heat-stressed breeder hens. A: Biological process, B: Molecular function, and C: Cellular component. The circle size in each term corresponds to the number of genes. The circle's color goes from blue to red, indicating a lower P value. Terms were detected from differentially expressed genes in the jejunal mucosa of breeder hens under heat stress conditions (n = 12) compared to thermoneutral conditions (n = 12).

Table 3.6 Kyoto encyclopedia of genes and genomes pathways possibly affected by heat stress in jejunal mucosa in breeding hens.

Pathway Term	Count	P value	Gene Symbols[1]
gga00100: Steroid biosynthesis	5	1.36E−06	*LOC100857622*↓, *SQLE*↓, *CYP51A1*↓, *FDFT1*↓, *HSD17B7*↓
gga00900: Terpenoid backbone biosynthesis	3	0.0013	*HMGCS1*↓, *IDI1*↓, *FDPS*↓
gga00140: Steroid hormone biosynthesis	3	0.0080	*CYP3A5*↑, *CYP7B1*↑, *HSD17B7*↓
gga04141: Protein processing in endoplasmic reticulum	6	0.0087	*HSPA8*↑, *HSPH1*↑, *HSP90AA1*↑, *HSPA2*↑, *LOC425431*↑, *DNAJA1*↑
gga00910: Nitrogen metabolism	2	0.0160	*CA2*↑, *CA6*↓
gga00650: Butanoate metabolism	2	0.0308	*HMGCS1*↓, *AACS*↓
gga03320: Peroxisome proliferator-activated receptor signaling pathway	3	0.0357	*PPARA*↑, *HMGCS1*↓, *CD36*↑
gga04920: Adipocytokine signaling pathway	3	0.0372	*IRS2*↑, *PPARA*↑, *CD36*↑
gga03030: DNA replication	2	0.0492	*RFC5*↓, *MCM5*↓

[1]Up and down arrows indicate the upregulated and downregulated genes, respectively, in the jejunal mucosa in heat-stressed breeder hens.

3.4.5 Protein interaction analysis

In this study, we used protein–protein interaction (PPI) analysis to explore potential functional relationships and interactions among proteins encoded by DEGs. Six networks were identified, including two large networks, each containing 10 or more proteins (Figure 3.3). The largest network included several HSPs and proteins whose

genes were identified in KEGG pathways (PPARA, CD36, carbonic anhydrase 6, and mini-chromosome maintenance complex component 5) (Table 3.5). Three other networks contained some of the top 20 upregulated and downregulated DEGs (ATP binding cassette subfamily A member 13, N-myc downstream regulated 1, ubiquitin specific peptidase (USP18), and isopentenyl-diphosphate delta isomerase 1), as listed in Table 3.6.

