EFFICACY OF DIETARY ANTIOXIDANTS ON GUT FUNCTION AND HEALTH IN HEAT-STRESSED BREEDER HENS

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ผลของสารต้านอนุมูลอิสระในอาหารต่อการทำงานและสุขภาพลำไส้ในไก่แม่พันธุ์ที่เครียดจากความร้อน

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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คำสำคัญ: ความเครียดจากความร้อน/ไก่แม่พันธุ์/ทรานสคริปโตม/สารต้านอนุมูลอิสระในอาหาร/สุขภาพลำไส้

ความเครียดจากความร้อน ส่งผลกระทบต่อความแข็งแรงของเยื่อบุลำไส้และการดูดซึมสารอาหารของไก่แม่พันธุ์โดยเฉพาะในไก่แม่พันธุ์ที่ไวต่อความร้อน การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาการตอบสนองระดับโมเลกุลของลำไส้เล็กในไก่แม่พันธุ์ที่ทนและไวต่อความร้อนด้วยเทคนิคทรานสคริปโตมิกส์ และประเมินประสิทธิภาพของสารต้านอนุมูลอิสระสังเคราะห์ (ประกอบด้วยวิตามินซี วิตามินอี ซีลีเนียม และแอลคาร์นีทีน) และสารไฟโตจีนิก (ประกอบด้วยกานพลู กากชาเขียว และผักแพว) ในการลดผลกระทบจากความร้อน ประกอบด้วย 3 การทดลอง ดังนี้

การทดลองที่ 1 ประกอบด้วย 2 การทดลองย่อย การทดลองย่อยที่ 1 มีวัตถุประสงค์เพื่อศึกษาการตอบสนองระดับโมเลกุลของลำไส้เล็กต่อความร้อนแบบเฉียบพลันในไก่แม่พันธุ์ที่ไวต่อความร้อน ใช้ไก่อายุ 28 สัปดาห์ จำนวน 50 ตัว แบ่งเป็นกลุ่มควบคุม (23°C) และกลุ่มที่ได้รับความเครียดจากความร้อน (อุณหภูมิ 36°C เป็นเวลา 6 ชั่วโมง) กลุ่มละ 25 ตัว เลี้ยงแยกเดี่ยวในกรง ผลการศึกษาพบยีนที่มีการแสดงออกแตกต่างกันทั้งหมด 138 ยีน แบ่งเป็นยีนที่มีการแสดงออกเพิ่มขึ้น 75 ยีน และลดลง 63 ยีน การวิเคราะห์หน้าที่ของยีนด้วยการจัดจำแนกตาม gene ontology (GO) การวิเคราะห์ Kyoto Encyclopedia of Genes and Genomes (KEGG) และการวิเคราะห์เครือข่ายปฏิสัมพันธ์ของโปรตีน (protein interaction, PPI) พบยีนสำคัญหลายกลุ่ม ได้แก่ ยีนที่เกี่ยวข้องกับการตอบสนองต่อความร้อน (HSPA8 และ HSPA2) การรักษาสมดุลของพลังงานและเมแทบอลิซึมของไขมัน (PDK4 PPARA และ CD36) โดยยีน HSPA2 HSPB9 IL-18BP และ CD36 อาจใช้เป็นยีนเครื่องหมายของการตอบสนองต่อความเครียดจากความร้อนในเยื่อบุลำไส้เล็กส่วนกลางได้ งานทดลองย่อยที่ 2 เพื่อศึกษาการแสดงออกของยีนเครื่องหมาย (HSPA2 HSPB9 IL-18BP และ CD36) ในไก่แม่พันธุ์ที่ไวต่อความร้อนที่ได้รับสารต้านอนุมูลอิสระสังเคราะห์หรือไฟโตเจนนิก ใช้ไก่แม่พันธุ์อายุ 33 สัปดาห์ จำนวน 100 ตัว สุ่มเลี้ยงในสภาวะอุณหภูมิปกติ (23 °C) และสภาวะเครียดจากความร้อน (36°C 4 ชั่วโมง/วัน) แบ่งกลุ่มการทดลองออกเป็น 4 กลุ่ม คือ 1) อาหารพื้นฐานเลี้ยงภายใต้สภาวะปกติ กลุ่ม 2) อาหารพื้นฐาน เลี้ยงภายใต้สภาวะเครียดจากความร้อน 3) อาหารพื้นฐานเสริมสารต้านอนุมูลอิสระสังเคราะห์ ภายใต้สภาวะเครียดจากความร้อน และ 4) อาหารพื้นฐานเสริมด้วยสารต้านอนุมูลอิสระไฟโตเจนนิก ภายใต้สภาวะเครียดจากความร้อน ผลการทดลองพบว่า การ

เสริมสารต้านอนุมูลอิสระทั้งสองแหล่ง ส่งผลให้การแสดงออกของยีน CD36 ในลำไส้เล็กส่วนกลางเพิ่มขึ้น ขณะที่การแสดงออกของยีน HSPB9 HSPA2 และ IL18BP ลดลง เมื่อเปรียบเทียบกับกลุ่มที่ได้รับความเครียดจากความร้อนโดยไม่เสริมสารต้านอนุมูลอิสระ

การทดลองที่ 2 ใช้การวิเคราะห์ทรานสคริปโตมิกส์เพื่อเปรียบเทียบการแสดงออกของยีนในลำไส้เล็กส่วนกลางของไก่แม่พันธุ์ที่ปรับตัวได้ดีต่อความร้อนและไก่แม่พันธุ์ที่ไวต่อความร้อนภายใต้สภาวะเครียดจากความร้อนแบบเฉียบพลัน (36°C เป็นเวลา 6 ชั่วโมง) ใช้ไก่อายุ 28 สัปดาห์ จำนวน 50 ตัว (25 ตัวต่อสายพันธุ์) พบยีนที่แสดงออกแตกต่างกัน 284 ยีน (เพิ่มขึ้น 155 ยีน และลดง 129 ยีน) การวิเคราะห์ GO พบการเพิ่มขึ้นอย่างมีนัยสำคัญใน 555 หมวดหมู่ ขณะที่การวิเคราะห์ KEGG พบยีนที่มีการแสดงออกเพิ่มขึ้นเกี่ยวข้องการส่งสัญญาณ VEGF และ MAPK การสังเคราะห์สตียรอยด์ การปฏิสัมพันธ์ระหว่างลิแกนด์และตัวรับในระบบประสาท และวัฏจักรของเซลล์ ส่วนยีนที่ลดการแสดงออกเกี่ยวข้องกับโมเลกุลการยึดเกาะระหว่างเซลล์ จากการวิเคราะห์ PI พบว่า PLK1 CDC7 CDC20 HSPA2 IL6 SLC22A19A LBFABP และ SLC2A2 เป็นยีนหลักที่มีบทบาทสำคัญในการควบคุมผลกระทบความเครียดจากความร้อนต่อการแบ่งเซลล์ การทำงานของระบบภูมิคุ้มกัน กระบวนการเมแทบลิซึมของพลังงานและไขมัน และการขนส่งสารอาหาร

การทดลองที่ 3 ใช้ไก่แม่พันธุ์ที่ไวต่อความร้อน จำนวน 100 ตัว อายุ 38-52 สัปดาห์ (ใช้ไก่ชุดเดียวกันกับการทดลองย่อยที่ 2) พบว่าในสภาวะเครียดจากความร้อน สารต้านอนุมูลอิสระทั้งสองแหล่งช่วยเพิ่มการแสดงออกของยีนที่เกี่ยวกับสารต้านอนุมูลอิสระ (SOD and GSH-Px) โปรตีนไทด์จังชัน (CLDN1) และไซโตไคน์ต้านการอักเสบ (IL-10) ในลำไส้เล็กส่วนกลาง รวมทั้งเพิ่มความเข้มข้นของกรดไขมันสายสั้นรวม และลดการแสดงออกของยีนที่เกี่ยวข้องกับโปรตีนฮีทช็อก (HSP70 และ HSP90) ภูมิคุ้มกัน (IL-6, TNF-**α**, NF-**K**B และ TLR4) และการผลิตแอมโมเนีย นอกจากนี้ยังพบการเสริมสารต้านอนุมูลอิสระทำให้แบคทีเรียที่ผลิตกรดไขมันสายสั้น เช่น *Firmicutes Lachnospiraceae Ruminococcaceae* และ *Megamonas* เพิ่มขึ้นเมื่อเปรียบเทียบกับกลุ่มที่ไม่ได้รับการเสริม

โดยสรุป การศึกษาครั้งนี้ให้ข้อมูลเชิงลึกที่มีคุณค่าเกี่ยวกับการตอบสนองระดับโมเลกุลของไก่แม่พันธุ์ต่อความเครียดจากความร้อน และแสดงให้เห็นถึงศักยภาพของสารต้านอนุมูลอิสระทั้งชนิดสังเคราะห์และไฟโตจีนิกในการลดผลกระทบจากความเครียดจากความร้อนในสัตว์ปีก

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Heat stress (HS) negatively affects intestinal integrity and nutrient absorption in breeder hens. This study aimed to explore the molecular response of the small intestine in heat-adapted and heat-sensitive breeder hens using transcriptomic techniques, and to evaluate the efficiacy of synthetic (a combination of vitamin E, vitamin C, Se, and L-carnitine) and phytogenic (a combination of clove, green tea pomace, and Vietnamese coriander) antioxidants in mitigating the adverse effects of HS. This study was comprised of three experiments as follows:

Experiment 1 consisted of two trials. Trial 1: This trial aimed to explore the molecular response of the small intestine to acute HS in heat-sensitive breeder hens. A total of fifty hens, aged 28 weeks, were used and divided into two groups: a control group (maintained at 23°C) and a heat-treated group (exposed to 36°C for a 6-hour), with 25 birds each. A total of 138 differentially expressed genes (DEGs) were identified, comprising 75 upregulated and 63 downregulated genes. Functional analysis through gene ontology (GO) classification, pathway mapping via the Kyoto Encyclopedia of Genes and Genomes (KEGG), and protein interaction (PPI) networks revealed several key regulatory genes involved in thermal response (HSPA8 and HSPA2), and energy homeostasis and fat metabolism (PDK4, PPARA, and CD36). The following genes were identified as candidate biomarker genes in the jejunum for HS response: HSPA2, HSPB9, IL-18BP, and CD36. Trial 2 aimed to investigate the expression of candidate genes (HSPA2, HSPB9, CD36, and IL18BP) in heat-sensitive hens supplemented with synthetic or phytogenic antioxidants. One hundred hens, aged 33 weeks, were randomly housed under thermoneutral (TN; 23°C) or HS conditions (36°C, 4 h/d). The experiment was divided into four groups: 1) basal diet under TN; 2) basal diet under HS; 3) basal diet supplemented with synthetic antioxidants under HS; and 4) basal diet supplemented with phytogenic antioxidants under HS. Results showed that supplementation with

either antioxidant sources increased the expression of the CD36 gene in the jejunum, while the expression of the HSPB9, HSPA2, and IL18BP genes decreased compared to the HS group without supplementation.

Experiment 2, this study used transcriptomic analysis to compare gene expression jejunum of heat-adapted and heat-sensitive hens under acute HS (36°C for 6 hours). Fifty hens (28 week-old, 25 of each breed) were used. A total of 284 DEGs (155 upregulated and 129 downregulated genes) were identified. GO analysis revealed significant enrichment in 555 categories, while KEGG pathway revealed that upregulated genes were involved in VEGF signaling, MAPK signaling, steroid biosynthesis, neuroactive ligand-receptor interaction, and cell cycle pathways. Downregulated genes were related to cell adhesion molecules. The PPI analysis identified PLK1, CDC7, CDC20, HSPA2, IL6, SLC22A19A, LBFABP, and SLC2A2 as key core nodes, which may play crucial roles in regulating the effects of HS on cell division, immune function, energy and lipid metabolism, and nutrient transport.

Experiment 3: One hundred heat-sensitive hens aged 38 to 52 weeks (the same hens used in trial 2). The results showed that under HS condition, , both antioxidant sources increased the expression of genes related to antioxidants (SOD and GSH-Px), tight-junction protein (CLDN1), and anti-inflammatory cytokine (IL-10) in the jejunum. In addition, the total concentration of SCFAs increased, while the expression of HSPs (HSP70 and HSP90), immunity-related genes (IL-6, TNF-α, NF-κB, and TLR4), and ammonia production were reduced. Furthermore, antioxidant supplementation led to an increase in SCFA-producing bacteria, such as *Firmicutes*, *Lachnospiraceae*, *Ruminococcaceae*, and *Megamonas*, compared to the non-supplemented group.

