CHAPTER IV
EFFECT OF HEAT STRESS ON TRANSCRIPTOME PROFILING ANALYSIS AND PROTECTIVE EFFICACY OF DIETARY ANTIOXIDANTS IN JEJUNAL MUCOSAL TISSUE IN BREEDER HENS

4.1 Abstract

Heat stress impairs intestinal integrity in poultry, disrupting nutrient digestion and absorption, but the molecular mechanisms remain unclear. This study aimed to examine the gene expression profile in the jejunum of heat-adapted (HA) and heat-sensitive (HS) breeder hens under heat stress (36°C for a 6-h). Fifty 28-week-old breeder hens were randomly assigned to HA and HS groups (25 hens each). After exposure to heat stress for 6 hours, jejunal mucosa samples were collected for RNA sequencing (RNA-seq). RNA-seq analysis identified 284 differentially expressed genes (DEGs), with 155 genes upregulated and 129 downregulated in the HS group compared to the HA group. Gene ontology (GO) analysis revealed significant enrichment in 555 GO terms. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis identified five pathways enriched in upregulated DEGs (VEGF signaling pathway, MAPK signaling pathway, steroid biosynthesis, neuroactive ligand-receptor interaction, and cell cycle) and one pathway enriched in downregulated DEGs (cell adhesion molecules). Protein-protein interaction (PPI) network identified key genes (PLK1, CDC7, CDC20, HSPA2, IL6, SLC22A19A, LBFABP, and SLC2A2), involved in cell division, immune function, energy, lipid metabolism, and nutrient transport. Nine candidate genes, including HSPB9, HSPA2, RAG2, CD36, CLDN15, LBFABP, SLC22A19A, SLC2A2, and IL18BP, may play key roles in the regulation of the jejunal mucosa of breeder hens with acute heat stress. The findings suggest that acute heat stress might affect the cell cycle, immunity, and organic acid, glucose, and amino acids transport mechanisms in the jejunal mucosa of breeder hens. The upregulation of HSPs appears to serve as a protective mechanism, potentially preserving intestinal nutrient processing capacity under acute heat stress. These findings provide foundational knowledge for further investigation into the

molecular mechanisms governing heat stress responses in avian intestinal function and may inform strategies for maintaining gut health in commercial poultry operations exposed to environmental challenges.

Keywords: Transcriptome analysis; Breeder hen; Heat stress; Jejunal mucosa.

4.2 Introduction

As global ambient temperatures rise, heat stress has emerged as a prevalent environmental stressor with significant impacts on animal growth and health. Among livestock, poultry are particularly sensitive to elevated temperatures due to their limited capacity to regulate heat loss through evaporation (Cahaner, 2008). Heat stress induces multiple adverse effects, including growth retardation, oxidative stress, and intestinal inflammation, leading to serious problems in poultry production (Wang et al., 2022; Yang et al., 2024). Moreover, heat stress can damage intestinal integrity and barrier function, which in turn initiates an inflammatory response and affects nutrient absorption and transport (Pearce et al., 2013; Varasteh et al., 2015). Previous study has reported that heat stress-induced reduction in mesenteric blood flow causes intestinal epithelial damage (Zhang et al., 2020), subsequently alternating the absorption and transport of essential nutrients such as glucose, amino acids, and lipids in the jejunum of chickens (Sun et al., 2015; Wang et al., 2022). However, there remains limited information regarding the specific effects of heat stress on jejunal barrier function, nutrient transport mechanisms, and immune responses in breeder hens.

The intestine, particularly the jejunum, plays a crucial role in nutrient digestion and absorption, is recognized as the primary target of heat stress (Chauhan et al., 2021). In response to heat stress, cells activate protective mechanisms, notably through the upregulation of heat shock proteins (HSPs) (Beere, 2004). The HSPs are essential for cell survival under stress conditions and maintain cellular homeostasis by preventing protein misfolding and facilitating the removal of damaged proteins (Gupta et al., 2010). Among HSPs, HSP70 and HSP90 are the most extensively studied and serve as biomarkers of cellular stress (Yu et al., 2021). Research has shown that heat stress upregulates the expression of HSP70 mRNA in the jejunal mucosa (Hao et al., 2012), activates the intestinal MAPK signaling pathway, and mitigates both structural and

oxidative damage to the intestinal mucosa induced by high temperature (Yu et al., 2021). In addition, heat stress regulates genes involved in nutrient absorption and transport (Goel et al., 2021). Studies have demonstrated that heat stress significantly reduces the expression levels of key transport proteins, including glucose transporter 2 (GLUT-2), fatty-acid-binding protein (FABP), and cluster of differentiation 36 (CD36) (Sun et al., 2015). Furthermore, heat stress has been shown to impair immune responses in the small intestine (Farag and Alagawany, 2018), with heat stress leading to increased expression of interleukin-6 (IL6), and tumor necrosis factor-alpha (TNF-α) in the jejunum of broilers (Al-Zghoul et al., 2019). However, there is limited data on how heat stress affects changes and interactions in genes related to immune function, intestinal barrier, and nutrient transport in breeder hens.

Genetics plays a critical role in shaping the host's response to heat stress in poultry (Felver-Gant, 2012). Indigenous chicken breeds in tropical regions, such as the slow-growing Thai native chicken Leung Hang Khao (Katemala et al., 2022), have been shown to exhibit greater tolerance to heat stress compared to other breeds (Soleimani and Zulkifli, 2010). Moreover, fast-growing broilers are more susceptible to heat stress than slow-growing broiler strains (Yunis and Cahaner, 1999). Previous studies have reported that gene identification based on breed-specific expression in commercial and Indigenous chickens revealed candidate genes and molecular pathways related to metabolism, immune system, and heat stress response (Perini et al., 2020; Sadr et al., 2023). However, the impact of heat stress on the gut health of commercial and Indigenous breeder hens remains unclear.

Transcriptomic technology provides valuable insights into the adaptation mechanisms of resilient breeds (Shashank et al., 2024) and aids in comparing the transcriptome profile among breeds (Pareek et al., 2019). In our previous study using RNA-seq, we identified DEGs associated with steroid biosynthesis, terpenoid backbone biosynthesis, steroid hormone biosynthesis, endoplasmic reticulum protein processing, PPAR signaling pathway, and DNA replication in the jejunal mucosa of HS breeder hens under acute heat stress (Zhu et al., 2025). However, there is limited research on the molecular mechanisms by which heat stress affects the intestinal health of both HA and HS breeder hens. Therefore, this study aims to investigate the physiological

responses of HA and HS breeder hens under heat stress, identify candidate biomarkers for genetic selection to enhance heat tolerance.

4.3 Materials and methods

4.3.1 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 number SUT-IACUC-012/2020).

4.3.2 Housing, birds, and sample collection

A total of fifty HS breeds (SUT breeder hens) and HA breeds (Leuang Hang Kao breeder hens) at 22 weeks of age, 25 hens per strain, were raised in individually housed in cages with a size of 40 × 45 × 40 cm^3 (width × depth × height) and acclimated for 5 weeks in thermoneutral (23±1°C) room by using air conditioner. Leuang Hang Kao breeder hens are a Thai native breed, SUT breeder hens represent a synthesized commercial line developed for producing Thai indigenous crossbred chickens. The breeder hens were fed 140 g/day of corn-soy basal diets, formulated following the guidelines of the National Research Council 1994 (NRC, 1994) and Aviagen (2016) recommendations (2,800 kcal of metabolizable energy/kg and 15% crude protein), with water available ad libitum, and were maintained on a 16-hour light cycle daily. At 28 weeks of age, the hens were divided into two groups, i.e., HA and HS groups, each consisting of 25 hens, using a completely randomized design. All breeder hens were moved to a heat stress room with a controlled temperature at 36 °C with a humidity of 40-70% for 6 hours using a gas heater with thermostat-controlled equipment according to the modified method (Duangjinda et al., 2017). After the hens were exposed to heat stress for 6 hours, 12 breeder hens from each strain were randomly 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 further transcriptome analysis and gene validation analysis.

4.3.3 Extraction of total RNA

Total RNA was extracted from 12 jejunal mucosal tissue samples from each strain (HA and HS breeds) using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and

subsequently purified with a QIAamp spin column (Qiagen), following the manufacturer's protocol. The RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and its quality was assessed through 1% agarose gel electrophoresis, employing 0.5× TAE buffer and an electric current of 100 V for 25 min. The three pooled RNA samples (each pool consisting of four individual jejunal mucosa samples) from each strain were used to construct an RNA-seq library. Capillary electrophoresis using a QIAxcel Connect (Qiagen) system was employed to assess the RNA integrity number (RIN), with RNA samples having a RIN ≥ 7 selected for long RNA library construction.

4.3.4 Library construction and data processing

The cDNA library construction and RNA-seq were conducted by BGI Co., Ltd. (BGI, Shenzhen, China). Six libraries were sequenced on the DNBSEQ platform. Sequencing data were processed using SOAPnuke Version v1.5.2 (Cock et al., 2010) to generate clean reads. The clean reads were then aligned to the chicken reference genome (GCF_000002315.6_GRCg6a) using HISAT2 v2.0.4 (Kim et al., 2015), and gene expression levels were calculated using RSEM Version v1.2.8 (Li and Dewey, 2011).