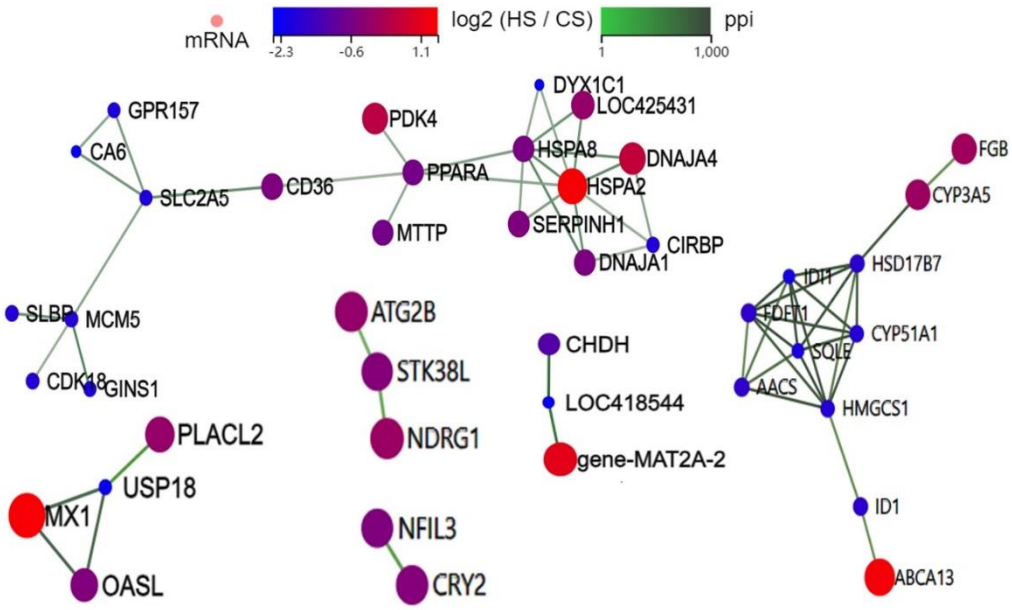

Figure 3.3 Protein–protein interaction network of differentially expressed genes in the jejunal mucosa in heat-stressed breeder hens. The color of the 'lines' becoming greener indicates a greater number of connected genes and associations with other genes. The size of the circle represents the values of log2 fold change. Red and blue nodes indicate the upregulated and downregulated genes, respectively.

3.4.6 Validation of RNA-seq results by real-time PCR

We validated the expression levels of six upregulated transcripts (HSPB9, PDK4, HSPA2, HSPH1, DNAJA4, and IL18BP) and two downregulated transcripts (GBP7 and CAMK1G) in the jejunal mucosa for the validation by qPCR (Figure 3.4). All genes showed similar expression trends in both the qPCR and RNA-seq. These results demonstrated the reliability and accuracy of our RNA-seq data in this study.