In conclusion, this study provides valuable insights into the distinct molecular responses of breeder hens to HS. Both synthetic and phytogenic antioxidants demonstrate potential as strategic interventions to mitigate heat-induced damage in poultry.

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LIST OF ABBREVIATIONS

HS	=	Heat stress
TN	=	Thermoneutral zone
T	=	Treatment
RNA-seq	=	Transcriptome sequencing
mg	=	Milligram
µg	=	Microgram
µL	=	Microliter
g	=	Gram
min	=	Minute
s	=	Second
mL	=	Milliliter
°C	=	Degree Celsius
h	=	Hour
FC	=	Fold change
FPKM	=	Fragments per kilobase of transcript per million mapped reads
DEG	=	Differentially expressed gene
FC	=	Fold change
GO	=	Gene ontology
KEGG	=	Kyoto Encyclopedia of Genes and Genomes
PPI	=	Protein-Protein Interaction
PDK4	=	Pyruvate dehydrogenase kinase 4
HSPA2	=	Heat shock protein family A (Hsp70) member 2
HSPB9	=	Heat shock protein family B (small) member 9
DNAJA4	=	Heat shock 40kDa protein (HSP40)
IL18BP	=	Interleukin 18 binding protein
CD36	=	Cluster of differentiation 36
CLDN15	=	Claudin 15
RAG2	=	Recombination activating gene 2

LIST OF ABBREVIATIONS (Continued)

SOD	=	Superoxide dismutase
GSH-Px,	=	Glutathione peroxidase
HSP70	=	Heat shock protein 70
HSP90	=	Heat shock protein 90
IL6	=	Interleukin 6
IL10	=	Interleukin 10
TNF-α	=	Tumor necrosis factor-alpha
TLR4	=	Toll-like receptor 4
NF-ΚB	=	Nuclear factor-ΚB
CLND1	=	Claudin-1
ZO1	=	Zona occludens 1
GAPDH	=	Glyceraldehyde-3-phosphate dehydrogenase
SCFA	=	Short-chain fatty acid
PCoA	=	Principal coordinate analysis

CHAPTER I

INTRODUCTION

1.1 Introduction

The impact of heat stress (**HS**) on poultry is a major cause of economic losses in the poultry industry (Shehata et al., 2020). All homeothermic animals have a thermoneutral zone, and when the temperatures exceed this zone, animals cannot maintain a stable body temperature, resulting in HS. HS can be categorized as either acute (sudden, short-duration exposure to extremely high ambient temperatures and humidity) or chronic (extended period of elevated ambient temperatures with increased humidity) (Loyau et al., 2015). Both chronic and acute HS can compromise intestinal function through multiple mechanisms: reducing blood flow, increasing intestinal permeability, impairing intestinal barrier integrity, and causing microbiota dysbiosis, all of which affect nutrient digestion and absorption (Zhang et al., 2015; Gupta et al., 2017; Rostagno, 2020). In addition, HS triggers various pathophysiological changes, including immune dysregulation, overproduction of reactive oxygen species (ROS), decreased energy metabolism, and cell membrane lipid peroxidation. These changes ultimately increase susceptibility to pathogens and impair overall health (Liu et al., 2016; Shi et al., 2019). To combat the deleterious effects of HS in chickens, various genetic, management, and nutritional strategies have been implemented (Saeed et al., 2019). While genetic and management approaches may involve increased costs, technical complexity, and reduced genetic diversity, nutritional interventions offer a more feasible solution. These interventions, particularly the use of phytogenic compounds, vitamins (C and E), and minerals like selenium (Se), are gaining popularity as remedies for heat-stressed poultry (Kumar et al., 2021). Therefore, understanding the intrinsic physiological mechanisms of HS-induced intestinal damage in poultry and investigating the anti-HS effects of dietary phytochemicals and synthetic antioxidants, along with their underlying mechanisms, represents a critical area of research for improving intestinal barrier function and overall poultry health.

Similar to other animals, indigenous chickens demonstrate better adaptation to high temperatures compared to commercial high-performance broilers (Malila et al., 2024). Previous studies comparing the HS responses between slow-growing and fast-growing chickens have suggested that increased growth rates negatively correlate with birds' tolerance (Soleimani et al., 2011). Two breeder strains, heat-adapted (Leung Hang Kaeo breeder line) and heat-sensitive (Suranaree University of Technology (SUT) breeder line), are preserved by the avian research center of SUT in Thailand. These strains are used to produce crossbred meat chicken (Korat chicken) for the local niche market. Under elevated temperatures, the SUT breeder line exhibits significant signs of heat stress susceptibility and reduced productive performance, while the local breed shows minimal adverse effects. The investigation of gene biomarkers through transcriptomic analysis holds promise for optimizing feed modulation with antioxidant bioactive substances. These approaches could enhance HS adaptation while promoting gut health and increasing both efficiency and genetic gain (Gvozdanović et al., 2023).

The gut is the primary organ affected by HS (Chauhan et al., 2021). HS reduces blood flow to the gut, causing damage to the epithelial tissue and generating free radicals that impair nutrient digestion and absorption. The jejunum, which plays a critical role in nutrient digestion and absorption, is particularly sensitive to HS (Song et al., 2013), and is commonly used as a model in intestinal studies (Abdelli et al., 2021; Wang et al., 2021). Under HS conditions, birds activate defense mechanisms such as heat-shock proteins (HSPs) to alleviate or reduce the negative effects (Belhadj Slimen et al., 2016; Emami et al., 2021). The expression of *HSP70* and *HSP90* is upregulated to protect and repair cells (Varasteh et al., 2015). HSPs also play a regulatory role during cellular stress by inhibiting inflammatory cytokine production (Ferat-Osorio et al., 2014) and modulating tight junction proteins (Dokladny et al., 2008), thereby mitigating the detrimental effects of HS on gut health. Beyond molecular-level responses, various nutritional strategies are being tested, with dietary intervention emerging as a particularly cost-effective approach (Greene et al., 2021). Antioxidant supplementation combining vitamins E and C, minerals (Se, manganese, and zinc), and phytogenic bioactive compounds has demonstrated synergistic efficacy in enhancing antioxidant activity, reducing oxidative stress, strengthening immune function, and gut dysbiosis regulation (Ghazi Harsini et al., 2012; Kumbhar et al., 2018), while mitigating HS and

lipid peroxidation in poultry (Leskovec et al., 2019). In addition, L-carnitine, a potent antioxidant, plays a crucial role in scavenging free radicals and protecting tissues from ROS-induced oxidative damage (Agarwal et al., 2018). Phytogenic compounds, rich in bioactive chemicals like polyphenols and flavonoids, can improve antioxidant capacity, and immunity, enhance gut microbiota and health, and reduce oxidative/inflammatory pathways (Yang et al., 2021; Reith et al., 2022). These properties contribute to increased resistance to external stress, garnering significant attention in recent research (Mahasneh et al., 2024). Notable herbs of interest include *Camellia sinensis* (green tea), which contains major antioxidant catechins (particularly epigallocatechin gallate), *Syzygium aromatum* (clove), which is rich in eugenol, and *Persicaria odorata* (Vietnamese coriander), which contains gallic acid and quercetin. These herbs have shown potential as feed additives to mitigate HS, providing several beneficial functionalities (Erener et al., 2011; Hosseinzadeh et al., 2014; El-Maati et al., 2016; Aziz-Aliabadi et al., 2023; Saracila et al., 2023). However, the role of synthetic and phytogenic antioxidants in gut health and production, along with their underlying mechanisms in heat-stressed breeder hens, remains to be fully explored.

Transcriptome sequencing (RNA-seq) serves as a powerful tool for analyzing molecular mechanisms and revealing intrinsic cellular biological regulatory mechanisms underlying various physiological conditions in animals (Wang et al., 2019). This technology has been successfully employed across species, including poultry, cattle, and pigs, to identify genes that play key roles in responses to high ambient temperatures (Coble et al., 2014; Srikanth et al., 2017). Previous RNA-seq studies have revealed differential expression of genes in the jejunal mucosa of heat-stressed animals. In broilers subjected to chronic HS, differentially expressed genes (DEGs) were associated with immune response, glutathione metabolism, defense mechanisms, and detoxification of xenobiotics (Kim et al., 2022). Similarly, in breeder chickens under acute HS, RNA-seq analysis demonstrated DEGs involved in steroid biosynthesis, steroid hormone biosynthesis, protein processing in endoplasmic reticulum, the peroxisome proliferator-activated receptor signaling pathway, and the adipocytokine signaling pathway (Zhu et al., 2025). While these studies have provided valuable insights into the gene expression profiles of poultry under HS, there remains a critical need to investigate the long-term effects of HS on gut health at the molecular level. Such

research could reveal novel mechanisms and potential therapeutic targets for improving heat tolerance in poultry.

Therefore, this study pursued a comprehensive investigation with two interconnected objectives. The first objective was to conduct transcriptomic analysis in the jejunal mucosal tissue of breeder hens exposed to HS, aiming to identify relevant gene markers by comparing heat-adapted and heat-sensitive breeds and track progressive changes in selected candidate genes among heat-sensitive breeder hens under HS conditions. The second objective was to evaluate the efficacy of two antioxidant sources in breeder hens' diets under HS conditions: 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). The evaluation encompassed antioxidants genes (SOD, GSH-Px), HSPs (HSP70, HSP90), immune-related genes (IL-10, IL-6, TNF-α, NF-κB and TLR4), and TJ protein genes (ZO-1, CLND1), as well as parameters such as short-chain fatty acids (SCFAs) concentration, ammonia production, and microbiota composition.

1.2 Research objectives

The objectives of this study were:

1.2.1 To identify heat tolerance mechanisms in breeder hens by comparing jejunal transcriptome profiles between heat-adapted and heat-sensitive hens, and tracking expression changes of selected candidate genes in heat-sensitive hens supplemented with dietary antioxidants under HS conditions.

1.2.2 To evaluate the efficacy of dietary supplementation, either with synthetic or phytogenic antioxidant mixtures, on alleviating the deleterious impact of HS in heat-sensitive breeder hens by measuring gut health parameters, including gene expression related to intestinal function, antioxidant capacity, short-chain fatty acid (SCFAs) and ammonia concentrations, and microbial populations.

1.3 Research hypotheses

1.3.1 Heat-adapted and heat-sensitive breeder hens exhibit distinct jejunal transcriptome profiles under HS conditions, with differentially expressed genes and

biological pathways that can explain the variation in heat tolerance. Dietary antioxidant supplementation in heat-sensitive breeder hens under HS can normalize the expression of selected candidate genes.

1.3.2 Dietary antioxidant supplementation with synthetic or phytogenic antioxidant mixtures in heat-sensitive breeder hens under HS can improve expression of the genes related to gut barrier integrity (ZO-1, CLDN1), anti-inflammatory cytokine (IL-10) and antioxidant capacity (SOD, GSH-Px), reduce pro-inflammatory cytokines (IL-6, TNF-α, NF-κB and TLR4) and HSPs (HSP70, HSP90) expression, increase cecal SCFAs content, reduce ammonia production, and modify microbial population.

1.4 Scope of the study

In this study, two 28-week-old broiler breeder strains (heat-adapted [Leung Hang Kaeo breeder line] and heat-sensitive [Suranaree University of Technology (SUT)] breeder line) were used to acquire a deeper understanding of their transcriptomic responses to HS. In addition, this study aims to leverage these findings to develop innovative strategies for mitigating HS impacts by evaluating the effects of dietary 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) supplementation in heat-sensitive breeders under HS conditions. This study was assessed various gut health parameters, including the expression of antioxidant enzymes (SOD and GSH-Px), HSPs (HSP70 and HSP90), immune-related genes (IL-10, IL-6, TNF-α, NF-κB and TLR4), TJ protein genes (ZO-1, CLDN1), cecal short-chain fatty acid (SCFAs) concentration, ammonia production, and the composition of the cecal microbiota. By integrating transcriptomic analysis with gut health evaluations, this study seeks to identify molecular and physiological mechanisms underlying heat resilience and propose targeted nutritional interventions to enhance poultry health and performance under HS conditions.

1.5 Expected benefits

1.5.1 The knowledge obtained from transcriptomic profiling of two breeding strains with different tolerance to HS can provide a deeper understanding of the

molecular responses and biological pathways involved in heat adaptation, which is crucial for developing targeted feed additive interventions to cope with HS in poultry production systems.