4.3.5 Differentially expressed gene screening and functional enrichment

Differential gene expression was analyzed using DESeq2 (v1.4.5) (Love et al., 2014). Differentially expressed genes (DEGs) were identified based on a fold-change (FC) of ≥ 1 and an adjusted value of $P < 0.05$. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted, with GO terms and KEGG pathways having $P < 0.05$ considered significantly enriched.

4.3.6 Validation of DEGs and marker genes via quantitative polymerase chain reaction (qPCR)

The primer sequences for heat shock protein family B (small) member 9 (HSPB9) and heat shock protein family A (Hsp70) member 2 (HSPA2), cluster of differentiation 36 (CD36), claudin 15 (CLDN15), recombination activating gene 2 (RAG2), interleukin 18 binding protein (IL18BP), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are presented in Table 4.1. Design qPCR-specific primers using NCBI's online primer design software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). These genes were analyzed in the jejunal mucosa tissues of HA and HS breeder hens using quantitative real-time polymerase chain reaction (qRT-PCR). Then, the cDNA samples

from HA and HS breeds, T1, T2, T3, and T4, were used to analyze the DEGs validation and marker gene confirmation. For reverse transcription, 2 μg of total RNA from each sample was used with the SuperScript III RNase H-Reverse Transcriptase Kit (Toyobo, Osaka, Japan) and random primers (Promega, Madison, WI, USA), following the manufacturer's instructions. RT-qPCR was performed using the QuantiNova SYBR Green PCR Kit (Qiagen, Hilden, Germany). Briefly, the 10 μL reaction mix was prepared containing 5 μL of SYBR Green, 0.4 μL of forward primer, 0.4 μL of reverse primer, 2 μL of cDNA, and 2.2 μL of nuclease-free water. The parameters of PCR cycles included the following phases: initial heat activation at 94℃ for 10 min, followed by 40 cycles of denaturation at 95℃ for 10 s, annealing at 60℃ for 30 s, and final extension at 72℃ for 30 s. Relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method, with GAPDH as the internal control.

Table 4.1 Primer sequences used for real-time PCR.

Gene	Primer sequences	Gene accession number
CLDN15	F-5'-AATATACTCGAGGGCCCATGT-3'	XM_040679248.2
	R-5'-AAATCCTCCCGTGACAGCAA-3'	
RAG2	F-5'-CTGCTTCTTCCAACAGATACCG-3'	XM_040700890.2
	R-5'-CAGGATCTCTTCGGCCATCC-3'	
IL18BP	F-5'-CTTCTGCTGCCACTGCTCT-3'	XM_015280902.4
	R-5'-CTCACGTTGCTGCCCATCT-3'	
CD36	F-5'-CAACCTCGCTGTTGTTGCTG-3'	NM_001030731.1
	R-5'-GGTCCAAGGGAAAGGGAACC-3'	
HSPB9	F-5'-CAAGTACGAGGTGCTGAAGCG-3'	NM_001010842.3
	R-5'-TGACAGCTCCATCCTTGGCT-3'	
HSPA2	F-5'-CCGTGGAGTTCCTCAGATCG-3'	NM_001006685.1
	R-5'-GCTAAGGCGACCCTTGTCAT-3'	
GAPDH	F-5'- AGAACATCATCCCAGCGT-3'	K01458
	R-5'- AGCCTTCACTACCCTCTTG-3'	

F, forward; R, revers; *HSPB9*, heat shock protein family B (small) member 9; *HSPA2*, heat shock protein family A (Hsp70) member 2; *CD36*, cluster of differentiation 36; *CLDN15*,

claudin 15; *RAG2*, recombination activating gene 2; *IL18BP*, interleukin 18 binding protein; and *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

4.4 Results

4.4.1 Quality of RNA-Seq reads

RNA sequencing libraries were prepared from the jejunal mucosa of HA and HS breeder hens and sequenced on the DNBSEQ platform. The RNA-seq data quality metrics are presented in Table 4.2. RNA-seq of jejunal samples from HA and HS breeder hens yielded an average of 43.52 million raw reads and 42.30 million clean reads per sample. The sequencing quality was high, with Q20 and Q30 percentages exceeding 97.76% and 93.13%, respectively. GC content across all samples ranged from 46.76% to 47.41%. Alignment of clean reads to the chicken reference genome resulted in mapping rates of 95.38% to 95.82%.

Table 4.2 RNA-sequencing metrics for jejunal mucosa transcriptome analysis of HA and HS breeder hens under heat stress.

Sample[1]	Raw reads (M)	Clean Reads (M)	Clean Bases (GB)	Q20 (%)[2]	Q30 (%)[2]	GC content (%)	Total Mapping (%)
HA1	45.44	44.09	6.61	98.06	94.05	47.14	95.38
HA2	44.15	43.06	6.46	97.76	93.13	46.76	95.82
HA3	45.44	43.85	6.58	97.89	93.68	47.41	95.66
HS1	40.39	39.33	5.93	97.91	93.64	47.01	95.49
HS2	40.78	39.76	5.96	97.84	93.38	47.13	95.55
HS3	44.94	43.73	6.56	97.91	93.65	47.24	95.65
Average	43.52	42.30	6.35	97.90	93.59	47.12	95.59

[1]Jejunal mucosa samples from four individual hens were pooled for each of the three replicates (n=3) in both HA and HS groups under heat stress

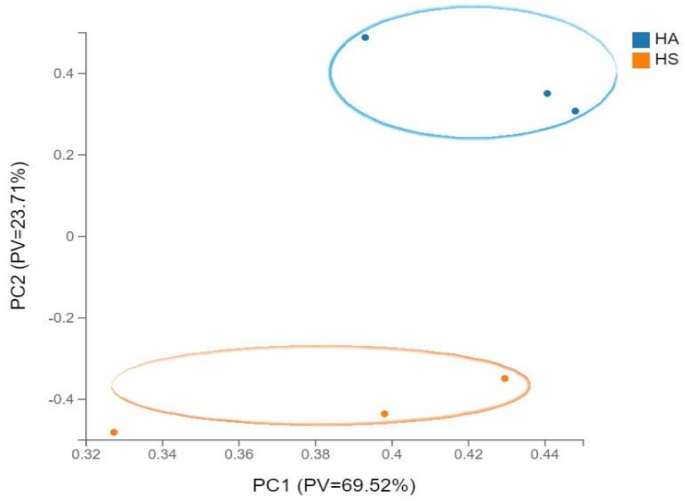
[2]Q20 and Q30 indicate the percentage of bases with P value ≥ 20 and 30, respectively.

4.4.2 Differentially expressed genes analysis

To identify differentially expressed genes (DEGs) in the jejunal mucosa of HA and HS breeder hens under heat stress, RNA-sequencing and subsequent

bioinformatic analysis were performed. Principal component analysis (PCA) of jejunal mucosa transcriptomics revealed a clear separation between HA and HS hens, indicating distinct mRNA expression profiles (Figure 4.1A). Hierarchical clustering of DEGs, based on FPKM values, further confirmed this separation, with samples clustering by groups and distinct gene expression patterns observed between HA and HS hens (Figure 4.1B). A total of 15,258 genes were identified across both groups. Of these, 397 genes were uniquely expressed in the HA group, and 347 genes were uniquely expressed in the HS group (Figure 4.2A). Differential expression analysis identified 284 DEGs, with 155 up-regulated and 128 down-regulated DEGs in HS compared to HA (Figure 4.2B and Table S4.1). Upregulated genes in HS hens included HSPB9, RAG2, HSPA2, and heat shock protein family B (small) member 1 (HSPB1), while downregulated genes included CLDN15 and liver basic fatty acid binding protein (LBFABP).

A



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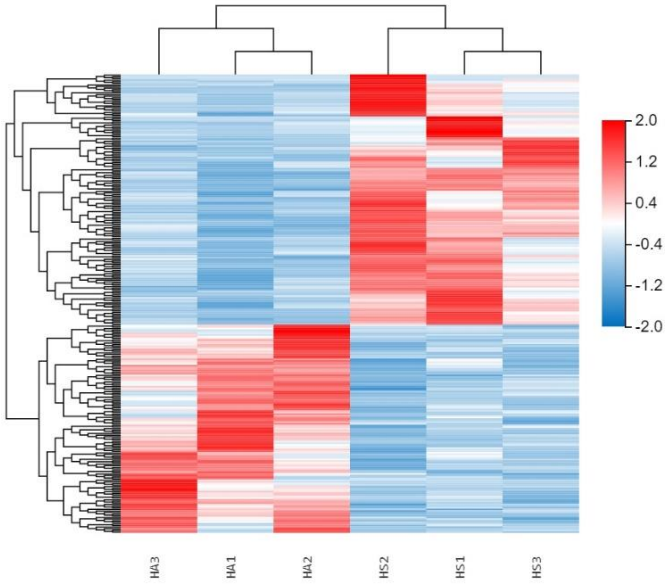

Figure 4.1 DEGs analysis in jejunal mucosa between HA and HS breeder hens under heat stress. (A) PCA of DEGs. Blue and orange nodes represent individuals from HA and HS breeder hens, respectively. (B) Hierarchical clustering heatmap of DEGs. Each row represents DEGs, and the column represents the sample name. The color gradient from blue to red indicates log2 fold-change values, with red representing upregulated genes and blue representing downregulated genes.