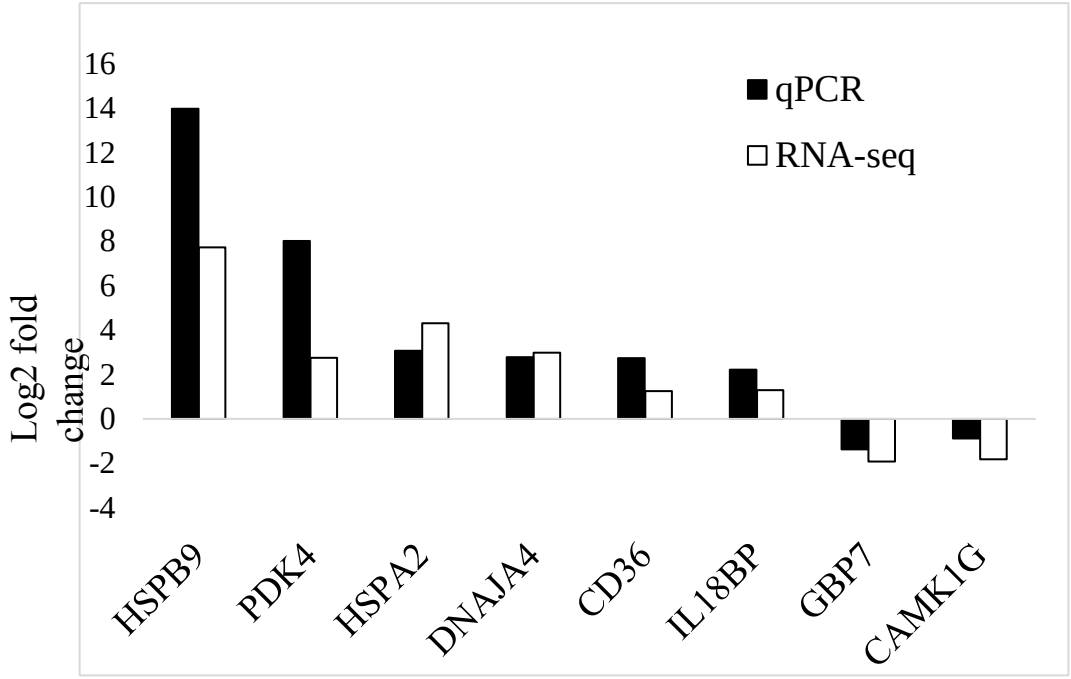

Figure 3.4 Quantitative polymerase chain reaction validation of 8 differentially expressed genes identified using RNA-sequencing. The x-axis represents the genes, and the y-axis represents their mRNA expression levels expressed in fold-change values. Expression levels determined via quantitative polymerase chain reaction (qPCR) and RNA-sequencing (RNA-seq) are represented by black and white fill columns, respectively. Expression levels were examined in jejunal mucosae of breeder hens under thermoneutral conditions (n = 12) and heat stress conditions (n = 12).

3.4.7 Effect of dietary antioxidants on altering gene markers in jejunal mucosal tissue

To evaluate whether antioxidants could mitigate the adverse effects of HS by modulating the expression of candidate genes identified from RNA-seq, such as HSPB9, HSPA2, IL18BP, and CD36. We investigated the impact of antioxidant supplementation in the form of synthetic (a combination of vitamin C, vitamin E, Se, and L-carnitine) and phytogenic substances (a combination of clove, green tea pomace, and Vietnamese coriander) in HS breeder hens' diets on altering these gene markers in the jejunum, is shown in Table 3.7. Based on orthogonal contrasts, the HS challenge

significantly altered the mRNA expression in the jejunal mucosa of genes related to HSPs (HSPB9, HSPA2) and fat metabolism (CD36) compared to the TN group ($P < 0.05$). Either synthetic or phytogenic antioxidant supplementation significantly increased the expression of CD36, while decreasing HSPB9, HSPA2, and IL18BP compared to the heat-stressed group without supplementation ($P < 0.05$). Tukey's multiple comparison tests indicated that the expression levels in heat stress non-supplementation significantly increased the expression of HSPB9, HSPA2, and IL18BP compared to the TN condition ($P < 0.05$). Either synthetic or phytogenic antioxidant supplementation significantly increased the expression of CD36 compared to the TN condition ($P < 0.05$).

Table 3.7 Effect of dietary antioxidant supplementation on relative gene markers in jejunal mucosa in breeder hens under heat stress conditions.

Items	Treatments[1]				Pooled	Contrasts[2]		
	T1	T2	T3	T4	SEM	1	2	3
HSPB9	0.91[b]	1.63[a]	0.95[b]	0.90[b]	0.083	0.002	<0.001	0.938
HSPA2	0.63[b]	1.57[a]	0.67[b]	0.74[b]	0.107	0.003	<0.001	0.926
IL18BP	1.55[b]	2.17[a]	1.56[b]	1.49[b]	0.091	0.205	0.004	0.976
CD36	1.09[b]	1.29[b]	2.03[a]	1.92[a]	0.112	<0.001	<0.001	0.193

[a-b]Means within each row with different superscripts are significantly different ($P < 0.05$).

[1]T1, thermoneutral zone (23±1°C) + basal diet without supplementation; T2, heat stress condition (36±1°C, 4 h/day) + basal diet without supplementation; T3, heat stress condition (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress condition (36±1°C, 4 h/day) + basal diets with phytogenic antioxidants.

[2]Orthogonal contrasts: 1= thermoneutral (T1) vs. heat stress condition (T2, T3, T4); 2, non-supplement (T2) vs. supplement (T3, T4); 3, synthetic antioxidants (T3) vs. phytogenic antioxidants (T4). Abbreviation: HSPB9, heat shock protein family B (small) member 9; HSPA2, heat shock protein family A (Hsp70) member 2; CD36, cluster of differentiation 36; IL18BP, interleukin 18 binding protein.