1.5.2 Dietary supplementation with either synthetic or phytogenic antioxidants can be applied to breeder hen diets and can be further studied for potential applications in other animal production systems facing HS challenges.

1.5.3 The marker genes identified in this study can serve as monitoring tools to evaluate the efficacy of feed additives designed to mitigate HS effects in poultry and potentially other livestock species. While the primary focus of this study is on dietary supplementation effects, the identified candidate genes may also provide preliminary insights for future genetic selection research.

1.6 References

Akbarian, A., MiAbdelli, N., Ramser, A., Greene, E. S., Beer, L., Tabler, T. W., Orlowski, S. K., Pérez, J. F., Solà-Oriol, D., Anthony, N. B., & Dridi, S. (2021). Effects of Cyclic Chronic Heat Stress on the Expression of Nutrient Transporters in the Jejunum of Modern Broilers and Their Ancestor Wild Jungle Fowl. **Frontiers in Physiology**, 12 (23), 733134.

Agarwal, A., Sengupta, P., & Durairajanayagam, D. (2018). Role of L-carnitine in female infertility. **Reproductive Biology and Endocrinology**, 16(1), 5.

Aziz-Aliabadi, F., Noruzi, H., & Hassanabadi, A. (2023). Effect of different levels of green tea (Camellia sinensis) and mulberry (Morus alba) leaves powder on performance, carcass characteristics, immune response, and intestinal morphology of broiler chickens. **Veterinary Medicine and Science**, 9(3), 1281–1291.

Belhadj Slimen, I., Najar, T., Ghram, A., & Abdrrabba, M. (2016). Heat stress effects on livestock: Molecular, cellular and metabolic aspects, a review. **Journal of Animal Physiology and Animal Nutrition**, 100(3), 401–412.

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.

Coble, D. J., Fleming, D., Persia, M. E., Ashwell, C. M., Rothschild, M. F., Schmidt, C. J., & Lamont, S. J. (2014). RNA-seq analysis of broiler liver transcriptome reveals novel responses to high ambient temperature. **BMC Genomics**, 15(1), 1084.

Dokladny, K., Ye, D., Kennedy, J. C., Moseley, P. L., & Ma, T. Y. (2008). Cellular and Molecular Mechanisms of Heat Stress-Induced Up-Regulation of Occludin Protein Expression. **The American Journal of Pathology**, 172(3), 659–670.

El-Maati, M. F. A., Mahgoub, S. A., Labib, S. M., Al-Gaby, A. M. A., & Ramadan, M. F. (2016). Phenolic extracts of clove (Syzygium aromaticum) with novel antioxidant and antibacterial activities. **European Journal of Integrative Medicine**, 8(4), 494–504.

Emami, N. K., Greene, E. S., Kogut, M. H., & Dridi, S. (2021). Heat Stress and Feed Restriction Distinctly Affect Performance, Carcass and Meat Yield, Intestinal Integrity, and Inflammatory (Chemo)Cytokines in Broiler Chickens. **Frontiers in Physiology**, 12, 707757.

Erener, G., Ocak, N., Altop, A., Cankaya, S., Aksoy, H. M., & Ozturk, E. (2011). Growth Performance, Meat Quality and Caecal Coliform Bacteria Count of Broiler Chicks Fed Diet with Green Tea Extract. **Asian-Australasian Journal of Animal Sciences**, 24(8), 1128–1135.

Ferat-Osorio, E., Sánchez-Anaya, A., Gutiérrez-Mendoza, M., Boscó-Gárate, I., Wong-Baeza, I., Pastelin-Palacios, R., Pedraza-Alva, G., Bonifaz, L. C., Cortés-Reynosa, P., Pérez-Salazar, E., Arriaga-Pizano, L., López-Macías, C., Rosenstein, Y., & Isibasi, A. (2014). Heat shock protein 70 down-regulates the production of toll-like receptor-induced pro-inflammatory cytokines by a heat shock factor-1/constitutive heat shock element-binding factor-dependent mechanism. **Journal of Inflammation**, 11(1), 19.

Ghazi Harsini, S., Habibiyan, M., Moeini, M. M., & Abdolmohammadi, A. R. (2012). Effects of Dietary Selenium, Vitamin E, and Their Combination on Growth, Serum Metabolites, and Antioxidant Defense System in Skeletal Muscle of Broilers Under Heat Stress. **Biological Trace Element Research**, 148(3), 322–330.

Greene, E. S., Emami, N. K., & Dridi, S. (2021). Research Note: Phytobiotics modulate the expression profile of circulating inflammasome and cyto(chemo)kine in whole blood of broilers exposed to cyclic heat stress. **Poultry Science**, 100(3), 100801.

Gupta, A., Chauhan, N. R., Chowdhury, D., Singh, A., Meena, R. C., Chakrabarti, A., & Singh, S. B. (2017). Heat stress modulated gastrointestinal barrier dysfunction: Role of tight junctions and heat shock proteins. **Scandinavian Journal of Gastroenterology**, 52(12), 1315–1319.

Gvozdanović, K., Kralik, Z., Radišić, Ž., Košević, M., Kralik, G., & Djurkin Kušec, I. (2023). The Interaction between Feed Bioactive Compounds and Chicken Genome. **Animals**, 13(11), 1831.

Hosseinzadeh, H., Alaw Qotbi, A. A., Seidavi, A., Norris, D., & Brown, D. (2014). Effects of Different Levels of Coriander (Coriandrum sativum) Seed Powder and Extract on Serum Biochemical Parameters, Microbiota, and Immunity in Broiler Chicks. **The Scientific World Journal**, 2014, 1–11.

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.

Kumar, M., Ratwan, P., Dahiya, S. P., & Nehra, A. K. (2021). Climate change and heat stress: Impact on production, reproduction and growth performance of poultry and its mitigation using genetic strategies. **Journal of Thermal Biology**, 97, 102867.

Kumbhar, S., Khan, A. Z., Parveen, F., Nizamani, Z. A., Siyal, F. A., El-Hack, M. E. A., Gan, F., Liu, Y., Hamid, M., Nido, S. A., & Huang, K. (2018). Impacts of selenium and vitamin E supplementation on mRNA of heat shock proteins, selenoproteins and antioxidants in broilers exposed to high temperature. **AMB Express**, 8(1), 112.

Leskovec, J., Levart, A., Perić, L., Đukić Stojčić, M., Tomović, V., Pirman, T., Salobir, J., & Rezar, V. (2019). Antioxidative effects of supplementing linseed oil-enriched diets with **α**-tocopherol, ascorbic acid, selenium, or their combination on carcass and meat quality in broilers. **Poultry Science**, 98(12), 6733–6741.

Liu, L., Fu, C., Yan, M., Xie, H., Li, S., Yu, Q., He, S., & He, J. (2016). Resveratrol modulates intestinal morphology and HSP70/90, NF-**K**B and EGF expression in the jejunal mucosa of black-boned chickens on exposure to circular heat stress. **Food & Function**, 7(3), 1329–1338.

Loyau, T., Bedrani, L., Berri, C., Métayer-Coustard, S., Praud, C., Coustham, V., Mignon-Grasteau, S., Duclos, M. J., Tesseraud, S., Rideau, N., Hennequet-Antier, C., Everaert, N., Yahav, S., & Collin, A. (2015). Cyclic variations in incubation conditions induce adaptive responses to later heat exposure in chickens: A review. **Animal**, 9(1), 76–85.

Mahasneh, Z. M. H., Abuajamieh, M., Abedal-Majed, M. A., Al-Qaisi, M., Abdelqader, A., & Al-Fataftah, A.-R. A. (2024). Effects of medical plants on alleviating the effects of heat stress on chickens. **Poultry Science**, 103(3), 103391.

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.

Reith, R. R., Sieck, R. L., Grijalva, P. C., Swanson, R. M., Fuller, A. M., Diaz, D. E., Schmidt, T. B., Yates, D. T., & Petersen, J. L. (2022). Transcriptome analyses indicate that heat stress-induced inflammation in white adipose tissue and oxidative stress in skeletal muscle is partially moderated by zilpaterol supplementation in beef cattle. **Journal of Animal Science**, 100(3), skac019.

Rostagno, M. H. (2020). Effects of heat stress on the gut health of poultry. **Journal of Animal Science**, 98(4), skaa090.

Saeed, M., Abbas, G., Alagawany, M., Kamboh, A. A., Abd El-Hack, M. E., Khafaga, A. F., & Chao, S. (2019). Heat stress management in poultry farms: A comprehensive overview. **Journal of Thermal Biology**, 84(12), 414–425.

Saracila, M., Panaite, T. D., Predescu, N. C., Untea, A. E., & Vlaicu, P. A. (2023). Effect of Dietary Salicin Standardized Extract from Salix alba Bark on Oxidative Stress Biomarkers and Intestinal Microflora of Broiler Chickens Exposed to Heat Stress. **Agriculture**, 13(3), 698.

Shehata, A. M., Saadeldin, I. M., Tukur, H. A., & Habashy, W. S. (2020). Modulation of Heat-Shock Proteins Mediates Chicken Cell Survival against Thermal Stress. **Animals**, 10(12), 2407.

Shi, D., Bai, L., Qu, Q., Zhou, S., Yang, M., Guo, S., Li, Q., & Liu, C. (2019). Impact of gut microbiota structure in heat-stressed broilers. **Poultry Science**, 98(6), 2405–2413.

Soleimani, A. F., Zulkifli, I., Omar, A. R., & Raha, A. R. (2011). Physiological responses of 3 chicken breeds to acute heat stress. **Poultry Science**, 90(7), 1435–1440.

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.

Srikanth, K., Lee, E., Kwan, A., Lim, Y., Lee, J., Jang, G., & Chung, H. (2017). Transcriptome analysis and identification of significantly differentially expressed genes in Holstein calves subjected to severe thermal stress. **International Journal of Biometeorology**, 61(11), 1993–2008.

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.

Wang, G., Li, X., Zhou, Y., Feng, J., & Zhang, M. (2021). Effects of Heat Stress on Gut-Microbial Metabolites, Gastrointestinal Peptides, Glycolipid Metabolism, and Performance of Broilers. **Animals**, 11(5), 1286.

Wang, Q., Li, J., & Guo, H. (2019). Transcriptome analysis and discovery of genes involved in immune pathways in Solen strictus (Gould, 1861) under Vibrio anguillarum. **Fish & Shellfish Immunology**, 88(12), 237–243.

Yang, C., Luo, P., Chen, S., Deng, Z., Fu, X., Xu, D., Tian, Y., Huang, Y., & Liu, W. (2021). Resveratrol sustains intestinal barrier integrity, improves antioxidant capacity, and alleviates inflammation in the jejunum of ducks exposed to acute heat stress. **Poultry Science**, 100(11), 101459.

Zhang, K., Hornef, M. W., & Dupont, A. (2015). The intestinal epithelium as guardian of gut barrier integrity: The epithelium as a barrier to infection. **Cellular Microbiology**, 17(11), 1561–1569.

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

CHAPTER II

LITERATURE REVIEW

2.1 The impact of heat stress on the gut health of chickens

It is well-established that an increase in the energy requirements for maintenance leads to heat stress (HS) in poultry (Johnson et al., 2018). As a metabolically active organ, intestinal tissue, particularly the jejunum, is highly susceptible to the effects of HS (Calik et al., 2022). High temperatures can induce oxidative stress and cause a cellular imbalance between the production of reactive oxygen species (ROS) and antioxidant protective systems, thus further stimulating ROS production, which is responsible for various types of oxidative damage, such as lipids and protein oxidation, and ultimately contributes to tissue damage (Hidayat et al., 2023). Excessive production of ROS can compromise intestinal barrier function, induce aberrant immune responses, and increase intestinal permeability (Varasteh et al., 2015). These alterations facilitate the translocation of antigens, toxins, and pathogens across the tight junction (TJ) barrier, subsequently activating the immune system through Toll-like receptor (TLR) signaling, cytokines, and heat shock proteins (HSPs) (Wallin et al., 2002; Turner, 2009). This cascade of events ultimately leads to modifications in the intestinal mucosal microstructure, triggering inflammation and tissue damage. HS can significantly impair microbiota and metabolites in the intestinal of poultry, disrupt the dynamic balance between beneficial and pathogenic bacteria, and result in dysbiosis of the intestinal flora (Wang et al., 2022). In addition, HS impairs nutrient transport and digestion by decreasing enzymatic activity in the digesta, reducing the absorptive surface area, and modulating the expression of related nutrient transporters, proteins, and genes (Patra & Kar, 2021). It seems that many physiological alterations in immunity, barrier function, and nutrient transport within the intestines appear to result from heat stress-induced imbalances in the gut microbiome, which may subsequently provoke a systemic response, and thus impair intestinal health.