A

B

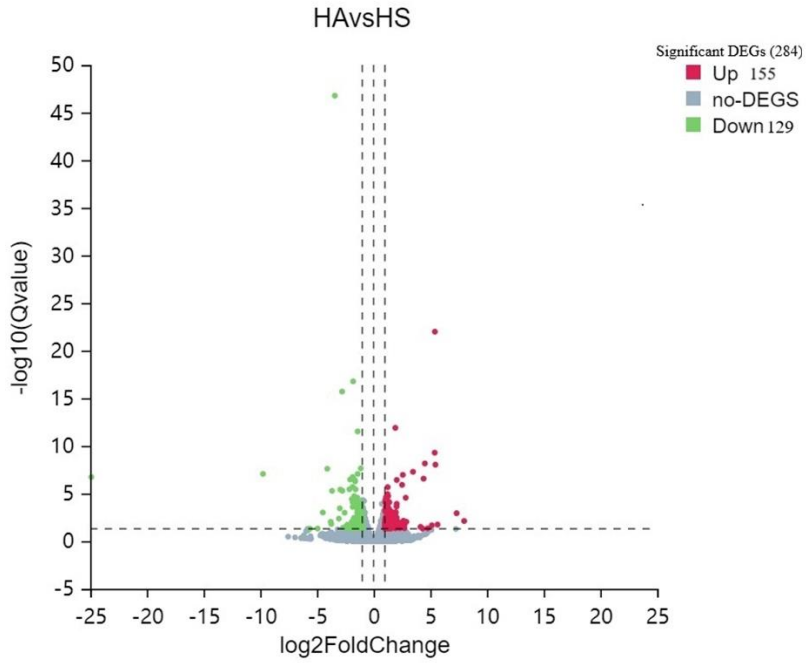

Figure 4.2 DEGs analysis was performed on jejunal mucosa tissues between HA and HS breeder hens under heat stress. (A) Venn diagram illustrating the distribution of DEGs identified in the jejunal tissues. (B) Volcano plot displaying the DEGs between the HA and HS groups. Red and green dots represent significantly upregulated and downregulated genes, respectively (adjusted $P < 0.05$, $|\log2\ FC| \geq 1$). Gray dots indicate genes that did not significance threshold. The x-axis represents log2 fold-changes, and the y-axis shows log10 (adjusted P-value).

4.4.3 Gene ontology (GO) annotation analyses of DEGs

To elucidate the molecular mechanism underlying heat stress, GO annotation analysis was performed on the DEGs. The analysis categorized the DEGs into three main functional groups: biological processes (BP), molecular functions (MF), and cellular components (CC). The analysis revealed significant enrichment in 555 GO terms ($P < 0.05$, log2 FC ≥ 1), comprising 371 BP, 110 MF, and 74 CC (Figure 4.3, Table S4.2). Within the BP category, including cell division, chromosome segregation, cell cycle, fructose transmembrane transport, and response to heat pathway. The MF revealed prominent enrichment in various transport-related activities, especially microtubule

binding, fructose transmembrane transport, short-chain fatty acid transmembrane transport, glucose transmembrane transport, and neutral amino acid transmembrane transport, etc. In terms of CC, the DEGs were predominantly enriched in chromosomes, centromeric regions, extracellular space, brush border membrane, plasma membrane, and extracellular regions, etc.

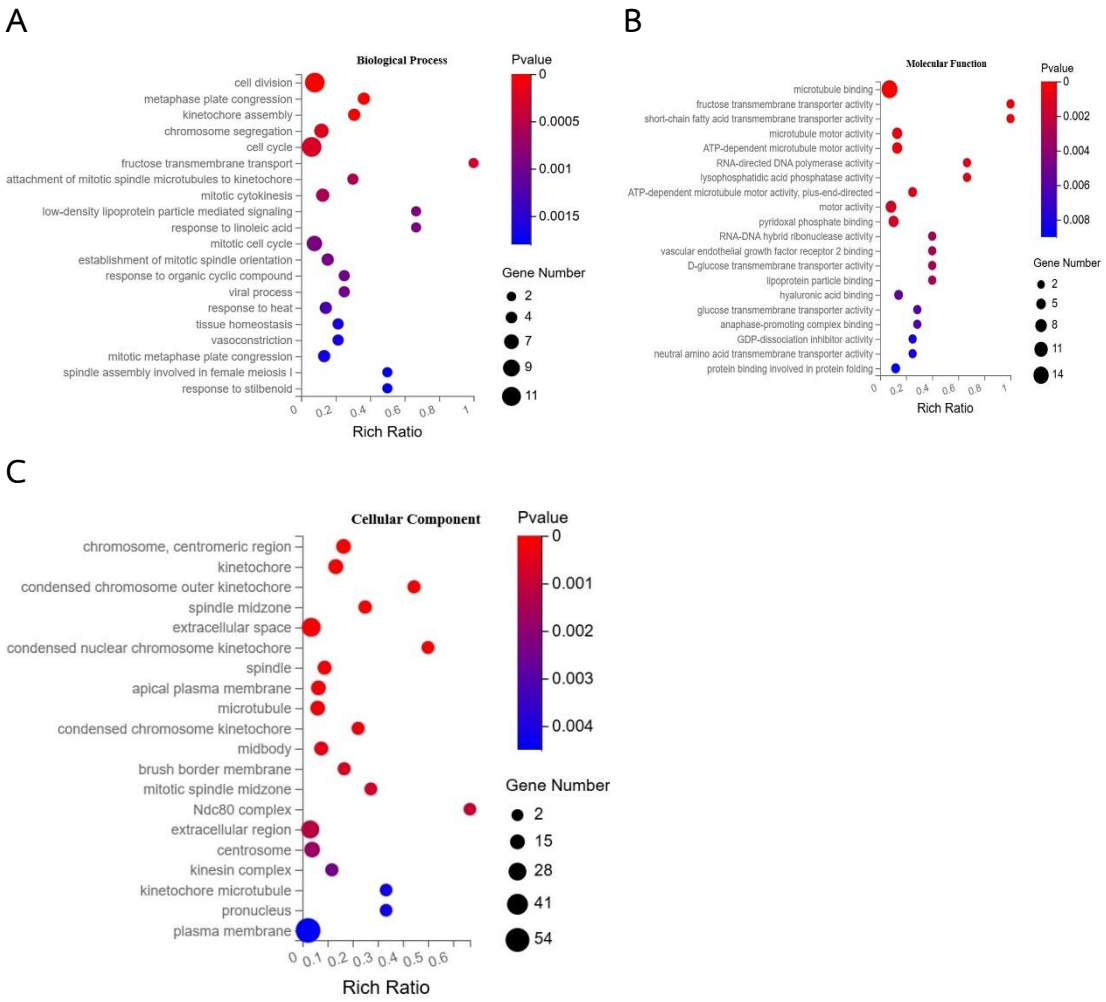

Figure 4.3 Functional enrichment analysis of identified DEGs in jejunal mucosa between HA and HS breeder hens. The top 20 enriched GO terms of DEGs are shown for (A) biological process, (B) molecular function, and (C) cellular component categories. The x-axis represents the richness ratio, and the y-axis shows the functional categories.

4.4.4 KEGG pathway analyses of DEGs

To identify the biological pathways involved in breeder hens under heat stress, the DEGs from both groups were mapped to the KEGG pathway database. KEGG pathway enrichment analysis revealed significant alterations in multiple signaling cascades following acute heat stress exposure. The analysis identified six significantly enriched pathways ($P < 0.05$, log2 FC ≥ 1): VEGF signaling pathway, MAPK signaling pathway, cell adhesion molecules, steroid biosynthesis, neuroactive ligand-receptor interaction, and cell cycle (Table 4.3). These enriched pathways suggested a complex cellular response to acute heat stress, involving multiple regulatory mechanisms and cellular processes.

Table 4.3 Significantly enriched KEGG pathways in jejunal mucosa tissues between HA and HS breeder hens under heat stress.

KEGG[1] Pathway Term	Count	P value	Gene Symbols[2]
gga04370: VEGF signaling pathway	2	7.82E−03	HSPB1↑, PLA2G4EL2↑
gga04010: MAPK signaling pathway	3	0.0208	HSPH1↑, HSPA2↑, PLA2G4EL2↑
gga04514: Cell adhesion molecules	2	0.0345	HLA-F10AL4↓, CLDN15↓
gga00100: Steroid biosynthesis	1	0.0413	LIPML5↑
gga04080: Neuroactive ligand-receptor interaction	3	0.0430	TAC1↑, HTR1B↑, RLN3↑
gga:04110 Cell cycle	5	0.0483	BUBIB↑, CDK1↑, PLK1↑, CDC7↑, CAC20↑

[1]KEGG, Kyoto Encyclopedia of Genes and Genomes

[2]Up and down arrows (↑ ↓) indicate upregulated and downregulated genes, respectively, in jejunal mucosa between HA and HS breeder hens under heat stress.