3.5 Discussion

Chickens cope with higher body temperatures by increasing their heart rate and cardiac output, which facilitates the transfer of large amounts of blood to the skin capillaries (Yahav, 2009; Chang et al., 2018). The elevated heart rate and body temperature are physiological stress responses in heat-stressed poultry, and cloacal temperature is an overall indicator of these reactions (Cândido et al., 2020). Our results indicated a significant increase in heart rate and cloacal temperature in breeder hens subjected to HS. High ambient temperatures can impair intestinal health, damage the intestinal mucosal barrier, and reduce the digestion and absorption of nutrients by breeder hens (Goel et al., 2021). We performed RNA-seq analysis on the jejunal mucosa to further characterize the molecular changes occurring in heat-stressed breeder hens and gain a comprehensive understanding of the molecular mechanisms underlying the effects of acute HS on the intestinal health of breeder hens. RNA-seq analysis revealed 138 DEGs, with 75 upregulated and 63 downregulated DEGs, in heat-stressed breeder hens.

HSPs are produced in response to stressors, such as high temperature, and play a crucial role in protecting the gut epithelium from oxidative stress and inflammation (Liu et al., 2014). In the present study, all the detected HSP transcripts (HSPB9, HSPA2, DNAJA4, HSPH1, HSP90AA1, and HSPA8) were upregulated in the jejunal mucosa of heat-stressed breeder hens. Previous studies have shown the upregulation of various HSPs, including HSP40 (DNAJA1), HSP70 (HSPA2 and HSPA8), and HSP90 (HSP90AA1) in the jejunal mucosa of chronically heat-stressed broiler chickens (Kim et al., 2022). Among the upregulated HSP transcripts in our study, the HSPB9 transcript was the most upregulated (FC = 7.73), followed by HSPA2 and DNAJA4 (FC = 4.32 and 2.99, respectively). HSPB9, also known as HSP25, belongs to the small HSP family and functions by stabilizing unfolded proteins and preventing their precipitation within cells (Katoh et al., 2004). Moreover, HSPA2, a 70 kDa protein, has been linked to mRNA expression levels and heat tolerance and can serve as an effective marker for the selection of heat-tolerant chickens. Under stressful conditions, HSP70 protects cells from damage by enhancing protein expression (Oyake et al., 2006; Zhong et al., 2010). HSP70 can reduce cell damage and protect the intestinal mucosa from HS damage by improving the antioxidant capacity of chickens and inhibiting lipid peroxidation (Gu et al., 2012; Mountzouris et al., 2020). Furthermore, overexpression of HSP70 may increase

intestinal alkaline phosphatase activity, suggesting that it plays a key role in maintaining normal intestinal barrier function (Hao et al., 2012). The HSP40 (DnaJ HSP) family is a key molecular chaperone in the HSP superfamily. As an auxiliary molecular chaperone to HSP70, HSP40 not only regulates the ATPase activity of HSP70 but also promotes the binding activity of HSP70 with protein substrates, thereby increasing the active domain of HSP70 (Hageman et al., 2010). Previous studies have indicated that overexpression of HSP40 may be partially responsible for increased thermotolerance in animals (Shi et al., 2019). In the present study, the HSP90 transcript (HSP90AA1) was upregulated in the jejunal mucosal tissue of heat-stressed breeder hens (FC = 2.15). As an ATP-dependent molecular chaperone, HSP90 binds to ATP and hydrolyzes it at its N-terminus (Meyer et al., 2003). Previous studies have shown that HSP90 levels in the jejunum and ileum increased significantly after broiler chickens were continuously exposed to a high-temperature environment at 39°C for 8 hours daily for 5 days (Varasteh et al., 2015). Moreover, the expression of HSP90 in the intestinal mucosa of silky chickens was upregulated under HS conditions (Liu et al., 2016). These findings suggested that breeder hens alleviate HS by inducing HSP70 and HSP90 expression, and that the expression levels of HSP70 and HSP90 can be used as indicators of the intestinal health of heat-stressed breeder hens. In addition to the upregulation of HSPs, the expression of PDK4 is also upregulated in the jejunal mucosa of heat-stressed breeder hens (FC = 2.76). The protein encoded by the PDK4 gene is a member of the pyruvate dehydrogenase kinase family and plays an important role in regulating lipolysis, glycolysis, and energy homeostasis metabolism (Honda et al., 2017; Wen et al., 2021; Forteza et al., 2023). PDK4 regulates the irreversible conversion of pyruvate to acetyl-CoA by affecting pyruvate dehydrogenase complex activity. PDK4 expression is upregulated in the liver and breast muscle of broilers under HS (Lu et al., 2017; Barreto Sánchez et al., 2022). The elevated mRNA levels of PDK4 correspond to increased lactic dehydrogenase activity and reduced citrate synthase activity, suggesting that glucose flow into the tricarboxylic acid cycle is diminished and that the cell relies on anaerobic glycolysis (Zhang et al., 2012; Lu et al., 2017). The upregulation of PDK4 in the jejunal mucosa of heat-stressed breeder hens in this study suggested that HS may rely on anaerobic glycolysis and affect glucose uptake, leading to energy deficiency and potentially causing further damage to the jejunal mucosa, as previously