2.2 Heat stress and antioxidant defense system

Under HS, the oxidative and antioxidant systems in poultry lose their dynamic balance, leading to the overproduction of ROS. This imbalance results in inflammatory neutrophil infiltration and increased protease secretion (Habashy et al., 2017). Active oxygen refers to a group of free radicals that exist independently and contain unpaired electrons. These include superoxide anion ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2) (Goncalves et al., 2020). The production of ROS occurs in specific parts of the mitochondrial electron transport chain, mainly in complexes I and III (Quijano et al., 2016). The main way of generating $O_2^{\bullet-}$ in the electron transport chain is auto radiation caused by the reaction between the reduced flavin protein and oxygen, under the condition of HS, the related enzymes in the complex undergo thermal denaturation, and the auto radiation rate of flavin increases in a temperature-dependent manner, increasing ROS generation (Messner & Imlay, 1999). In addition, high temperature down-regulated the expression of uncoupling proteins in the inner membrane of poultry mitochondria, hampering mild uncoupling in heat-stressed birds, which is a major contributor to the overproduction of ROS (Kikusato & Toyomizu, 2013). Under HS conditions, the overproduction of transitional metal ions will also increase the production of the Fenton reaction [$H_2O_2 + Fe^{2+} \longrightarrow OH^{\bullet} + (OH)^- + Fe^{3+}$], leading to increased ROS production. There are mainly antioxidant enzyme systems in animals, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). SOD can convert high-activity $O_2^{\bullet-}$ to low-activity H_2O_2; CAT can reduce H_2O_2 to H_2O (Nagami et al., 2005). GPx, with selenocysteine as its active center, reduces H_2O_2 and various organic hydroperoxides (Liochev & Fridovich, 1999) (Figure 2.1).

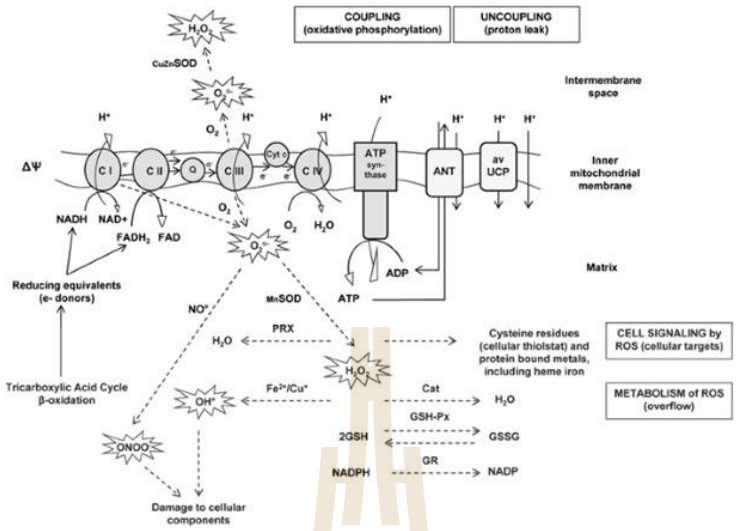


Figure 2.1 Mitochondrial energy transduction and pathophysiology of oxidative stress upon heat stress (Akbarian et al., 2016).

2.3 Heat stress on antioxidant status, barrier integrity, morphology, immunity, and production performance of chickens

HS has emerged as an important issue in the poultry production industry (Abdelnour et al., 2019). HS can be categorized into acute and chronic HS, depending on its duration and severity (Saeed et al., 2019). HS can adversely affect production performance, reproductive performance, gut health, economic traits, and poultry welfare (Tajima et al., 2007; Lara & Rostagno, 2013). In addition, HS induces a variety of negative effects on intestinal morphology, immunity, barrier integrity, digestive enzyme secretion, antioxidant status, and HSP expression in chickens, as summarized in Table 2.1.

Table 2.1 Effects of heat stress on antioxidant status, barrier integrity, morphology, immunity, and production performance in the gut of chickens.

Stains	TN vs. HS conditions with RH	Results	Reference
Broiler	TN (21°C) vs. HS (32°C) with 64% RH	HS decreased BW, ADG and increased FCR HS decreased villus length, villus surface area, and epithelium cell area.	(Al-Fataftah & Abdelqader, 2014)
Broilers	TN (20°C) vs. HS (30°C) with 70% RH	HS decreased BW, ADG and increased FCR Increased lipopolysaccharide, corticosterone, TNF-α, and IL2 in blood.	(Alhenaky et al., 2017)
Broilers	TN (23°C) vs. HS (30°C) with 67% RH	HS decreased crypt depth but did not affect villus height.	(Burkholder et al., 2008)
Wenchang chickens	TN (25.7°C, 88% RH) vs. HS (40.5°C, 52.4% RH)	HS reduced in villus length, mucosa thickness, intestinal wall thickness, and crypt depth in all three segments. Mucosal epithelia were detached with ruptured.	(Patra & Kar, 2021)
Broilers	TN (22°C) vs. HS (32°C) with (50 ± 10%) RH	HS decreased villus height the VH-to-crypt depth (CD) ratio (VCR), and antioxidant enzymes activity, while MDA content increased. HS reduced *Parabacteroides*, *Saccharimonas*, *Romboutsia*, and *Weissella* abundance.	(Liu et al., 2020)

Table 2.1 (continued).

Stains	TN vs. HS conditions with RH	Results	Reference
Cobb chicks	TN (24°C) vs. HS (33°C) with (50 ± 5%) RH	HS decreased the final BW, downregulated the expression of *GPX3*, *IL4*, and *CLDN2,* and decreased *Ruminococcus*, *Ocillospira*, and *Lactobacillus* abundance.	(Chaudhary et al., 2023)
Indigenous broilers	TN (21°C) vs. HS (32°C) with (55–70%) RH	HS decreased the BWG. HS had lower T-AOC, GSH-Px and SOD in serum and jejunum. HS downregulated *Occludin, Claudin-1, Claudin-4, ZO-1, Mucin-2* in the jejunum.	(Liu et al., 2022)
Broilers	TN (21°C) vs. HS (31°C) with (70%) RH	HS increased *Proteobacteria* abundance and decreased *Firmicutes* abundance.	(Liu et al., 2023)
Broilers	TN (24-26°C) vs. HS (334-38°C)	HS increased concentrations of HSP70 and cortisol. HS increased *Firmicutes*, *Tenericutes*, and *Proteobacteria* and decreased *Bacteroidetes* and *Cyanobacteria* abundance.	(Shi et al., 2019)
Broilers	TN (20°C) vs. HS (32°C)	HS decreased ADG, ADFI, and FCR. HS reduced villi height and VCR in the jejunum and ileum. HS reduced mRNA levels of jejunal *MUC2* and *OCLN*, and ileal *MUC2*, *ZO1*, *OCLN*, and *CLDN3*.	(Gu et al., 2012)

Table 2.1 (continued).

Stains	TN vs. HS conditions with RH	Results	Reference
Broilers	TN (20°C) vs. HS (27.8°C) with 53.0% RH	HS decreased BW, ADG, ADFI, and feed efficiency. HS did not affect jejunal gene expressions of *OCLN*, *ZO1*, *CLDN1*, and *JAM2*.	(Koch et al., 2019)
Broilers	TN (23°C) vs. cyclic HS (28°C–35°C–28°C)	HS decreased ADFI, ADG, and FCR. HS reduced villus height and VCR in the jejunum. HS increased serum TNFα, IL6, and IL1β levels, but decreased anti-inflammatory cytokine IL10 levels.	(Patra, 2018)
Broilers	TN (26°C) vs. HS (34°C)	HS decreased FI and BW. HS decreased gene expressions of *CLDN3* and *OCLN* but not *CLDN1*. HS increased gene expressions of *HSPA1A*, *HSPD1*, and *HSPB1*.	(Jimoh et al., 2018)
Broilers	TN (22°C) vs. HS (38°C)	HS increased *HSF-3*, *HSP70*, *HSP90*, *CLDN*, *CLDN5*, *ZO1*, *TLR-4*, *IL6*, *IL8*, and *HSP70* mRNA expression in jejunum.	(Varasteh et al., 2015)
Layers	TN (21°C, RH 62%) vs. HS (35°C, 64% RH)	HS decreased duodenal, jejunal, and ileal villus height, crypt depth, and absorptive epithelial cell area.	(Abdelqader et al., 2017)

Table 2.1 (continued).

Stains	TN vs. HS conditions with RH	Results	Reference
White Leghorn hens	TN (20°C–22°C, 50%–60% RH) vs. HS (30°C–33°C, 70%–80% RH)	HS decreased egg weight, eggshell thickness, percentage, and density. HS decreased calcium-binding protein (calbindin) in the ileum, cecum, and colon.	(Shakeri et al., 2019)
Commercial laying hens	TN (26°C) vs. HS (33°C), with 60%–70%	HS reduced egg production rate, feed intake, and egg weight while increasing the feed-to-egg ratio, broken egg ratio, and mortality—down-regulated expression levels of *OCLN*, *ZO1*, and *JAM-A* in ileum and cecum.	(Tajima et al., 2007)

TN, thermoneutral; HS, heat stress; RH, relative humidity; BW, body weight; ADG, average daily gain; FCR, feed conversion ratio; TNF-α, tumor necrosis factor α; IL, interleukin; VCR, villus height to crypt depth ratio; ADFI, average daily feed intake; MDA, malondialdehyde; SOD, superoxide dismutase; CLDN, claudins; MUC, mucin; OCLN, occludin; ZO1, zonula occludens 1; HSP, heat shock protein; SGLT1, sodium-dependent glucose cotransporter 1; GPx, glutathione peroxidase; T-AOC, total antioxidant capacity; HSPA1A, heat shock protein family A (HSP70) member 1A; HSPD1, heat shock protein family D (HSP60) member 1, HSPB1, heat shock protein family B (small) member 1; HSF, heat shock factor; GLUT, facilitative glucose transporter; VH, villus height.

2.3.1 Heat stress on oxidative stress in the gut

HS typically induces oxidative stress. SOD, CAT, and GPx represent the primary components of the antioxidant defense system, playing a critical role in maintaining oxidative stability within the host. They work synergistically to eliminate superoxide anions and hydrogen peroxide within cells (Gu et al., 2012). HS resulted in a significant decrease in various oxidative stress markers, such as GPx, SOD, and CAT, total antioxidant capacity, and nuclear factor erythroid 2-related factor 2 (Nrf2), while increasing Kelch-like ECH-associated protein 1 (Keap1) transcripts and malondialdehyde (MDA) levels in broilers (Hidayat et al., 2023; Algothmi et al., 2024). Previous studies have found that chronic HS (20°C vs. 32°C–33°C 8 h/day) leads to increased MDA content in the jejunal mucosa and decreased SOD levels in the ileal mucosa of chickens (Gu et al., 2012). Similarly, after 2 weeks of cyclic HS (22°C, 24 h/d vs. 32°C, 10 h/d), chickens showed increased MDA content and decreased GPx levels in the ileum and jejunum (Roushdy et al., 2018). The Nrf2-mediated antioxidant response pathway plays a crucial role in maintaining cellular redox balance by promoting the transcription of various cytoprotective genes (Liu et al., 2016). Moreover, Du et al. (2022) reported that HS resulted in reduced SOD levels in jejunal mucosa and increased MDA content in the serum, liver, and intestinal of heat-stressed broilers. In conclusion, HS causes redox dysfunction and hemostasis imbalance by decreasing the activities of SOD, GPx, and CAT, as well as the total antioxidant capacity in jejunal and ileal tissues and serum, while simultaneously elevating the levels of oxidative markers such as MDA and H_2O_2 levels.