4.4.5 Protein-protein interaction network analysis of DEGs

Protein-protein interaction (PPI) network analysis for the DEGs identified three distinct networks. The largest network comprised 30 protein-coding genes, with CDK1, PLK1, CDC7, and CDC20 positioned as core nodes. These core proteins showed primary enrichment in the cell cycle pathway within the jejunum (Table 4.3 and Figure 4.4). Notably, most proteins in this network were upregulated, as indicated by red nodes, with a few exceptions, such as KIF33 and CDK18, shown in blue. The second network contained 13 interaction proteins, including HSPA2 and IL6, all of which were upregulated (red nodes). The third network consisted of 10 proteins, all of which were downregulated (blue nodes), with SLC22A13L serving as the core node of the network.

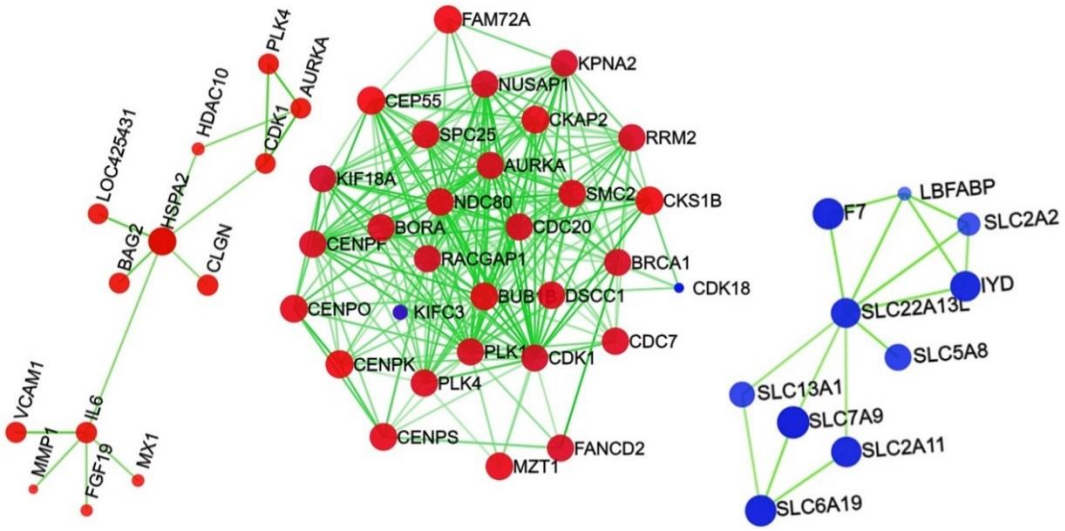

Figure 4.4 PPI network of DEGs in jejunal mucosa between HA and HS breeder hens under heat stress. The size of the circle represents the values of log2 fold change. Red and blue nodes indicate the upregulated and downregulated genes, respectively.

4.4.6 Validation of DEGs and marker genes by real-time PCR

To validate the RNA-seq results, six DEGs with representative function or notably altered expression profiles in response to acute heat stress were selected for quantitative polymerase chain reaction (qPCR) analysis. The validation included four upregulated genes (HSPB9, RAG2, HSPA2, and IL18BP) and two downregulated genes

(CLDN15 and CD36) in the jejunal mucosa between HA and HS breeder hens (Figure 4.5). The expression patterns were observed through qPCR (Figure 4.5). The expression patterns observed through qPCR were consistent with RNA-seq data.

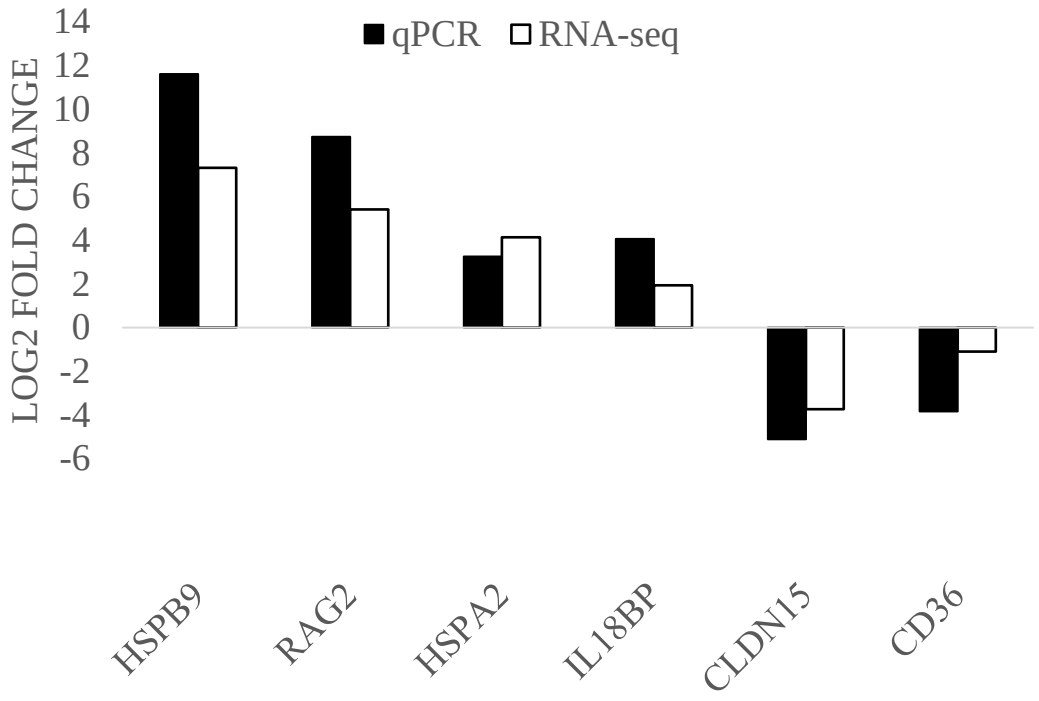

Figure 4.5 Expression of 6 DEGs was detected using either RNA-seq or RT-qPCR. The x-axis represents the genes, and the y-axis represents their mRNA expression levels expressed in fold-change values. Expression levels determined via qPCR and RNA-seq are represented by black and white fill columns, respectively. HSPB9, heat shock protein family B (small) member 9; HSPA2, heat shock protein family A (Hsp70) member 2; CD36, cluster of differentiation 36; CLDN15, claudin 15; RAG2, recombination activating gene 2; IL18BP, interleukin 18 binding protein.

4.5 Discussion

The small intestine plays a crucial role in the digestion and absorption of dietary nutrients (Madara, 1991). However, heat stress can compromise the structural integrity of the intestinal mucosa (Habashy et al., 2017), impair nutrient absorption and transport (Brake, 1998), and downregulate genes involved in processes (Sun et al., 2015). To

evaluate these effects, jejunal mucosal tissue from HA and HS breeder hens was selected for RNA-Seq analysis. This analysis identified 284 DEGs, including 155 upregulated and 129 downregulated genes, between the two groups, and identified some candidate genes, including HSPB9, HSPA2, RAG2, IL18BP, CLDN15, and CD36. Heat stress challenge significantly altered the mRNA expression of HSPB9, HSPA2, and CD36 compared to the TN group, whereas 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. By linking the transcriptomic findings with the antioxidant study, we aim to explore how dietary interventions can alleviate the adverse effects of HS on gene expression and intestinal health. These findings suggest that antioxidant supplementation can alleviate these molecular disruptions caused by heat stress, providing potential dietary interventions to enhance heat tolerance in poultry.

The primary defense against heat stress involves HSPs, which function as intracellular molecular chaperones by binding to misfolded proteins and preventing their aggregation (Wang et al., 2020; Johnston et al., 2021). Recent research has identified significant differences in HSP expression patterns between HA and HS breeds, particularly in the jejunal mucosa tissue under heat-stress conditions. This study reveals the upregulation of several key HSP family members, including HSPA2 (HSP70), HSPB1 (HSP27), and HSPB9 (HSP25). The expression of HSPA2 (HSP70) was significantly upregulated, with a fold change of 4.4 being observed under acute heat stress conditions. This finding was consistent with previous studies by Kim et al. (2022) and Zhu et al. (2025), where HSPA2 upregulation was also documented in heat-stressed poultry. HSPB9 (HSP25) expression patterns were characterized by an initial low expression followed by a gradual increase over time (Xu et al., 2019). The protein's role as a molecular chaperone was confirmed through its involvement in cellular homeostasis maintenance and protein denaturation prevention. HSPB1 was also found to be elevated in the jejunal mucosa. Its functionality was demonstrated through multiple protective mechanisms, including protein stability maintenance and oxidative stress protection (Rogalla et al., 1999; Santoro, 2000). The protein's interaction with cytosolic cytochrome C was observed to regulate apoptotic pathways (Vidyasagar et al., 2012), while its involvement in lipid clearance was also documented (Na et al.,

2018). Both HSPB1 and HSPB2 were observed to form large multimeric complexes that were involved in preventing protein aggregation and maintaining cytoskeletal integrity during heat stress (Georg et al, 2020). In addition to HSP-related changes, immune response modifications were detected through the upregulation of the RAG2 gene in heat-stressed breeder hens' jejunal tissue. The gene's involvement in V(D)J (recombination and lymphocyte development was confirmed (Ru et al., 2015), suggesting its role in maintaining immune function under heat stress conditions. An indirect activation of PPAR-γ by RAG2 was also observed, which was associated with increased adipogenesis in the jejunum of heat-stressed breeder hens (Gellert, 2007). These molecular adaptations were found to be more pronounced in HA breeds compared to HS breeds, indicating the development of a more sophisticated cellular protection system in the former group. The observed changes were noted to encompass multiple aspects of cellular function, including protein stability maintenance, metabolic regulation, and immune system functionality.