reported by Garriga et al. (2006). However, further measurements of jejunal morphology are needed to determine whether the mRNA level of PDK4 can be used as an indicator of intestinal health under HS in chickens.

Genes associated with nutrient absorption and transport are downregulated in the jejunum of heat-stressed chickens (Sun et al., 2015). In this study, breeder hens raised under HS conditions showed decreased expression of solute carrier family 10 member 2 (SLC10A2) in the jejunum mucosa (FC = −2.48). SLC10A2 and solute carrier family 10 member 1 are major members of the solute carrier family 10, also known as the Na+-dependent bile acid (BA) transporter family. BA acts as an emulsifier that enhances fat digestibility by improving micelle formation and aiding the absorption of fat-soluble nutrients in the intestinal lumen (Yin et al., 2021a). BA is primarily synthesized in the liver and reabsorbed in the ileum (Jia et al., 2021; Cai et al., 2022). SLC10A2 plays an essential role in the absorption of BA from the intestinal lumen (Miyata et al., 2011) and is expressed on the apical brush border membrane of ileal epithelial cells (Shneider, 2001). Markedly high expression levels of SLC10A2 have been found in the ileum of chickens, whereas very low expression levels have been detected in the jejunum (Nakao et al., 2015), suggesting that the main role of SLC10A2 in the jejunum is the transport of BA, rather than its absorption. Chronic HS disrupts the homeostasis of BA metabolism in broiler chickens, and several genes related to BA metabolism in the liver and ileum are altered; however, it has no significant effect on the expression level of SLC10A2 in the ileum (Zhang et al., 2023). These findings suggested that acute HS reduces the absorption of fat-soluble nutrients associated with BA into the jejunal mucosa. BA as a nutritional strategy has some potential to alleviate HS (Yin et al., 2021b; Li et al., 2023). SLC10A2, which was downregulated in the present study, may be involved in HS alleviation in the jejunum.

Biological processes within cells rely on the coordination of various gene systems. GO analysis of genes can provide a deeper understanding of their biological functions. In this study, 138 DEGs were significantly enriched among 352 GO terms. A previous transcriptome study on jejunal mucosal tissue in broilers subjected to chronic HS reported significant enrichment of 16 GO terms (Kim et al., 2022), of which we identified 8 GO terms (response to heat, protein refolding, progesterone receptor binding, ATP binding, ATPase activity, extracellular space, receptor complex, and extracellular

region). These findings suggested that acute HS affects many functions in breeder hens and that the eight GO terms detected in both HS conditions may play an important role in the adaptive response to HS in the jejunal mucosa of breeder hens.