2.3.2 Heat stress on immunity in the gut

HS can induce the release of proinflammatory cytokines and mediators (such as nuclear factor **κ**B [NF-kB], Toll-like receptor [TLR], and interleukin [**IL**]) in broilers, leading to decreased growth rate and weakened immune system in most broilers. In general, pro-inflammatory mediators contribute to inflammatory damage, while anti-inflammatory mediators help alleviate inflammation and facilitate the healing process in response to environmental triggers (Bamias et al., 2014). Studies have shown that HS (38°C - 39°C, 8 hours per day, for 5 consecutive days) resulted in upregulating of *TLR4*, *IL6*, and *IL8* levels in the jejunum of chickens (Varasteh et al., 2015). The transcription factor NF-**κ**B plays a crucial role in the regulation of genes activated by inflammatory cytokines, pathogens, and oxidative stress (Sanz Fernandez et al., 2014). HS leads to

increased levels of proinflammatory cytokines (IL-1β, IL-6, and tumor necrosis factor-alpha [TNF-α]) by affecting the redox-sensitive downstream pathways of NF-κB (Fang et al., 2023). In addition, IL-10 is a key anti-inflammatory mediator that plays a pivotal role in regulating the inflammatory response. Several studies have highlighted IL-10 as one of the most important cytokines involved in various pathophysiological conditions, where it inhibits the production of pro-inflammatory mediators (Hidayat et al., 2023). TNF-α is a proinflammatory mediator that is widely used in animal models (Abdelnour et al., 2019). In addition, TNF-α is also an important mediator of early liver injury (Hoek & Pastorino, 2002). However, it is well known that HS is an important environmental factor leading to liver injury. Therefore, elevated TNF-α levels in the liver or serum may lead to liver dysfunction (Li et al., 2023). Notably, IL-6 exhibits both pro- and anti-inflammatory properties, influencing both metabolic and inflammatory pathways (Su et al., 2013). Additionally, IL-6 impacts the tight junctions in the gastrointestinal tract, while TNF-α is known to increase intestinal permeability (Su et al., 2013). Furthermore, HS can trigger inflammation through the release of inflammatory markers, including IL-2, TNF-α, and IL-4 (He et al., 2019). TLRs, particularly TLR4, serve as key biosensors for stress. TLR4 activation can stimulate NF-κB, a central nuclear transcription factor in inflammatory and immune responses, influencing the expression of various inflammatory markers such as IL-6, TNF-α, and IL-1β (He et al., 2019). Previous studies have shown that HS increases the expression of both NF-κB and TLR4 (Cheng et al., 2019). Therefore, the activation of inflammatory signaling pathways may serve as a key factor in the disruption of innate immunity and the initiation of the inflammatory response during HS.

2.3.3 Heat stress on barrier integrity in the gut

HS-induced hypoxia impairs the balance of antioxidants and immune responses, resulting in epithelial damage and compromised barrier integrity (Lian et al., 2020). The preservation of intestinal mucosal integrity is crucial for optimal nutrient absorption and preventing the paracellular passage of harmful antigens. Tight junction (TJ) proteins, which seal the spaces between adjacent epithelial cells, are particularly vulnerable to the effects of heat stress (Varasteh et al., 2015). The junctional transmembrane proteins, occludins (OCLN), claudins (CLDN), junctional adhesion molecules (JAMs), and tricellular interact with the intracellular scaffolding protein zona

occludens (ZO), which is anchored to the actin cytoskeleton (Lee, 2015). However, oxidative damage caused by heat stress impairs digestion and absorption of nutrients in poultry (Mishra & Jha, 2019). Previous research has shown that HS significantly increases the expression of E-cadherin, claudin-1, claudin-5, and ZO-1 proteins in the small intestine of broilers. Furthermore, HS-induced upregulation of HSF-1 enhances occludin expression by binding to its promoter, thereby promoting its involvement in the junctional complex (Dokladny et al., 2008). Consequently, the elevated levels of HSPs may be linked to the increased mRNA levels of TJ proteins under HS conditions (Lian et al., 2020). In addition, HS compromises the gut barrier integrity, leading to increased intestinal permeability, which can trigger chronic systemic inflammation and reduce disease resistance in broilers (Zhang et al., 2022; Saracila et al., 2023). Also, studies have shown that HS can reduce the number of goblet cells in the intestinal mucosa of chickens (Yu et al., 2010) and mucin 2 mRNA levels (Pearce et al., 2014). The above studies indicate that HS induces intestinal dysfunction by changing TJ proteins, leading to increased pathogen invasion, increased susceptibility to the mucosa, and reduced nutrient absorption, thus causing growth retardation in broilers.

2.3.4 Heat stress on heat shock protein in the gut

HSPs are well-known stress response proteins and molecular chaperones. They safeguard cells by aiding in protein folding, repair, localization, and degradation. HSPs facilitate the production of proteins involved in highly conserved cellular response mechanisms under stress conditions (Zilaee et al., 2014). The most prevalent HSPs induced by HS are *HSP70* and *HSP90*, which play crucial roles in cell protection. Among HSPs, the *HSP70* family is the most conserved and abundant protein in organisms (Milarski & Morimoto, 1989). Under HS conditions, *HSP70* is activated to eliminate denatured or misfolded proteins within cells, thereby enhancing cell viability and increasing resistance to heat stress (Bhat et al., 2016). *HSP70* has been detected in the pectoral muscle, liver, heart, and lungs, with its concentration in the brain being 2 to 5 times higher than in other embryonic tissues. This suggests that the elevated expression of *HSP70* in various embryonic tissues serves as an adaptive mechanism to mitigate stress conditions (Leandro et al., 2004). HSP expression serves as a potent antioxidant defense mechanism in the chicken intestine (Surai et al., 2019). However, under HS conditions, the overexpression of *HSP70* did not influence intestinal morphology. Notably, a significant

correlation was observed between *HSP70* expression and digestive enzyme activity in hens (Hao et al., 2012). In addition, Studies have found that inducing *HSP70* expression can protect the intestinal mucosa from HS damage by increasing the activity of antioxidant enzymes, preventing lipid peroxidation, and enhancing the antioxidant capacity of broilers (Gu et al., 2012). The *HSP90* family, a crucial group of chaperones downstream of *HSP70*, aids in the final structural maturation and conformational changes of proteins, thereby preserving homeostasis and cellular integrity under heat stress conditions (Dangi et al., 2014). It has been reported that HS increased HSP90 levels, which helps correct the folding, stability, and function of other proteins (Hong et al., 2013). In addition, HSP90 is involved in various cellular processes such as cell survival, cell cycle regulation, and other signal transduction pathways (Jackson, 2012). Calik et al. (2022) found that HS significantly upregulated *HSP70*, *HSP90* levels in the jejunum of broilers. To sum up, the findings of the above studies, the enhanced expression of *HSP70* and *HSP90* is one of the important defense responses to avoid or cope with the adverse changes in protein function and structure caused by different stresses, effectively inhibiting lipid peroxidation, improving antioxidant capacity, and playing an important role in protecting the integrity of the intestinal mucosa from HS damage, thereby contributing to cell function under stress conditions. However, more research is needed to better understand the molecular mechanisms of *HSP70* and *HSP90* regulation in avian organisms.

2.3.5 Heat stress on microbiota in the gut

The gut microbiota plays a crucial role in providing nutrients from the diet and regulating both the digestive and immune systems; therefore, maintaining a healthy gastrointestinal microbiome is essential for animals (Obianwuna et al., 2024). Studies have shown that heat stress affects the structure of the intestinal flora, which may be a key factor in the health of host animals. The distribution of intestinal flora can also affect the host's stress response (Chen et al., 2021). HS altered the profile of the cecal microbiome, with increased abundances of *Firmicutes* and *Tyzzerella*, and decreased abundances of *Bacteroidetes* and the genera *Bacteroides*, *Parabacteroides*, and *Romboutsia* (Liu et al., 2020). Wang et al. (2018) reported that HS (31 ± 1 °C) significantly increased the α diversity (observed species, PD of the whole tree, and Chao 1) of the ileal microbiota of 42-day-old broilers. In addition, HS induces oxidative stress and produces excessive ROS, leading to an imbalance between endogenous antioxidant

defense mechanisms. Excessive ROS production can lead to the invasion of facultative anaerobic bacteria and cause membrane permeability damage (Tomasello et al., 2016). Fang et al. (2023) demonstrated that HS led to a reduction in the relative abundance of *Firmicutes*, while the abundance of *Bacteroidetes* also decreased. These findings indicate that heat stress disrupts the homeostatic balance of the intestinal microbiota. Dysbiosis refers to an imbalance in the intestinal microbiota, characterized by an overgrowth of harmful microorganisms or a reduction in beneficial bacteria, which disrupts the delicate equilibrium between the host and its gastrointestinal microbiota (Walker, 2017; Ducatelle et al., 2018). It is commonly linked to nutrient maldigestion, compromised intestinal barrier function, and gastrointestinal inflammation (Chen et al., 2015). Despite the availability of advanced analytical methods for studying the gastrointestinal microbiota (Borda-Molina et al., 2018), the alterations in the structure, composition, and function of the microbiota in heat-stressed chickens are not yet fully understood (He et al., 2021; Liu et al., 2022).

2.4 Effect of dietary vitamin C, vitamin E, selenium, L-carnitine, and their combined supplementation in poultry under heat stress conditions

With the advancement of modern farming, the requirements for equipment and management technology are getting higher and higher, such as chicken houses, ventilation, cooling systems, and management technology have been greatly improved, which has a positive effect on poultry HS management. However, the high cost of this method and the rapid update rate of equipment make this technology not always feasible. Therefore, it is necessary to start from a nutritional strategy and consider inexpensive and nutritious antioxidant additives to alleviate HS in poultry. Therefore, during the HS, it is urgent to find efficient and feasible methods to enhance the technical effect, to alleviate or reduce the heat stress of poultry. At present, most studies have shown that different nutritional strategies can alleviate the negative effects of HS, thereby improving the breeding efficiency and production performance of poultry in high-temperature environments, such as supplementing the diet with plant-derived antioxidants (polyphenols, EGCG) minerals (selenium, zinc), vitamins (vitamin C, vitamin E), electrolytes, phytobiotics, probiotics, fats and amino acids can effectively relieve HS (Kumar et al., 2021; Calik et al., 2022).

2.4.1 Vitamin C

Vitamin C, also known as L-ascorbic acid, is a water-soluble antioxidant compound that is usually synthesized in the liver and kidneys of poultry, but environmental stressors can affect its synthesis and utilization, supplementing vitamin C in poultry diets can help enhance immunity, which can scavenge ROS to prevent cellular damage caused by oxidative stress. Vitamin C may act as a co-antioxidant with other antioxidants by synergistic effects, to relieve HS. The antioxidant activity of vitamin E increased in the presence of vitamin C through reducing tocopheroxy radicals back to their active form of vitamin E (Calik et al., 2022). A number of studies have found that vitamin C supplementation of 150-500 mg/kg in broiler diets under high temperature conditions can effectively improve various production factors and growth performance, and reduce the expression of *HSP70* (Farooqi et al., 2005; Attia et al., 2011; Kumar et al., 2017). Under HS conditions, the poultry's own endogenous synthesis of vitamin C is hindered, and the demand for vitamin C will increase at this time (Ghazi Harsini et al., 2012). Furthermore, the authors found that vitamin C supplementation in the diets of heat-stressed layer hens improved laying performance and egg quality, significantly increased the length, width and diameter of intestinal crypts, and protected intestinal epithelial cells from HS caused oxidative damage (Ajakaiye et al., 2011). This indicates that higher concentrations of vitamin C in broiler diets can effectively combat HS.

2.4.2 Vitamin E

Vitamin E contains four tocopherols (α-、β-、γ- and δ-), of which α-tocopherol is biologically active and able to meet the needs of animals. Vitamin E is a group of compounds containing both tocopherols and tocotrienols, which are the first line of defense against lipid peroxidation caused by HS in poultry, it is a biological antioxidant and free radical scavenger (Ajakaiye et al., 2011). Dietary supplementation with vitamin E at various levels (150–500 mg/kg) improved poultry performance (Ghazi Harsini et al., 2012), and reduced the negative effects of HS (Maini et al., 2007). Additionally, vitamin E increased the secretion of growth hormone, which may be responsible for improved growth performance (Khan et al., 2013). Habibian et al. (2014) reported that vitamin E supplementation increased the production of antibodies against different diseases in heat-stressed poultry, which improved their immunity. The literature showed that supplementation with vitamin E at an average concentration of 250 mg/kg

is a viable protective measure to reduce HS symptoms and the best possible broiler performance (Maini et al., 2007). Supplementation with 200 mg/kg vitamin E to grain could significantly increase the activity of SOD, CAT and GSH, and reduce the concentration of MDA in blood and liver of heat-stressed broilers (Calik et al., 2022). Vitamin E supplementation had positive effects on laying hens' egg production and egg quality (Ajakaiye et al., 2011). Sahin et al. (2006) found that supplementing 250mg/kg of vitamins in heat-stressed quail diets significantly improved growth performance, increased blood vitamins E and A concentrations, and decreased MDA concentrations.