Heat stress has emerged as a critical factor compromising intestinal barrier integrity in poultry production (Song et al., 2014). The intestinal barrier is maintained by tight junction proteins such as occludin (OCLN) and claudin (CLDN), which regulate paracellular permeability and are essential for maintaining gut health (Lee, 2015). Our transcriptomic analysis revealed that claudin 15 (CLDN15) gene expression was significantly downregulated (FC = −3.74) in the jejunal mucosa of heat-stressed breeder hens. CLDN15 serves as a critical tight junction protein that forms a cation-selective channel, facilitating Na+-dependent nutrient transport and maintaining Na+ homeostasis (Wada et al., 2013). This Na+ gradient is fundamental for various transport processes, indicating that Na+-dependent uptake of bile acids into enterocytes (Keating and Keely, 2009) and the absorption of essential nutrients such as glucose and amino acids (Nakayama et al., 2020). Moreover, CLDN15 has been shown to promote the proliferation of intestinal cryptic cells (Tamura et al., 2008), which are vital for continuous epithelial renewal and barrier maintenance. The heat stress-induced downregulation of CLDN15 suggests a compromised intestinal barrier function that may significantly impair nutrient absorption and utilization in breeder hens.

Heat stress effects extend beyond barrier functions to impact lipid metabolism and transport mechanisms in the intestine (Goel et al., 2021). Fatty acid-binding

proteins (FABPs) are crucial facilitators of long-chain fatty acid uptake and transport from intestinal chyme into intestinal epithelial cells, where they support triglyceride synthesis (Prows et al., 1995). Our findings demonstrated significant downregulation (FC = -2.42) of liver basic fatty acid-binding protein (LBFABP) in the jejunal mucosa of heat-stressed breeder hens, aligning with previous studies reporting decreased FABP expression in heat-stressed chicken intestine (Sun et al., 2015; Al-Zghoul et al., 2019). While LBFABP (also known as FABP10) is dominantly expressed in liver tissue (Murai et al., 2009), its presence in intestinal tissue plays a vital role in the efflux and transport of various lipids, including cholesterol and bile acids (Nichesola et al., 2004; McIntosh et al., 2012). Recent research has further emphasized LBFABP's significance in energy and lipid metabolism (Sun et al., 2023). Heat stress compromises intestinal barrier integrity in broilers (Song et al., 2013). The downregulation of LBFABP expression during heat stress can be attributed to heat-induced structural damage to the intestine epithelial and subsequent cell loss (Garriga et al., 2006). This damage disrupts both lipid re-esterification within intestinal cells and their transport through the lymphatic system, reducing long-chain fatty acid absorption, decreased plasma triglyceride levels, and compromised energy availability in chickens (Xie et al., 2015). The reduced lipid absorption capacity in the jejunum, caused by LBFABP downregulation, potentially creates an energy deficit that threatens metabolic homeostasis in breeder hens. Furthermore, the compromised intestinal barrier function may exacerbate these effects by allowing increased translocation of harmful substances into the bloodstream (Schreier et al., 2022). Further research directions should focus on elucidating the molecular mechanism underlying heat stress-induced LBFABP downregulation and developing targeted nutritional or management interventions to mitigate these effects. Understanding these mechanisms could lead to more effective strategies for maintaining intestinal function and barrier integrity during periods of heat stress, ultimately improving the productivity and welfare of breeder hens.

GO analysis of transcriptomic data revealed significant enrichment in 555 GO terms in the jejunal mucosa of heat-stressed breeder hens. Three GO terms- response to heat, extracellular space, and extracellular region were particularly noteworthy, as they align with findings from Kim et al. (2022) in their analysis of chronic heat stress responses in hen jejunal mucosa. In addition, the comparison between HA and HS hens

highlighted GO terms related to fructose and glucose transmembrane transport, brush-border membrane, and motor activity in the jejunum. These findings are particularly relevant given that the jejunum is the primary site for the absorption of amino acids, carbohydrates, and fatty acids (Montoro-Huguet et al., 2021; McQuilken, 2024), with these molecules being transported across the intestinal brush border membrane via specific transporters (Shibata et al., 2020).

KEGG pathway analysis revealed six enriched pathways influenced by acute HS in the jejunal mucosal tissues of breeder hens (Table 4). Notable among these were the vascular endothelial growth factor (VEGF) signaling pathways and neuroactive ligand-receptor interaction, which have been previously identified in heat-stressed and immune-stressed broilers' jejunum (Kim et al., 2022; Hu et al., 2024). The VEGF signaling pathway plays a crucial role in maintaining metabolic homeostasis, cell proliferation, migration, and vascular architecture (Malila et al., 2024). Heat stress-induced intestinal mucosal damage leads to intestinal hypoxia and triggers VEGF regulation through hypoxia-inducible factor-1α (HIF-1α) activation (Li et al., 2024). While VEGF signaling promotes angiogenesis to restore oxygen and nutrient delivery to heat-stressed tissues, excessive VEGF activity under prolonged heat stress can lead to vascular hyperpermeability and enhanced tissue inflammation (Huang et al., 2017). In addition, VEGF signaling may suppress T cell development, potentially contributing to immune suppression under heat-stress conditions (Ohm et al., 2003).

The mitogen-activated protein kinase (MAPK) signaling pathway emerged as another significant pathway affected by acute heat stress in the jejunal mucosa, particularly differing between HA and HS breeder hens. This pathway’s activation during heat stress in the broiler jejunal has been previously documented (Huang et al., 2024). MAPK signaling regulates various physiological functions, including oxidative stress responses, inflammation, cell multiplication, apoptosis, and autophagy (Murai et al., 2010; Liu et al., 2022). Our analysis revealed the upregulation of two HSPs, HSPH1 and HSPA2, within the MAPK signaling pathway. HSPH1, a member of the Hsp110 family, shows increased expression to prevent cell death and promote survival under heat-stress conditions (Balakrishnan et al., 2023). Similarly, HSPA2, encoding an HSP70 family member, helps alleviate structural and oxidative damage to intestinal mucosa during heat stress (Hao et al., 2012). Previous research has shown that heat stress upregulates

HSP70 expression in chicken jejunal mucosa and activates the intestinal MAPK signaling pathway, suggesting a protective mechanism through MAPK signaling pathway activation (Yu et al., 2021). The coordinated upregulation of HSPH1 and HSPA2 represents an important adaptive response to heat stress, with HSPH1 promoting cell survival and HSPA2 mitigating intestinal mucosal damage through MAPK pathway activation. These molecular responses appear to be critical for heat stress adaptation in breeder hens. Furthermore, the potential roles of HSPA2 and HSPH1 in the modulation of immune function under heat stress conditions warrant further investigation, as their involvement in immune regulation may represent an important aspect of the heat stress responses (Beere, 2004).

PPI network analysis revealed several up-regulated cell cycle-related genes in the jejunum of heat-stressed breeder hens, including CDK1, PLK1, CDC7, and CDC20. Cyclin-dependent kinase 1 (CDK1), a key member of the cyclin-dependent kinase family, is a serine/threonine kinase that influences both the Wnt and fibroblast growth factor signaling pathways, thereby affecting cell proliferation (Yang et al, 2020; Wang et al., 2023; Liu et al., 2023). The polo-like kinase 1 gene (PLK1), another serine/threonine kinase, regulates cell division and DNA replication, with its overexpression enabling cells to bypass cell cycle checkpoints (Van Vugt et al., 2004). Through interaction with cell division cycle 7 (CDC7), PLK1 induces diaphragm formation and mitotic exit (Donaldson et al., 2001). CDC7 serves as a critical cell cycle regulator, as demonstrated in studies showing that its inactivation leads to S-phase arrest and P53-dependent apoptosis in mouse embryonic stem cell culture (Kim, 2002). Notably, previous research in Illinois broilers under heat stress also reported upregulation of PLK1, CDC7, and CDC20 (Zhang et al., 2017), suggesting a conserved response to heat stress across different chicken breeds. These findings suggest that PLK1, CDC7, and CDC20 play pivotal roles in cellular adaptation to heat-induced DNA damage, with increased expression of polo-like kinases between HA and HS breeder hens potentially reducing cell-cycle arrest and apoptosis under heat stress. The second largest network identified comprised 13 upregulated genes, with HSPA2 and interleukin-6 (IL6) emerging as key core nodes related to the immune response, showing 6 and 5 interactions, respectively. HSPs are intricately linked to immune system functions (Tsan and Gao, 2009). In particular, HSP70 (HSPA2) induces calcium flux, exhibits high-affinity binding to the plasma membrane, and activates

nuclear factor (NF)-κB (Asea et al., 2000). The pro-inflammatory cytokine IL6 plays an important role in innate and acquired immunity (Wigley and Kaiser, 2003). Multiple studies have indicated increased IL6 expression following heat stress exposure (Varasteh et al., 2015), including elevated levels in the jejunal mucosae of thermal manipulation chicks under chronic heat stress (Al-Zghoul and Mohammad Saleh, 2020). The relationship between HSPA2 and IL-6 is particularly noteworthy. While elevated IL-6 levels may be associated with increased HSPA2 expression, the upregulation of HSP70 serves as a protective mechanism by inhibiting pro-inflammatory cytokine expression (Yoo et al., 2000; Stocki and Dickinson, 2012). Research has shown that heat shock factor (HSF) induces both HSP70 and IL-6 expression in heat-stressed chickens, suggesting IL-6 may act as a heat-shock gene (Prakasam et al., 2013). These findings indicate that heat stress suppresses innate immunity in the jejunal mucosa of breeder hens while simultaneously triggering protective mechanisms through HSPA2 upregulation. The concurrent elevation of IL6 expression suggests an inflammatory response, likely resulting from heat-induced tissue damage.