Furthermore, KEGG pathway analysis identified nine pathways affected by acute HS in the jejunal mucosal tissues of breeder hens. A previous study on the jejunal mucosa of broilers under chronic HS reported six KEGG pathways related to immune signaling and energy metabolism, including cytokine-cytokine receptor interaction, glutathione metabolism, influenza A, NOD-like receptor signaling pathway, neuroactive ligand–receptor interaction and protein processing in endoplasmic reticulum (Kim et al., 2022). Of the six KEGG pathways identified in a previous study, we identified only protein processing in endoplasmic reticulum with upregulated HSPs (HSPA8, HSPH1, HSP90AA1, HSPA2, and DNAJA1). HS disrupts the function of the endoplasmic reticulum, which is crucial for the processing and folding of cellular proteins (Tokutake et al., 2022). Therefore, upregulation of HSPs in the jejunal mucosa may initiate an unfolded protein response, clearing misfolded proteins to protect the cell. These findings suggested that protein processing in endoplasmic reticulum is a key pathway involved in acute and chronic HS in breeder hens.

In the present study, three KEGG pathways involved in sequential reactions were identified (terpenoid backbone biosynthesis, steroid biosynthesis, and steroid hormone biosynthesis) (Figure S3.1). Among these pathways, the steroid biosynthesis pathway was the most significant (P = 1.36E–06) and cholesterol is the final product of this pathway. Cholesterol is an important component of the membrane and plasma lipoproteins of vertebrates and regulates membrane fluidity and permeability (Chen et al., 2023). The steroid biosynthesis pathway with downregulated genes identified in this study may decrease cholesterol content and affect membrane fluidity and permeability in the jejunum of heat-stressed breeder hens. However, in the serum and liver, HS exposure increases total cholesterol levels (Lan et al., 2022). Cholesterol is also a precursor of steroid hormones and BA (Chen et al., 2023). Cortisol (a glucocorticoid) and corticosterone are steroid hormones derived from cholesterol and are considered the main stress hormones in birds (Oluwagbenga et al., 2022). Oluwagbenga and Fraley (2023) have reported that the effects of HS on the bursa, spleen, and thymus of birds can be verified by measuring cortisol levels. It can be

inferred that the effect of HS on the small intestine can also be verified using cortisol as an HS indicator. However, there are no previous reports on cholesterol and cortisol levels and steroid and steroid hormone biosynthesis capacity of the jejunum in chickens under HS. The actions of the terpenoid backbone, steroid, and steroid hormone biosynthesis pathways, along with many downregulated DEGs in the jejunum of breeder hens under acute HS, remain unknown.

The PPAR signaling pathway is considered a key pathway that promotes fatty acid oxidation and enhances cellular energy metabolism (Ni et al., 2022). Adipocytokine signaling is regularly utilized as a mechanism for stress adaptation by activating energy metabolism in response to HS in broilers (Ma et al., 2021). In this study, we identified that the PPAR and adipocytokine signaling pathways were affected by acute HS, including two upregulated genes (PPARA and CD36) in the jejunal mucosa tissue of heat-stressed breeder hens. The PPAR family comprises three isoforms (α, γ, and β/δ), all of which play significant roles in animal lipid metabolism and energy metabolism (Wahli and Michalik, 2012). Of these, PPARα plays an important role in fatty acid oxidation and synthesis (Nguyen et al., 2008). The mRNA expression levels of PPARα in the liver and breast muscles of chickens raised under HS (31 ± 1°C) for 14 days were decreased compared with those chickens raised under normal temperature (21 ± 1°C) (Li et al., 2024). Previous research indicated that chronic HS altered the expression of lipid metabolism-related genes, including downregulated PPARα genes in the liver of laying hens, suggesting disturbances in lipid metabolism and induction of fat deposition (Yin et al., 2023). These findings indicated the importance of the PPARA gene in lipid metabolism, and the upregulation of this gene in jejunal mucosa under acute HS may reduce fat deposition. CD36 is involved in fatty acid uptake and transport. Downregulation of CD36 expression in the jejunal tissue of Arbor Acres broilers after 7 days of exposure to 32 ± 1°C for 10 hours daily was reported (Sun et al., 2015). Abdelli et al. (2021) reported that CD36 gene expression was unchanged in modern broiler chickens subjected to 26 days of chronic HS (36°C for 8 hours daily); however, it was upregulated in their ancestor heat-tolerant wild jungle fowl. These findings suggested that CD36 gene expression was affected by HS and specific strains of experimental birds, and the upregulation of CD36 genes in this study may be an early response to

acclimatization to HS. Future studies are required to determine the associations between mRNA of PPARA and CD36 levels and lipid deposition or fatty acid uptake and transport functions to use as an indicator of intestinal health of heat-stressed chickens.