2.4.3 Selenium

In recent years, the role of selenium (Se) in poultry nutrition has been studied more and more, especially the supplementation of selenium to reduce or alleviate the HS of poultry, because the main form of glutathione peroxidase is selenium-dependent. (Calik et al., 2022). In addition, Se can act as a cofactor of glutathione peroxidase and superoxide dismutase, play a major role in the antioxidant system, and can scavenge free radicals to protect poultry from HS damage (Shakeri et al., 2020). When dietary Se is deficient, poultry exhibit poor appetite and inefficient utilization, which negatively affects growth performance (Wasti et al., 2020). Studies have shown that supplementation of Se can increase the feed intake and body weight of heat-stressed broilers and reduce the feed conversion ratio (Niu et al., 2009; Ghazi Harsini et al., 2012). In addition, supplementation with 0.2-1 mg/kg Se in poultry diets can improve heat stress-induced antioxidant status (Khoi et al., 2021). Supplementation with Se (0.3-0.5 mg/kg) in heat-stressed broiler diets increased the activities of SOD and GPx and decreased MDA in serum (Xu et al., 2014). Sahin et al. (2008) found that supplementation with 0.3 mg/kg Se in heat-stressed quail diets improved growth performance and decreased serum and liver MDA concentrations. A study showed that supplementation with 0.3 mg/kg Se in diets of broiler chickens under HS increased the levels of TNF-α, IL-4, TNF-γ and IL-2 (Habibian et al., 2015). Moreover, nicotinamide adenine dinucleotide phosphate (NADPH) levels were elevated due to Se supplementation, which further promotes glutathione reductase activation, resulting in increased GSH-Px production (Zhao et al., 2018).

2.4.4 L-carnitine

L-carnitine is a water-soluble product found in animals, plants, and microorganisms. It is synthesized from two important amino acids, lysine and methionine. In essence, L-carnitine plays an intermediary role in metabolism, promotes cell energy metabolism, regulates the concentration of coenzyme A in cytoplasm and mitochondria, and plays an important role in glycolipid metabolism. In addition, L-carnitine reduces liver toxicity and enhances antioxidant capacity (Abu-El-Zahab et al., 2019). Dietary supplementation with 100-160 mg/kg L-carnitine can improve heat-stressed broiler feed intake and feed conversion ratio, and reduce serum triglyceride and cholesterol levels (Kuter & Onol, 2021; Qiao et al., 2021). In addition, Supplementation with 100 mg/kg L-carnitine reduced MDA in heart tissue of broilers raised under low temperature environment, and increased SOD and GSH-Px activities (Wang et al., 2013). In addition, supplementation with 50 mg/kg L-carnitine in the diet increased sheep red blood cell (SRBC) antibody titers in broiler chickens under HS (Rehman et al., 2017). Moreover, Yousefi et al. (2023) showed that dietary supplementation with 0.5g/kg L-carnitine increased average daily gain, reduced the concentration of MDA in serum and the depth of jejunal crypts in heat-stressed broilers.

2.4.5 The combination of selenium with vitamin E, or vitamin C

High temperature will affect the absorption of vitamins A, C, and E, and reduce the concentration of iron, zinc, and Se in tissues and blood; therefore, vitamins and trace elements should be supplemented in the diet at this time to maintain the normal requirements of the poultry body. However, based on the available literature, there is currently a limitation on the simultaneous supplementation of vitamins E, C, Se, and carnitine to alleviate HS in poultry. However, the combination of selenium with vitamin E, or with vitamin C, has been shown to have a positive facilitative effect in reducing the negative effects of heat stress in poultry. Studies have found that supplementing selenium and vitamin E (0.5mg/kg+250mg/kg) or (0.5mg/kg+150mg/kg) in heat-stressed broiler diets improved antibody responses to some diseases and improved performance production (FI, weight gain, and FCR) and antioxidant capacity (Ghazi Harsini et al., 2012; Habibian et al., 2014). In addition, Ajakaiye et al. (2011) reported that supplementation with 150 mg Vit C + 150 mg Vit E in heat-stressed layer diets significantly improved laying performance and egg quality. Supplementation with vitamin C (200 mg/kg

diet) and vitamin E (100 mg/kg diet) to broiler chickens diets increased total antioxidant capacity, SOD, and GPx enzyme activity under HS (Hosseini-Mansoub et al., 2010). Dietary supplementation with 15000IU Vit A + 30 mg Se increased feed intake and body weight and reduced feed conversion ratio and lipid peroxidation in heat-stressed broilers (Kucuk et al., 2003). In addition, Calik et al. (2022) found that supplantation with combination vitamins E (250 mg/kg) and Se (1 mg/kg) significantly downregulated the mRNA levels of HSPs in liver and jejunal tissues of the HS-challenged birds both on d 28 and d 35, while mRNA abundance of TLR2, TNFα, IFNγ, IL-1β, IL-10, and iNOS in the liver was significantly downregulated in birds fed the vitamin E, Se diet on d 35. Moreover, *Lachnospiraceae FE2018* and *Ruminococcaceae NK4A214* groups were enriched in the vitamin E, Se birds on day 35. The combination of vitamins E, C, and Se effectively reduces the impact of heat stress on poultry, and the effect is better when these additives are used alone.

2.5 Mechanism of action of phytogenic compounds to mitigate oxidative stress and heat stress

Phytogenic compounds can act in several ways to scavenge ROS. For example, Sandoval-Acuña et al. (2014) defined two kinds of ROS-scavenging activities: direct activities to scavenge ROS or indirect activities by inducing the synthesis of ROS-removing enzymes (e. g, SOD, CAT). Figure 2.2 schematically shows these activities. Polyphenols also have the ability to directly chelate transition metal ions, especially Fe^{2+} and Cu^{2+}, which can generate highly reactive oxygen radicals (Karamać, 2009).

It is generally known that HS can induce free radicals in the animal body, leading to oxidative stress and reduced production performance (Chauhan et al., 2021). The antioxidant properties of phytogenic additives can be attributed to their chemical structure, including the presence of hydroxyl groups attached to the benzene ring, which are good hydrogen donors (Saracila et al., 2021). In the case of polyphenols and flavonoids, it was reported that the B ring hydroxyl structure has a major role in the activity of free radical scavenging (Salehi et al., 2020). Polyphenols participate in the elimination of numerous ROS and RNS, such as hydroxyl radicals, peroxyl radicals, hypochlorous acids, superoxide anions, and proximities (Halliwell, 2006) by transferring the H atom from the

OH group (polyphenols) or a single electron to the free radical or a transition metal ion (Sandoval-Acuña et al., 2014; Papuc et al., 2017) as shown in Figure 2.3.

Phytogenic additives are rich in catechins, polyphenols, flavonoids and volatile oils, gallic acid, tannins, and flavonoids; this has been shown to alleviate the negative effects of HS (Chauhan et al., 2021). Most bioactive polyphenol compounds have been assessed partly through biological properties and bioavailability, especially strong radical scavenging activities for their antioxidant ability which is dependent on the quantity and quality of polyphenol compounds in each plant species that act as reducing agents, hydrogen donors, singlet oxygen quenchers, metal chelators and reductants of ferryl hemoglobin (Lee et al., 2016). Polyphenols elevate the expression of stress response proteins such as heat shock proteins and antioxidant enzymes, which can suppress ROS and interfere with many players of HS responses (Yin et al., 2021). Phytogenic compounds enhance the expression of antioxidant enzymes mainly by activating nuclear factor erythroid 2-related factor 2 (Nrf2) (Saracila et al., 2023). Normally, Nrf2 is combined with kelch-like epichlorohydrin-related protein 1 (Keap1) in the cytoplasm, where it is inactive at this time; however, after cells are treated with phytogenic additives, Nrf2 will separate from Keap1 and translocate to the nucleus, it plays a role in up-regulating the gene expression of antioxidant defense enzyme 1 (NQO1) and heme oxygenase 1 (HO1), thereby enhancing the expression of antioxidant enzymes, such as SOD, CAT, GSH-Px, GR, and GST, etc (Kurutas, 2015) (Figure 2.2 and 2.3). Firstly, SOD can scavenge $O_2^{\bullet-}$ free radicals, and secondly, in the presence of CAT, H_2O_2 can rapidly decompose to produce active oxygen such as $O_2^{\bullet-}$ and $HO^{\bullet}$, which scavenging free radicals; thirdly, glutathione peroxidase (the sulfhydryl group (-SH) in GSH-Px) provides reduced hydrogen, gives free radicals a paired electron, and makes free radicals lose their strong oxidative and erosive properties (Calik et al., 2022). Glutathione itself becomes the oxidation state of this disulfide bond, and then, through the reduced hydrogen, is converted into reduced glutathione, thus continuously circulating and continuing to exert its antioxidant effect (Georgiou-Siafis & Tsiftsoglou, 2023). On the other hand, due to the special molecular structure of the main active species, Fe^{2+} can be released by increasing the Fenton reaction to generate more $OH^{\bullet}$ and H_2O_2 (Zhou et al., 2021). In addition, since the main active substances of phytogenic are polyphenols, polyphenols (Phen-OH) react with free radicals ($R^{\bullet}$) to generate active oxygen, including superoxide ions ($O_2^{\bullet-}$) and hydroxyl

radicals ($OH^{\bullet}$), thus scavenging free radicals (Guo et al., 1999; Michalak, 2006) (Figure 2.3). HSP (HSP70) reduces the release of oxygen free radicals and increases the activity of SOD, thereby scavenging free radicals (Figure 2.2).

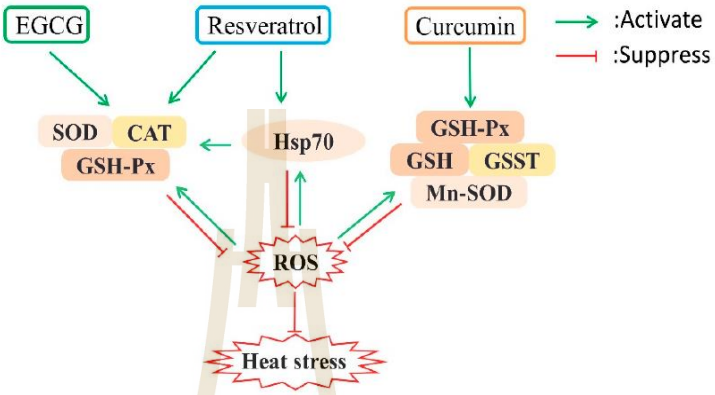


Figure 2.2 Potential mechanisms underlying the protective effect of polyphenols against heat stress (Saracila et al., 2021).

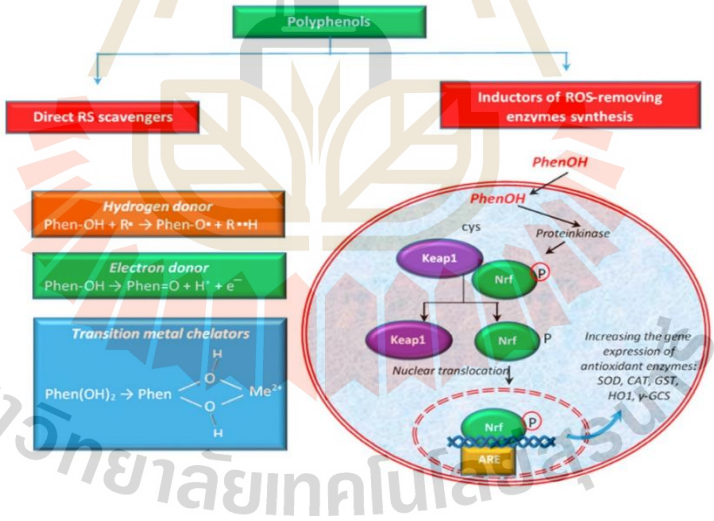


Figure 2.3 Role of polyphenols in scavenging reactive oxygen species and stimulating antioxidant enzymes (Saracila et al., 2021).