In addition, PPI analysis highlighted a network of ten downregulated DEGs associated with nutrient transport and metabolism, such as SLC22A13L, LBFABP, SLC2A2, and SLC6A19, consistent with previous reports of reduced nutrient absorption and transport gene expression in heat-stressed animals (Sun et al., 2015). Solute carrier family 22 member 13 (SLC22A13L), also known as organic anion transporter 10 (OAT10) (Vávra et al., 2024), is predominantly expressed in the apical membrane of proximal tubules in the kidneys. This transporter mediates urate reabsorption through the exchange of organic anions such as urate, nicotinate, and orotate for OH-anions or organic anions like lactate (Bahn et al., 2008; Toyoda et al., 2022). The downregulation of SLC22A13L in heat-stressed chicken jejunum may disrupt ion transport balance and metabolic regulation, potentially compromising nutrient absorption, cellular function, and tissue homeostasis (Garriga et al., 2006). This reduction could represent a protective mechanism to minimize cellular damage from oxidative stress or impaired cellular metabolism. The glucose transport gene SLC2A2 (GLUT2) plays a role in glucosamine transport necessary for glycosaminoglycan biosynthesis (Uldry et al., 2002). Previous research in broiler jejunum has demonstrated reduced GLUT2 expression under heat stress (Sun et al., 2015), suggesting disrupted intestinal glucose

transport. Similarly, solute carrier family 6 member 19 (SLC6A19), located in the apical membrane, encodes the B0AT protein responsible for high-affinity amino acid transport through electroneutral exchange coupled with the sodium co-transport (Bröer, 2008). The reduction in SLC6A19 expression under heat stress conditions may result in decreased amino acid levels in the jejunal apical membrane. These findings suggest that the downregulation of nutrient transport and metabolism genes in heat-stressed breeder hen jejunum may lead to impaired nutrient absorption, metabolic regulation, and cellular function. Additional research is needed to elucidate the precise mechanisms governing transporter regulation under heat stress conditions.

4.6 Conclusion

In this study, we identified 155 DEGs that were up-regulated and 128 DEGs that were down-regulated in the jejunal mucosa tissue between HA and HS breeder hens under heat stress using RNA-seq. Twelve DEGs associated with HSP, immune response, intestinal barrier integrity, lipids, organic acid, glucose, and amino acids transport, including HSPB9, HSPA2, HSPB1, RAG2, IL6, IL-18BP, CLDN15, LBFABP, CD36, SLC22A13L, SLC2A2, and SLC6A19 may play key roles in the regulation of jejunal mucosa of breeder hens under acute heat stress. The identified DEGs are associated with key processes related to response to heat, cell division, and glucose and amino acids transport. KEGG pathway enrichment analysis revealed that the main biological pathways were related to the VEGF signaling pathway, MAPK signaling pathway, cell adhesion molecules, neuroactive ligand-receptor interaction, and cell cycle. KEGG pathway and PPI analyses showed that acute heat stress may affect the cell cycle, immunity, and organic acid, glucose, and amino acids transport in the jejunal mucosa of breeder hens and that heat-stressed hens increase the expression of HSPs as a protective mechanism for their cells. The identified key pathways and candidate genes can be used as indicators to monitor acute HS responses in breeder hens and may inform strategies for developing heat-tolerant strains.

4.7 References

Al-Zghoul, M. B., Alliftawi, A. R. S., Saleh, K. M. M., & Jaradat, Z. W. (2019). Expression of digestive enzyme and intestinal transporter genes during chronic heat stress in the thermally manipulated broiler chicken. **Poultry Science**, 98(9), 4113–4122.

Al-Zghoul, M. B., & Mohammad Saleh, K. M. (2020). Effects of thermal manipulation of eggs on the response of jejunal mucosae to posthatch chronic heat stress in broiler chickens. **Poultry Science**, 99(5), 2727–2735.

Al-Zghoul, M. B., Sukker, H., & Ababneh, M. M. (2019). Effect of thermal manipulation of broilers embryos on the response to heat-induced oxidative stress. **Poultry Science**, 98(2), 991–1001.

Asea, A., Kraeft, S.-K., Kurt-Jones, E. A., Stevenson, M. A., Chen, L. B., Finberg, R. W., Koo, G. C., & Calderwood, S. K. (2000). HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. **Nature Medicine**, 6(4), 435–442.

Bahn, A., Hagos, Y., Reuter, S., Balen, D., Brzica, H., Krick, W., Burckhardt, B. C., Sabolić, I., & Burckhardt, G. (2008). Identification of a New Urate and High Affinity Nicotinate Transporter, hOAT10 (SLC22A13). **Journal of Biological Chemistry**, 283(24), 16332–16341.

Balakrishnan, K. N., Ramiah, S. K., & Zulkifli, I. (2023). Heat Shock Protein Response to Stress in Poultry: A Review. **Animals**, 13(2), 317.

Beere, H. M. (2004). `The stress of dying': The role of heat shock proteins in the regulation of apoptosis. **Journal of Cell Science**, 117(13), 2641–2651.

Berkmen, Y. M., & Lande, A. (1975). Chest roentgenography as a window to the diagnosis of Takayasu's arteritis. **The American Journal of Roentgenology, Radium Therapy, and Nuclear Medicine**, 125(4), 842–846.

Brake, J. (1998). Optimum dietary arginine: Lysine ratio for broiler chickens is altered during heat stress in association with changes in intestinal uptake and dietary sodium chloride. **British Poultry Science**, 39(5), 639–647.

Cahaner, A. (2008). Breeding fast-growing, high-yield broilers for hot conditions. In N. J. Daghir (Ed.), Poultry production in hot climates (2nd ed., pp. 30–47). **CABI**.

Chauhan, S. S., Rashamol, V. P., Bagath, M., Sejian, V., & Dunshea, F. R. (2021). Impacts of heat stress on immune responses and oxidative stress in farm animals and

nutritional strategies for amelioration. **International Journal of Biometeorology**, 65(7), 1231–1244.

Donaldson, M. M., Tavares, A. A. M., Hagan, I. M., Nigg, E. A., & Glover, D. M. (2001). The mitotic roles of Polo-like kinase. **Journal of Cell Science**, 114(13), 2357–2358.

Farag, M. R., & Alagawany, M. (2018). Erythrocytes as a biological model for screening of xenobiotic toxicity. **Chemico-Biological Interactions**, 279, 73–83.

Garriga, C., Hunter, R. R., Amat, C., Planas, J. M., Mitchell, M. A., & Moretó, M. (2006). Heat stress increases apical glucose transport in the chicken jejunum. **American Journal of Physiology-Regulatory, Integrative and Comparative Physiology**, 290(1), R195–R201.

Gellert, M. (2007). V(D)J Recombination: Mechanism and Consequences. In A. Aguilera & R. Rothstein (Eds.), **Molecular Genetics of Recombination** (Vol. 17, pp. 469–486). Springer Berlin Heidelberg.

Georg, R. C., Oshiquiri, L. H., Barbosa-Filho, J. R., & Gomes, S. L. (2020). Small heat shock protein genes are developmentally regulated during stress and non-stress conditions in Blastocladiella emersonii. **Fungal Biology**, 124(5), 482–489.

Goel, A., Ncho, C. M., & Choi, Y.-H. (2021). Regulation of gene expression in chickens by heat stress. **Journal of Animal Science and Biotechnology**, 12(1), 11.

Gupta, S. C., Sharma, A., Mishra, M., Mishra, R. K., & Chowdhuri, D. K. (2010). Heat shock proteins in toxicology: How close and how far? **Life Sciences**, 86(11–12), 377–384.

Habashy, W. S., Milfort, M. C., Adomako, K., Attia, Y. A., Rekaya, R., & Aggrey, S. E. (2017). Effect of heat stress on amino acid digestibility and transporters in meat-type chickens. **Poultry Science**, 96(7), 2312–2319.

Hao, Y., Gu, X. H., & Wang, X. L. (2012). Overexpression of heat shock protein 70 and its relationship to intestine under acute heat stress in broilers: 1. Intestinal structure and digestive function. **Poultry Science**, 91(4), 781–789.

Hu, W., Du, L., Shao, J., Qu, Y., Zhang, L., Zhang, D., Cao, L., Chen, H., & Bi, S. (2024). Molecular and metabolic responses to immune stress in the jejunum of broiler chickens: Transcriptomic and metabolomic analysis. **Poultry Science**, 103(5), 103621.