PPI network analysis revealed the two largest networks. Within the network containing HSP, we identified SLC2A5, whose mRNA was downregulated and encodes GLUT5. GLUT5 is essential for absorbing glucose and fructose from the intestinal lumen into the cytosol (Barone et al., 2009). The reduction in GLUT5 expression under HS conditions may lead to decreased levels of glucose in the jejunal cytosol. In addition to SLC2A5, other genes related to nutritional metabolism (PPARA and CD36 in fat metabolism and PDK4 in energy homeostasis) were also implicated in this network. Previous studies have consistently demonstrated that many HSP-encoding genes are upregulated during HS and respond to HS by increasing their transcription and translation (Lara and Rostagno, 2013; Kahl et al., 2015). Our results suggested that HS affects various metabolic pathways related to nutrient absorption and utilization in the jejunum and that the upregulation of HSPs improves these nutritional functions in heat-stressed breeder hens. However, the mutual regulatory relationships in this network in the jejuna of heat-stressed breeder chickens require further investigation. The second-largest network consisted of 11 transcripts, 7 of which were involved in three KEGG pathways representing a series of cholesterol-related responses. In addition, PPI analysis revealed a network containing 2'-5'-oligoadenylate synthetases like (OASL), MX dynamin like GTPase1 (MX1), and ubiquitin-specific peptidase 18 (USP18). Previous studies have reported that radical S-adenosyl methionine domain containing 2, OASL, epithelial-stromal interaction 1, cytidine/uridine monophosphate kinase 2, interferon induced with helicase C domain 1, interferon-induced protein with tetratricopeptide repeats 5, USP18, MX1, and signal transducer and activator of transcription 1 may contribute to the jejunal morphology in broilers (Li et al., 2022). Our results suggested that these genes (OASL, MX1, and USP18) could serve as valuable markers for assessing jejunal morphology in breeder hens with acute HS.

In this study, either synthetic or phytogenic antioxidant supplementation significantly altered gene expression patterns compared to the non-supplemented HS groups. Specifically, both antioxidant types increased CD36 expression while simultaneously decreasing the expression of HSPs (HSPA2 and HSPB9) and IL18BP in

the jejunum of heat-stressed hens. This finding is particularly significant given that CD36, a transmembrane protein receptor, plays a crucial role in dietary lipid absorption through its involvement in fatty acid uptake and transport (Chen et al., 2001). The modulation of CD36 expression is especially relevant in the context of HS, as ROS can induce structural modifications in macromolecules, such as fatty acids and glucose (Grüning et al., 2011). These oxidized products can subsequently interfere with normal glucose and lipid absorption and transport processes in the small intestine (Vital et al., 2018). Previous research has demonstrated that HS conditions lead to downregulated CD36 expression in the jejunum of wild jungle fowl (Abdelli et al., 2021), making the antioxidant-induced upregulation observed in this study particularly noteworthy. Previous research has demonstrated that antioxidants such as Vitamin E and lycopene increase mRNA and protein expression levels of CD36 (Meng et al., 2022). The observed upregulation of CD36 through antioxidant supplementation may enhance lipid homeostasis and mitigate heat stress-induced damage in breeder hens' jejunal tissue. The heat shock protein HSP70 (HSPA2) plays a crucial protective role during HS, with its expression typically induced to shield cells from temperature-related damage (Zhong et al., 2010). The observed reduction in both HSP70 and HSPB9 expression following antioxidant supplementation likely represents a balanced cellular response, and preservation of excessive activation of stress-related pathways while maintaining necessary protective functions (Chung et al., 2017). Various antioxidants like vitamins C and E, selenium, and L-carnitine have been demonstrated to reduce oxidative stress, potentially decreasing the need for elevated HSPs (HSP70 and HSP90) expression (Girisa et al., 2024; Khan et al., 2024). This is supported by findings that vitamins E, C, and Se supplementation downregulated the expression of HSP70 and HSP90 in the jejunum of heat-stressed broilers (Calik et al., 2022). HS significantly impacts gene expression in poultry, as evidenced by the downregulation of CD36 in the jejunum of wild jungle fowl (Abdelli et al., 2021). During HS conditions, the expression of HSPA2 and HSPB9 becomes dysregulated, leading to cellular stress and tissue dysfunction. IL18BP is a key regulator of the immune response, specifically by inhibiting the activity of interleukin-18 (IL-18), a cytokine involved in inflammation and immune activation (Ihim et al., 2022). Under HS conditions, pro-inflammatory cytokines, including IL-18, are elevated, contributing to systemic inflammation and immune dysfunction. Notably, dietary