2.6 Effects of phytogenic supplementation to mitigate the negative effects of heat stress in broilers.

HS increased intestinal inflammation, oxidative stress, and reduced antioxidant and immune markers in broilers. Phytogenic antioxidants in the diet help alleviate stress in broilers. Under stress, phytogenic antioxidants contain bioactive components that can improve immunity, antioxidant capacity, enhance gut microbiota and health, and reduce oxidative/inflammatory pathways (Yang et al., 2021; Reith et al., 2022), making animals more resistant to external stress (Al-Garadi et al., 2023). Previous studies found that most phytogenic antioxidant substances are mostly supplemented in both broiler and laying hen diets under HS and improved the total antioxidant capacity (T-AOC), GSH, GSH-PX, SOD and CAT, while lipid peroxidation, MDA and nitric oxide (NO) reduced in blood and tissues (El-Maaty et al., 2014; Hosseini-Vashan et al., 2016; Ibtisham et al., 2019; Reis et al., 2019). Studies have shown that the supplementation with *Artemisia annua* (0.75-1.25 g/kg) significantly increased body weight, reduce oxidative stress biomarkers (MDA, corticosterone), and improved liver function (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]) and antioxidant capacity (Wan et al., 2017). Olive oil or its leaf extract enhanced the health and redox status balance of chickens by increasing plasma SOD levels and decreasing MDA content (Lee et al., 2016). In addition, Fatima et al. (2022) explored the effect of fennel seeds (20-25 g/kg) on improving growth, antioxidant status, carcass characteristics, and immune responses in broiler chickens under HS conditions. Likewise, Khalil et al. (2020) demonstrated that supplementation with thyme essential oil (150 or 200 mg/kg) in broilers' diets resulted in enhanced growth performance, improved immune function, and favorable alterations in blood metabolites. Moreover, it contributed to a reduction in stress-related biomarkers, including corticosterone and malondialdehyde (MDA), under HS conditions. The beneficial impact of rosemary on productivity and the health of broilers under stress conditions can be ascribed to its potential to augment antioxidant activity (Hosseinzadeh et al., 2023), regulate the composition of intestinal microbiota (Liu et al., 2022), enhance intestinal morphology, boost immune function (Rostami et al., 2018), and improve plasma biochemical parameters in broilers (Torki et al., 2018). Mirzaei et al. (2023) found that fennel nanoemulsion significantly

improved the antioxidant capacity and immunity of stressed broilers. At the same time, it also led to a decrease in *Escherichia coli* levels and an increase in the levels of beneficial bacteria such as *Lactobacillus*. Song et al. (2017) showed that supplementation with 1 g of *Artemisia annua* to heat-stressed broilers (34 ± 1°C) reduced plasma diamine oxidase (DAO) activity, *HSP70* mRNA expression, and *TLR-4*, *IL-6*, *IL-1*β, and *IFN-*γ expression in intestinal tissues. In the intestinal mucosa, mature epithelial cells showed higher oxidase activity. Moreover, rosemary modulates intestinal microbiota (Liu et al., 2022), improves intestinal morphology, and enhances immune activity (Rostami et al., 2018), which may be attributed to its enhanced antioxidant activity (Hosseinzadeh et al., 2023). Phytogenics have demonstrated potential in mitigating the negative effects of HS in chickens by promoting gut health, preserving microbiota balance, attenuating inflammatory and oxidative stress pathways, enhancing immune function, boosting antioxidant capacity, and improving production performance. Nonetheless, additional research is required to explore the molecular mechanisms underlying phytogenics, as well as the interactions between their active components, gut microbiota, and the gut barrier. These studies may enhance broiler welfare and foster a more sustainable and efficient poultry industry. The supplementation of phytogenic substances to broilers to mitigate the negative effects of HS is summarized in Table 2.2.

Table 2.2 Effects of phytogenic supplementation to mitigate the negative effects of heat stress in broilers.

Spices and Conditions	Phytogenic substances	Results	Bioactive Compounds and References
Broilers, TN (24°C, 50% RH), HS (36°C, 78% RH, 2 h/day) for 0 days	Basal diets (control) Basal diets (HS) PFA-C 250 ppm (encapsulated essential oils, dried herbs and spices, saponins, and anticaking agents) under HS PFA-C 400 ppm (encapsulated essential oils, dried herbs and spices, saponins, and anticaking agents) under HS	PFA 250 upregulated the expression of SOD1 and downregulated GPX-3, but was unchanged for PFA-C400. PFA 400 decreased TNFα levels. HSP and HSF were unaffected by PFA. Total antioxidant capacity, MDA content was increased by HS, but were unchanged for PFA. Expression of IL-18 is unaffected by PFA.	Phenolic compounds PFA-C exhibited a significantly higher antioxidant capacity. 8.9- and 15.5-fold increase for PFA-C250 and PFA-C400, respectively. (Greene et al., 2021).
Broilers, TN (21°C, 55% RH), HS (34°C, 55% RH, 4h/day) for 28 days	Basal diets (control) Basal diets (HS) 3% Dried tomato pomace DTP under HS 5% Dried tomato pomace DTP under HS	Enhanced GSP-PX and SOD Reduced MDA in plasma by 5% DTP	3 and 5% of DTP contains 420 and 708 mg lycopene/kg diet, respectively. (Hosseini-Vashan et al., 2015).

Table 2.2 (continued).

Spices and Conditions	Phytogenic substances	Results	Bioactive Compounds and References
Broilers, HS (32±2°C, 50±5% RH 24h/day) for 14 days	Basal diets (control) Basal diets (HS) 50 (R1) and 100 (R2) mg/kg of rosemary leaves extract (RLE) under HS 50 (O1) and 100 (O2) mg/kg of oregano leaves extract (OLE) under HS	Total lipids and LDL-cholesterol levels decreased in the R2 and O2 groups. Total antioxidant capacity increased in the R1 and R2 groups. The mRNA expression of HSP70 and HSP90A was downregulated in R1, R2, O1, and O2 groups.	Ferruginol, triterpenoid diterpenoid, sesquiterpene, eucalyptol, camphor, and monoterpenoids. (Madkour et al., 2024).
Broilers, TN (24°C, 55% RH), HS (35°C, 50-60% RH 8h/day) for 28 days	Basal diets (control) Basal diets (HS) Mixture of thymol and carvacrol (60, 100, and 200 mg/kg) under HS	The addition of this mixture improved the GPx and SOD, pancreatic enzymes (trypsin, lipase, and protease) and immune markers.	Thymol, carvacrol, resin, tannins, steroids, saponins, flavonoids, and alkaloids. (Mahasneh et al., 2024).
Broilers, TN (22°C 24 h/day), HS (33°C, 60-70% RH 10h/day) for 21 days	Basal diets (control) (TN) Basal diets (HS) Inclusion of 400 mg/kg resveratrol under HS	The HS+ resveratrol increased serum IgY, IgA, and IL-10 contents, while lowering splenic TLR4, TNF-α, IL-1β, and NF-κB mRNA levels.	Resveratrol, extracted from a variety of plants. (Li et al., 2023).

Table 2.2 (continued).

Spices and Conditions	Phytogenic substances	Results	Bioactive Compounds and References
Broilers, TN (22-28°C), HS (32-38°C, 60-80% RH)	Basal diets (control) (TN) Basal diets (HS) HS+1% Ginger powder HS+0.332% Herbal medicine HS+0.1% Ginger powder + 0.332 g/kg Herbal medicine	Improved SOD, NO, T-AOC, MDA, and GSH-PX in serum by supplemented groups Improved CAT in serum by HS + Chinese herbal medicine and ginger powder + Chinese herbal medicine	Ginger: gingerol, gingerdione, and shogaols; Chinese herbal medicine: vitamins, lipids, amino acids, proteins, trace elements. (Ibtisham et al., 2019).
Broilers, TN (22°C), HS (34°C, 50-60% RH 8h/ day) for 20 days	Basal diets (control) (TN) Basal diets (HS) HS+1 g/kg *Artemisia annua* (EA)	EA decreased the expression of HSP70, TLR4, IL-6, IL-1β, and INF-γ in the intestine, whereas it increased jejunal zonula occludens-1 and occluding.	*Artemisia annua* contains antioxidant compounds such as flavonoids and phenolics. (Song et al., 2017).
Broilers, TN (24°C), HS (34°C, 60% RH 4h/ day)	Basal diets (control) (TN) HS+ probiotics 2 g/kg (PRO) HS+ clove essential oil 300 mg/kg (CEO) HS + PRO and CEO (PC)	decrease in TNF-α in all supplemented groups compared with the control. SOD and GPx levels were elevated and the MDA level value decreased.	The probiotic mixture L*actobacillus Acidophilus* and *Bacillus Subtilis*. eugenol, gallic acid, catechin. (Elbaz et al., 2023).

Table 2.2 (continued).

Spices and Conditions	Phytogenic substances	Results	Bioactive Compounds and References
Broilers, TN (28°C), HS (35°C, 70% RH 12h/ day)	Basal diets (control) (TN) HS+Epigallocatechin-3-gallate (EGCG) 0 mg/kg HS+EGCG 300 mg/kg HS+EGCG 600 mg/kg	Supplemented with EGCG increased VH, VH/CD (V/C), and the activities of GSH-Px, SOD and CAT, and decresed the crypt depth and MDA content. Supplementation increases the gene expression of *Nrf2*, *Claudin-1*, and Mucin 2, and reduces the *NF-κB*.	Epigallocatechin-3-gallate of green tea. (Song et al., 2019).

Abbreviation: TN, thermoneutral condition; HS, heat stress condition; PFA, phytogenic feed additive; SOD1, superoxide dismutase 1; GPX-3, glutathione peroxidase-3; HSP, heat shock proteins; HSF, heat shock factors; MDA, malondialdehyde; IL-18, interleukin-18; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor alpha; IL-1β, interleukin-1 beta; NF-κB, nuclear Factor kappa B; NO, nitric oxide; T-AOC, total Antioxidant Capacity; CAT, catalase; INF-γ, interferon-γ; Nrf2, nuclear factor-erythroid 2-related factor 2.

2.7 The application of the transcriptomic technique in animal research

The study of transcriptomes (RNA-seq)-the complete set of RNA molecules transcribed in an organism-provides insight into gene expression, regulation, and cellular processes. In recent years, RNA-seq (Nagalakshmi et al., 2008), a methodology for RNA profiling based on next-generation sequencing (NGS) (Shendure & Ji, 2008), is replacing microarrays for the study of gene expression. In animal research, transcriptomics technologies have facilitated the comprehensive analysis of gene activity under different conditions, such as disease states, environmental stressors, and genetic mutations (Qian et al., 2014). This has proven instrumental in understanding complex biological phenomena that are difficult to elucidate with traditional genomic approaches. Recent advancements in transcriptomics technologies, including RNA-Seq, microarray-based techniques, and single-cell sequencing, have significantly enhanced the scope and depth of animal research. Transcriptome sequencing has been used in several species, including poultry, cattle, and pigs, to identify genes that play key roles in responses to different conditions (Coble et al., 2014; Srikanth et al., 2017). RNA-seq has become the gold standard for transcriptomic analysis due to its ability to generate highly accurate and detailed gene expression profiles. Unlike microarrays, RNA-Seq does not rely on pre-existing knowledge of the genome and can identify novel transcripts, alternative splicing events, and rare RNA species. Studies utilizing RNA-Seq in animal models have provided critical insights into the molecular mechanisms underlying various physiological processes and diseases (Wang et al., 2009). Studies have used RNA-seq to investigate gene expression profiles in response to avian influenza (AI) and Newcastle disease (ND), providing insights into the molecular mechanisms of immunity in chickens (Wang et al., 2014). By profiling immune-related genes, researchers have been able to identify genetic markers associated with resistance to these diseases. In addition, RNA-seq analysis in poultry has also been used to study muscle development and fat deposition. For instance, the differential expression of genes involved in myogenesis and adipogenesis has been studied to identify potential targets for improving meat yield and quality in broilers (Malila et al., 2022). Transcriptomic studies in dairy cattle have provided insights into the molecular basis of lactation. RNA-Seq has been employed to investigate gene expression in mammary tissue during different stages of lactation, identifying key regulatory genes

involved in milk synthesis and secretion (Seo et al., 2016). This information has led to a better understanding of factors affecting milk yield and quality. Previous studies using RNA-Seq have revealed that genes such as *FLNC*, *COL1A1*, *NRAP*, *SMYD1*, *TNNI3*, *CRYAB* and *PDLIM3* played vital roles in the muscle growth, and genes such as *CCDC71L*, *LPIN1*, *CPT1A*, *UCP3*, *NR4A3* and *PDK4* played dominant roles in the lipid metabolism in Shaziling pigs (Zheng et al., 2024). Moreover, RNA-seq is also used in the animal models field, animal models are indispensable in understanding the molecular mechanisms of diseases such as cancer, neurological disorders, and metabolic diseases. For instance, RNA-Seq has been used to investigate the gene expression alterations in mouse models of Alzheimer's disease, offering new targets for therapeutic intervention (Wan et al., 2020). Similarly, transcriptomics has been used to uncover genetic factors involved in obesity and type 2 diabetes in rodent models (Agueda-Oyarzabal et al., 2025). However, one of the major challenges in transcriptomics is the sheer complexity of the data. For example, integrating transcriptomic data with other omics technologies (proteomics, metabolomics) remains a significant hurdle in comprehensive systems biology studies (Jendoubi, 2021). Despite its advantages, RNA-Seq is still associated with several technical challenges, such as bias in transcript quantification, particularly for low-abundance transcripts. As transcriptomics technologies continue to evolve, several advancements hold promise for the future of animal research. These include improvements in single-cell sequencing, better integration of multi-omics data, and advancements in spatial transcriptomics that allow the mapping of gene expression within intact tissues (Kleino et al., 2022).