Huang, L., Cao, C., Lin, X., Lu, L., Lin, X., Liu, H.-C., Odle, J., See, M. T., Zhang, L., Wu, W., Luo, X., & Liao, X. (2024). Zinc alleviates thermal stress-induced damage to the integrity and barrier function of cultured chicken embryonic primary jejunal epithelial cells via the MAPK and PI3K/AKT/mTOR signaling pathways. **Poultry Science**, 103(6), 103696.

Huang, S., Rehman, M. U., Lan, Y., Qiu, G., Zhang, H., Iqbal, M. K., Luo, H., Mehmood, K., Zhang, L., & Li, J. (2017). Tibial dyschondroplasia is highly associated with suppression of tibial angiogenesis through regulating the HIF-1α/VEGF/VEGFR signaling pathway in chickens. **Scientific Reports**, 7(1), 9089.

Johnston, C. L., Marzano, N. R., Paudel, B. P., Wright, G., Benesch, J. L. P., Van Oijen, A. M., & Ecroyd, H. (2021). Single-molecule fluorescence-based approach reveals novel mechanistic insights into human small heat shock protein chaperone function. **Journal of Biological Chemistry**, 296, 100161.

Katemala, S., Molee, A., Thumanu, K., & Yongsawatdigul, J. (2022). A comparative study of meat quality and vibrational spectroscopic properties of different chicken breeds. **Poultry Science**, 101(6), 101829.

Keating, N., & Keely, S. J. (2009). Bile acids in regulation of intestinal physiology. **Current Gastroenterology Reports**, 11(5), 375–382.

Kim, D. Y., Lim, B., Kim, J.-M., & Kil, D. Y. (2022). Integrated transcriptome analysis for the hepatic and jejunal mucosa tissues of broiler chickens raised under heat stress conditions. **Journal of Animal Science and Biotechnology**, 13(1), 79.

Kim, J. M. (2002). Inactivation of Cdc7 kinase in mouse ES cells results in S-phase arrest and p53-dependent cell death. **The EMBO Journal**, 21(9), 2168–2179.

Lee, S. H. (2015). Intestinal Permeability Regulation by Tight Junction: Implication on Inflammatory Bowel Diseases. **Intestinal Research**, 13(1), 11.

Li, H., Zhang, G., Liu, Y., Gao, F., Ye, X., Lin, R., & Wen, M. (2024). Hypoxia-inducible factor 1α inhibits heat stress-induced pig intestinal epithelial cell apoptosis through eif2α/ATF4/CHOP signaling. **Science of The Total Environment**, 924, 171649.

Liu, J., Zhao, Z. X., Li, B. K., & Zhao, Z. W. (2023). GRIK3 deficiency promotes non-small cell lung cancer progression by the regulation of the UBE2C/CDK1/Wnt signaling pathway. **American Journal of Cancer Research**, 13(11), 2066–2075.

Liu, L., Zhao, L., Liu, Y., Yu, X., & Qiao, X. (2022). Rutin Ameliorates Cadmium-Induced Necroptosis in the Chicken Liver via Inhibiting Oxidative Stress and MAPK/NF-**Κ**B Pathway. **Biological Trace Element Research**, 200(4), 1799–1810.

Madara, J. L. (1991). Functional Morphology of Epithelium of the Small Intestine. In R. Terjung (Ed.), Comprehensive Physiology (1st ed., pp. 83–120). **Wiley**.

Malila, Y., Uengwetwanit, T., Sanpinit, P., Songyou, W., Srimarut, Y., & Kunhareang, S. (2024). Thermal impacts on transcriptome of Pectoralis major muscle collected from commercial broilers, Thai native chickens and its crossbreeds. **Animal Bioscience**, 37(1), 61–73.

McIntosh, A. L., Atshaves, B. P., Storey, S. M., Landrock, K. K., Landrock, D., Martin, G. G., Kier, A. B., & Schroeder, F. (2012). Loss of liver FA binding protein significantly alters hepatocyte plasma membrane microdomains. **Journal of Lipid Research**, 53(3), 467–480.

McQuilken, S. A. 2024. Digestion and absorption. **Anaesthesia and Intensive Care**, 25(6), 293–296.

Montoro-Huguet, M. A., Belloc, B., & Domínguez-Cajal, M. (2021). Small and Large Intestine (I): Malabsorption of Nutrients. **Nutrients**, 13(4), 1254.

Murai, A., Furuse, M., Kitaguchi, K., Kusumoto, K., Nakanishi, Y., Kobayashi, M., & Horio, F. (2009). Characterization of critical factors influencing gene expression of two types of fatty acid-binding proteins (L-FABP and Lb-FABP) in the liver of birds. Comparative Biochemistry and Physiology Part A: **Molecular & Integrative Physiology**, 154(2), 216–223.

Murai, H., Hiragami, F., Kawamura, K., Motoda, H., Koike, Y., Inoue, S., Kumagishi, K., Ohtsuka, A., & Kano, Y. (2010). Differential Response of Heat-Shock-Induced p38 MAPK and JNK Activity in PC12 Mutant and PC12 Parental Cells for Differentiation and Apoptosis (1). **Okayama University Medical School**.

Na, W., Wu, Y.-Y., Gong, P.-F., Wu, C.-Y., Cheng, B.-H., Wang, Y.-X., Wang, N., Du, Z.-Q., & Li, H. (2018). Embryonic transcriptome and proteome analyses on hepatic lipid

metabolism in chickens divergently selected for abdominal fat content. **BMC Genomics**, 19(1), 384.

Nakayama, M., Ishizuka, N., Hempstock, W., Ikari, A., & Hayashi, H. (2020). Na+-Coupled Nutrient Cotransport Induced Luminal Negative Potential and Claudin-15 Play an Important Role in Paracellular Na+ Recycling in Mouse Small Intestine. **International Journal of Molecular Sciences**, 21(2), 376.

Nichesola, D., Perduca, M., Capaldi, S., Carrizo, M. E., Righetti, P. G., & Monaco, H. L. (2004). Crystal Structure of Chicken Liver Basic Fatty Acid-Binding Protein Complexed with Cholic Acid. **Biochemistry**, 43(44), 14072–14079.

Ohm, J. E., Gabrilovich, D. I., Sempowski, G. D., Kisseleva, E., Parman, K. S., Nadaf, S., & Carbone, D. P. (2003). VEGF inhibits T-cell development and may contribute to tumor-induced immune suppression. **Blood**, 101(12), 4878–4886.

Pareek, C., Sachajko, M., Jaskowski, J., Herudzinska, M., Skowronski, M., Domagalski, K., Szczepanek, J., Czarnik, U., Sobiech, P., Wysocka, D., Pierzchala, M., Polawska, E., Stepanow, K., Ogłuszka, M., Juszczuk-Kubiak, E., Feng, Y., & Kumar, D. (2019). Comparative Analysis of the Liver Transcriptome among Cattle Breeds Using RNA-seq. **Veterinary Sciences**, 6(2), 36.

Pearce, S. C., Mani, V., Weber, T. E., Rhoads, R. P., Patience, J. F., Baumgard, L. H., & Gabler, N. K. (2013). Heat stress and reduced plane of nutrition decreases intestinal integrity and function in pigs1. **Journal of Animal Science**, 91(11), 5183–5193.

Perini, F., Cendron, F., Rovelli, G., Castellini, C., Cassandro, M., & Lasagna, E. (2020). Emerging Genetic Tools to Investigate Molecular Pathways Related to Heat Stress in Chickens: A Review. **Animals**, 11(1), 46.

Prakasam, R., Fujimoto, M., Takii, R., Hayashida, N., Takaki, E., Tan, K., Wu, F., Inouye, S., & Nakai, A. (2013). Chicken IL-6 is a heat-shock gene. **FEBS Letters**, 587(21), 3541—3547.

Prows, D. R., Murphy, E. J., & Schroeder, F. (1995). Intestinal and liver fatty acid binding proteins differentially affect fatty acid uptake and esterification in L-cells. **Lipids**, 30(10), 907-910.

Rogalla, T., Ehrnsperger, M., Preville, X., Kotlyarov, A., Lutsch, G., Ducasse, C., Paul, C., Wieske, M., Arrigo, A.-P., Buchner, J., & Gaestel, M. (1999). Regulation of Hsp27 Oligomerization, Chaperone Function, and Protective Activity against Oxidative Stress/Tumor Necrosis Factor α by Phosphorylation. **Journal of Biological Chemistry**, 274(27), 18947–18956.

Ru, H., Chambers, M. G., Fu, T.-M., Tong, A. B., Liao, M., & Wu, H. (2015). Molecular Mechanism of V(D)J Recombination from Synaptic RAG1-RAG2 Complex Structures. **Cell**, 163(5), 1138–1152.

Sadr, A. S., Nassiri, M., Ghaderi-Zefrehei, M., Heidari, M., Smith, J., & Muhaghegh Dolatabady, M. (2023). RNA-Seq Profiling between Commercial and Indigenous Iranian Chickens Highlights Differences in Innate Immune Gene Expression. **Genes**, 14(4), 793.

Santoro, M. G. (2000). Heat shock factors and the control of the stress response. **Biochemical Pharmacology**, 59(1), 55–63.

Schreier, J., Rychlik, I., Karasova, D., Crhanova, M., Breves, G., Rautenschlein, S., & Jung, A. (2022). Influence of heat stress on intestinal integrity and the caecal microbiota during Enterococcus cecorum infection in broilers. **Veterinary Research**, 53(1), 110.