supplementation such as vitamin E and Se has been shown to decrease the mRNA levels of multiple pro-inflammatory cytokines, including IL-18, IL-6, and TNFα in the jejunal mucosa of heat-stressed broilers (Calik et al., 2022). This reduction in inflammatory markers suggests that antioxidants effectively alleviate HS-induced inflammation (Liu et al., 2021). The beneficial effects of antioxidants extend beyond inflammation control. By modulating IL-18 signaling pathways, antioxidants help reduce chronic inflammation and enhance immune system functionality in heat-sensitive hens. The observed changes in gene expression – especially, the upregulation of CD36 and downregulation of HSP70, HSPB9, and IL18BP – demonstrate how antioxidants comprehensively influence lipid metabolism, cellular stress responses, and immune regulation. These molecular adaptations support the use of dietary antioxidants as an effective strategy to mitigate HS-induced negative effects in heat-sensitive breeder hens, potentially leading to improved health outcomes and enhanced productivity.

3.6 Conclusions

The present study evaluated the transcriptome of the jejunal mucosal tissue of breeder hens exposed to acute HS and identified 138 DEGs. We found that the steroid biosynthesis pathway, steroid hormone biosynthesis pathway, protein processing in endoplasmic reticulum, PPAR signaling pathway, and adipocytokine signaling pathway were significantly enriched. KEGG pathway and PPI analyses showed that acute HS may affect energy metabolism, fat metabolism, and glucose transport in the jejunal mucosa of breeder hens and that heat-stressed hens restore the damage caused by HS to the jejunal mucosa by increasing the expression of HSPs. Nine candidate genes, including HSPA2, HSPB9, DNAJA4, HSP90AA1, PDK4, SLC10A2, PPARA, IL-18BP, and CD36, may play key roles in the regulation of the jejunal mucosa of breeder hens with acute HS. Our results contribute to a deeper understanding of the jejunal mucosal response in breeder hens to acute HS. The HSPB9, HSPA2, IL-18BP, and CD36 genes may serve as potential gene markers for heat stress effects in the jejunal mucosal tissue of HS breeder hens. Furthermore, supplementation with synthetic and phytogenic antioxidants has the potential to modulate the expression of HSPB9, HSPA2, IL-18BP, and CD36 genes in the jejunal mucosal tissue of breeder hens exposed to HS, which

indicates the ability of breeder hens to alleviate HS effects. These findings enhance our understanding of the molecular mechanisms underlying heat stress in breeder hens. The identification of these gene markers can provide valuable insights for developing guidelines on the use of dietary antioxidants to alleviate the effects of HS and protect gut health in the jejunal mucosa of breeder hens under HS. However, given that these transcriptome data are preliminary, further investigation is required to explore the functions of the DEGs. One limitation of this study is that we did not measure the indicators of oxidative stress and immune status, such as levels of reactive oxygen species, reactive nitrogen species, or differential white blood cell count. In the future, this information will be needed to validate how HS affects productivity.

3.7 References

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