Shashank, C. G., Sejian, V., Silpa, M. V., Devaraj, C., Madhusoodan, A. P., Rebez, E. B., Kalaignazhal, G., Sahoo, A., & Dunshea, F. R. (2024). Climate Resilience in Farm Animals: Transcriptomics-Based Alterations in Differentially Expressed Genes and Stress Pathways. **BioTech**, 13(4), 49.

Shibata, M., Takahashi, T., Endo, K., Kozakai, T., Azuma, Y., & Kurose, T. (2020). Age-related Regulation of Active Amino Acid Transport in the Ileum of Broiler Chickens. J. **Poultry Science**, 57(6), 131–137.

Soleimani, A. F., & Zulkifli, I. (2010). Effects of High Ambient Temperature on Blood Parameters in Red Jungle Fowl, Village Fowl and Broiler Chickens. **Journal of Animal and Veterinary Advances**, 9(14), 1201–1207.

Song, J., Jiao, L. F., Xiao, K., Luan, Z. S., Hu, C. H., Shi, B., & Zhan, X. A. (2013). Cello-oligosaccharide ameliorates heat stress-induced impairment of intestinal microflora, morphology and barrier integrity in broilers. **Animal Feed Science and Technology**, 185(3–4), 175–181.

Song, J., Xiao, K., Ke, Y. L., Jiao, L. F., Hu, C. H., Diao, Q. Y., Shi, B., & Zou, X. T. (2014). Effect of a probiotic mixture on intestinal microflora, morphology, and barrier integrity of broilers subjected to heat stress. **Poultry Science**, 93(3), 581–588.

Stocki, P., & Dickinson, A. M. (2012). The Immunosuppressive Activity of Heat Shock Protein 70. **Autoimmune Diseases**, 2012, 1–6.

Sun, X., Wang, Y., Wang, C., Wang, Y., Ren, Z., Yang, X., Yang, X., & Liu, Y. (2023). Genome analysis reveals hepatic transcriptional reprogramming changes mediated by enhancers during chick embryonic development. **Poultry Science**, 102(4), 102516.

Sun, X., Zhang, H., Sheikhahmadi, A., Wang, Y., Jiao, H., Lin, H., & Song, Z. (2015). Effects of heat stress on the gene expression of nutrient transporters in the jejunum of broiler chickens (Gallus gallus domesticus). **International Journal of Biometeorology**, 59(2), 127–135.

Tamura, A., Kitano, Y., Hata, M., Katsuno, T., Moriwaki, K., Sasaki, H., Hayashi, H., Suzuki, Y., Noda, T., Furuse, M., Tsukita, S., & Tsukita, S. (2008). Megaintestine in Claudin-15–Deficient Mice. **Gastroenterology**, 134(2), 523-534.e3.

Toyoda, Y., Kawamura, Y., Nakayama, A., Morimoto, K., Shimizu, S., Tanahashi, Y., Tamura, T., Kondo, T., Kato, Y., Ichida, K., Suzuki, H., Shinomiya, N., Kobayashi, Y., Takada, T., & Matsuo, H. (2022). OAT10/SLC22A13 Acts as a Renal Urate Re-Absorber: Clinico-Genetic and Functional Analyses with Pharmacological Impacts. **Frontiers in Pharmacology**, 13, 842717.

Tsan, M.-F., & Gao, B. (2009). Heat shock proteins and immune system. **Journal of Leukocyte Biology**, 85(6), 905–910.

Uldry, M., Ibberson, M., Hosokawa, M., & Thorens, B. (2002). GLUT2 is a high affinity glucosamine transporter. **FEBS Letters**, 524(1–3), 199–203.

Van Vugt, M. A. T. M., Brás, A., & Medema, R. H. (2004). Polo-like Kinase-1 Controls Recovery from a G2 DNA Damage-Induced Arrest in Mammalian Cells. **Molecular Cell**, 15(5), 799–811.

Varasteh, S., Braber, S., Akbari, P., Garssen, J., & Fink-Gremmels, J. (2015). Differences in Susceptibility to Heat Stress along the Chicken Intestine and the Protective Effects of Galacto-Oligosaccharides. **PLOS ONE**, 10(9), e0138975.

Varasteh, S., Braber, S., Garssen, J., & Fink-Gremmels, J. (2015). Galacto-oligosaccharides exert a protective effect against heat stress in a Caco-2 cell model. **Journal of Functional Foods**, 16, 265–277.

Vávra, J., Pavelcová, K., Mašínová, J., Hasíková, L., Bubeníková, E., Urbanová, A., Mančíková, A., & Stibůrková, B. (2024). Examining the Association of Rare Allelic Variants in Urate Transporters SLC22A11, SLC22A13, and SLC17A1 with Hyperuricemia and Gout. **Disease Markers**, 2024, 1–16.

Vidyasagar, A., Wilson, N. A., & Djamali, A. (2012). Heat shock protein 27 (HSP27): Biomarker of disease and therapeutic target. **Fibrogenesis & Tissue Repair**, 5(1), 7.

Wada, M., Tamura, A., Takahashi, N., & Tsukita, S. (2013). Loss of Claudins 2 and 15 From Mice Causes Defects in Paracellular Na+ Flow and Nutrient Transport in Gut and Leads to Death from Malnutrition. **Gastroenterology**, 144(2), 369–380.

Wang, L., Xu, X., Jiang, Z., & You, Q. (2020). Modulation of protein fate decision by small molecules: Targeting molecular chaperone machinery. **Acta Pharmaceutica Sinica B**, 10(10), 1904–1925.

Wang, Q., Bode, A. M., & Zhang, T. (2023). Targeting CDK1 in cancer: Mechanisms and implications. **Npj Precision Oncology**, 7(1), 58.

Wang, Z., Shao, D., Kang, K., Wu, S., Zhong, G., Song, Z., & Shi, S. (2022). Low protein with high amino acid diets improves the growth performance of yellow feather broilers by improving intestinal health under cyclic heat stress. **Journal of Thermal Biology**, 105, 103219.

Wang, Z., Shao, D., Wu, S., Song, Z., & Shi, S. (2022). Heat stress-induced intestinal barrier damage and dimethylglycine alleviates via improving the metabolism function of microbiota gut brain axis. **Ecotoxicology and Environmental Safety**, 244, 114053.

Wigley, P., & Kaiser, P. (2003). Avian cytokines in health and disease. **Revista Brasileira de Ciência Avícola**, 5(1), 1–14.

Xie, J., Tang, L., Lu, L., Zhang, L., Lin, X., Liu, H. C., Odle, J., & Luo, X. (2015). Effects of acute and chronic heat stress on plasma metabolites, hormones, and oxidant status in restrictedly fed broiler breeders. **Poultry Science**, 94(7), 1635–1644.

Yang, B., Gao, Y., Xi, K., Wang, H., Yan, M., Sun, H., Lin, Y., Zheng, X., Li, Y., Guo, S., & Liu, C. (2024). Effects of Ban Lian Zi Jin San on intestinal inflammation and barrier function of heat-stressed broilers. **Poultry Science**, 103(3), 103425.

Yang, Y., Zhu, X., Jia, X., Hou, W., Zhou, G., Ma, Z., Yu, B., Pi, Y., Zhang, X., Wang, J., & Wang, G. (2020). Phosphorylation of Msx1 promotes cell proliferation through the Fgf9/18-MAPK signaling pathway during embryonic limb development. **Nucleic Acids Research**, 48(20), 11452–11467.

Yoo, C. G., Lee, S., Lee, C. T., Kim, Y. W., Han, S. K., & Shim, Y.-S. (2000). Anti-Inflammatory Effect of Heat Shock Protein Induction Is Related to Stabilization of IκBα Through Preventing IκB Kinase Activation in Respiratory Epithelial Cells. **The Journal of Immunology**, 164(10), 5416–5423.

Yu, Z., Tian, J., Wen, J., & Chen, Z. (2021). Effects of Heat Stress on Expression of Heat Shock Proteins in the Small Intestine of Wenchang Chicks. Brazilian Journal of **Poultry Science**, 23(3), eRBCA-2020-1430.

Yunis, R., & Cahaner, A. (1999). The effects of the naked neck (Na) and frizzle (F) genes on growth and meat yield of broilers and their interactions with ambient temperatures and potential growth rate. **Poultry Science**, 78(10), 1347–1352.

Zhang, J., Schmidt, C. J., & Lamont, S. J. (2017). Transcriptome analysis reveals potential mechanisms underlying differential heart development in fast- and slow-growing broilers under heat stress. **BMC Genomics**, 18(1), 295.

Zhang, S., Ou, J., Luo, Z., & Kim, I. H. (2020). Effect of dietary β-1,3-glucan supplementation and heat stress on growth performance, nutrient digestibility, meat quality, organ weight, ileum microbiota, and immunity in broilers. **Poultry Science**, 99(10), 4969–4977.

Zhu, Y., Kubota, S., Pasri, P., Rakngam, S., Okrathok, S., Pukkung, C., Yang, S., & Khempaka, S. (2025). Transcriptome analysis of jejunal mucosal tissue in breeder hens exposed to acute heat stress. **Poultry Science**, 104(1), 104532.