2.8 Transcriptome responses to heat stress for gene marker identification in poultry research

HS can induce a change in the physiology and metabolism of poultry. In response to HS, poultry activate various stress response mechanisms at the molecular level, including changes in gene expression, protein synthesis, and metabolic pathways. Transcriptomics, the study of the complete set of RNA transcripts, has provided valuable insights into these molecular responses. Transcriptome analysis based on RNA-seq can contribute to an improvement in the current understanding of the

molecular and functional mechanisms of physiological changes in poultry exposed to HS conditions. Previous studies using RNA-seq have shown that HS leads to increased expression of genes related to various nutrient metabolism and HSPs in the liver of poultry (Xie et al., 2014). By sequencing the RNA content of cells under stress conditions, RNA-Seq allows for the identification of differentially expressed genes (DEGs), alternative splicing events, and the characterization of non-coding RNAs that contribute to stress responses (Wang et al., 2009). In poultry, RNA-Seq has been used to investigate the transcriptional changes in various tissues, including the liver, small intestine, muscle, and brain, under heat stress (Kim et al., 2022; Wu et al., 2024; Zhu et al., 2025). HS induces the upregulation of HSPs, which act as molecular chaperones to protect cellular proteins from denaturation. The transcriptomic analysis of broilers and breeder hens in jejunal mucosa under HS has highlighted key HSPs, including HSP40 (*DNAJA1*), HSP70 (*HSPA2* and *HSPA8*), HSP90 (*HSP90AA1*), and *HSP110*, as important markers of heat stress tolerance (Kim et al., 2022; Zhu et al., 2025). It has been reported that genes related to immune responses and glutathione metabolism in the small intestine are affected by HS. Kim et al. (2022) found that upregulated glutathione-S-transferases (*GSTA3* and *GSTA4*) and downregulated interleukin-1 beta (*IL1B*) and interleukin-1 receptor type 2 (*IL1R2*) in the jejunal mucosa of heat-stressed broilers, which suggests that HS conditions may impair mucosal integrity and functions and decrease the immune systems. Moreover, RNA-seq analysis between native and commercial chicken breeds to HS response found that the expression of *PT1A* and *ANGPTL4* genes in native chickens, and *HSP90B1* and *HSPA5* genes in commercial chickens could be potential candidate genes involved with HS. These genes exhibited enriched pathways related to metabolic activity and inflammatory reactions (Barreto Sánchez et al., 2022). Hosseinzadeh & Hasanpur (2023) found that under acute HS, the endoplasmic reticulum chaperone complex (*HSPA5*, *SDF2L1*) was inhibited, and *SSR1* and *SEC23B* genes were downregulated, suggesting that acute HS may lead to protein structure disruption, protein binding, protein transport, protein formation, and degradation of misfolded proteins. Furthermore, transcriptome analysis in the jejunal mucosa in breeder hens under thermoneutral (23°C) and HS (36°C for 6 h) indicated that the DEGs of *HSPA2*, *DNAJA4*, *HSP90AA1*, *PDK4*, *SLC10A2*, *PPARA*, and *CD36* were associated with steroid biosynthesis, steroid hormone biosynthesis, protein processing

in endoplasmic reticulum, the peroxisome proliferator-activated receptor signaling pathway, and the adipocytokine signaling pathway, which contribute to a deeper understanding of the jejunal mucosal response in breeder hens to acute HS (Zhu et al., 2025). Therefore, transcriptome sequencing provides a powerful tool to analyze the molecular mechanism of poultry that are exposed to HS conditions, and RNA-seq analysis identified key pathways and candidate genes that can be used as indicators to monitor acute or chronic HS responses in poultry and may provide strategies for the development of heat-resistant strains.

2.9 References

Abdelnour, S. A., Abd El-Hack, M. E., Khafaga, A. F., Arif, M., Taha, A. E., & Noreldin, A. E. (2019). Stress biomarkers and proteomics alteration to thermal stress in ruminants: A review. **Journal of Thermal Biology**, 79, 120–134.

Abdelqader, A. M., Abuajamieh, M., Hammad, H. M., & Al-Fataftah, A. R. A. (2017). Effects of dietary butyrate supplementation on intestinal integrity of heat-stressed cockerels. **Journal of Animal Physiology and Animal Nutrition**, 101(6), 1115–1121.

Abu-El-Zahab, H. S. H., Hamza, R. Z., Montaser, M. M., El-Mahdi, M. M., & Al-Harthi, W. A. (2019). Antioxidant, antiapoptotic, antigenotoxic, and hepatic ameliorative effects of L-carnitine and selenium on cadmium-induced hepatotoxicity and alterations in liver cell structure in male mice. **Ecotoxicology and Environmental Safety**, 173(22), 419–428.

Agueda-Oyarzabal, M., Isidor, M. S., Pluciński, K., Ingerslev, L. R., Dmytriyeva, O., Petersen, P. S. S., Laftih, S., Pontoppidan, A. B., Henningsen, J. B., Rupar, K., Brown, E. L., Schwartz, T. W., Barrès, R., Gerhart-Hines, Z., Schéele, C. C., & Emanuelli, B. (2025). Transcriptomic signatures of cold acclimated adipocytes reveal CXCL12 as a Brown autocrine and paracrine chemokine. **Molecular Metabolism**, 93(12), 102102.

Ajakaiye, J. J., Perez-Bello, A., & Mollineda-Trujillo, A. (2011). Impact of heat stress on egg quality in layer hens supplemented with l-ascorbic acid and dl-tocopherol acetate. **Veterinarski arhiv**, 100(3), 401–412.

Akbarian, A., Michiels, J., Degroote, J., Majdeddin, M., Golian, A., & De Smet, S. (2016). Association between heat stress and oxidative stress in poultry; mitochondrial dysfunction and dietary interventions with phytochemicals. **Journal of animal science and biotechnology**, 7(1), 1-14.

Al-Fataftah, A.-R., & Abdelqader, A. (2014). Effects of dietary Bacillus subtilis on heat-stressed broilers performance, intestinal morphology and microflora composition. **Animal Feed Science and Technology**, 198 (9), 279–285.

Al-Garadi, M. A., Suliman, G. M., Hussein, E. O., Al-Owaimer, A. N., Swelum, A. A., Almalamh, N. A., Alhotan, R. A., & Qaid, M. M. (2023). The effects of betaine and nano-emulsified plant-oil supplementation on growth performance and serum biochemistry indices of heat-stressed broiler chickens. **Italian Journal of Animal Science**, 22(1), 398–406.

Algothmi, K. M., Mahasneh, Z. M. H., Abdelnour, S. A., Khalaf, Q. A. W., Noreldin, A. E., Barkat, R. A., Khalifa, N. E., Khafaga, A. F., Tellez-Isaias, G., Alqhtani, A. H., Swelum, A. A., & Abd El-Hack, M. E. (2024). Protective impacts of mitochondria enhancers against thermal stress in poultry. **Poultry Science**, 103(1), 103218.

Alhenaky, A., Abdelqader, A., Abuajamieh, M., & Al-Fataftah, A.-R. (2017). The effect of heat stress on intestinal integrity and Salmonella invasion in broiler birds. **Journal of Thermal Biology**, 70 (6), 9–14.

Attia, Y. A., Hassan, R. A., Tag El-Din, A. E., & Abou-Shehema, B. M. (2011). Effect of ascorbic acid or increasing metabolizable energy level with or without supplementation of some essential amino acids on productive and physiological traits of slow-growing chicks exposed to chronic heat stress. **Journal of Animal Physiology and Animal Nutrition**, 95(6), 744–755.

Bamias, G., Arseneau, K. O., & Cominelli, F. (2014). Cytokines and mucosal immunity. **Current Opinion in Gastroenterology**, 30(6), 547–552.

Barreto Sánchez, A. L., Wang, Q., Thiam, M., Wang, Z., Zhang, J., Zhang, Q., Zhang, N., Li, Q., Wen, J., & Zhao, G. (2022). Liver Transcriptome Response to Heat Stress in Beijing You Chickens and Guang Ming Broilers. **Genes**, 13(3), 416.

Bhat, S., Kumar, P., Kashyap, N., Deshmukh, B., Dige, M. S., Bhushan, B., Chauhan, A., Kumar, A., & Singh, G. (2016). Effect of heat shock protein 70 polymorphism on thermotolerance in Tharparkar cattle. **Veterinary World**, 9(2), 113–117.

Burkholder, K. M., Thompson, K. L., Einstein, M. E., Applegate, T. J., & Patterson, J. A. (2008). Influence of Stressors on Normal Intestinal Microbiota, Intestinal Morphology, and Susceptibility to Salmonella Enteritidis Colonization in Broilers. **Poultry Science**, 87(9), 1734–1741.

Calik, A., Emami, N. K., Schyns, G., White, M. B., Walsh, M. C., Romero, L. F., & Dalloul, R. A. (2022). Influence of dietary vitamin E and selenium supplementation on broilers subjected to heat stress, Part II: Oxidative stress, immune response, gut integrity, and intestinal microbiota. **Poultry Science**, 101(6), 101858.

Chaudhary, A., Mishra, P., Amaz, S. A., Mahato, P. L., Das, R., Jha, R., & Mishra, B. (2023). Dietary supplementation of microalgae mitigates the negative effects of heat stress in broilers. **Poultry Science**, 102(10), 102958.

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.

Chen, F., Zhang, H., Zhao, N., Yang, X., Du, E., Huang, S., Guo, W., Zhang, W., & Wei, J. (2021). Effect of chlorogenic acid on intestinal inflammation, antioxidant status, and microbial community of young hens challenged with acute heat stress. **Animal Science Journal**, 92(1), e13619.

Chen, J., Tellez, G., Richards, J. D., & Escobar, J. (2015). Identification of Potential Biomarkers for Gut Barrier Failure in Broiler Chickens. **Frontiers in Veterinary Science**, 2(15), e00014.

Cheng, K., Yan, E., Song, Z., Li, S., Zhang, H., Zhang, L., Wang, C., & Wang, T. (2019). Protective effect of resveratrol against hepatic damage induced by heat stress in a rat model is associated with the regulation of oxidative stress and inflammation. **Journal of Thermal Biology**, 82(12), 70–75.

Coble, D. J., Fleming, D., Persia, M. E., Ashwell, C. M., Rothschild, M. F., Schmidt, C. J., & Lamont, S. J. (2014). RNA-seq analysis of broiler liver transcriptome reveals novel responses to high ambient temperature. **BMC Genomics**, 15(1), 1084.

Dangi, S. S., Gupta, M., Nagar, V., Yadav, V. P., Dangi, S. K., Shankar, O., Chouhan, V. S., Kumar, P., Singh, G., & Sarkar, M. (2014). Impact of short-term heat stress on

physiological responses and expression profile of HSPs in Barbari goats. **International Journal of Biometeorology**, 58(10), 2085–2093.

Dokladny, K., Ye, D., Kennedy, J. C., Moseley, P. L., & Ma, T. Y. (2008). Cellular and Molecular Mechanisms of Heat Stress-Induced Up-Regulation of Occludin Protein Expression. **The American Journal of Pathology**, 172(3), 659–670.

Du, M., Cheng, Y., Chen, Y., Wang, S., Zhao, H., Wen, C., & Zhou, Y. (2022). Dietary supplementation with synbiotics improves growth performance, antioxidant status, immune function, and intestinal barrier function in broilers subjected to cyclic heat stress. **Environmental Science and Pollution Research**, 30(7), 18026–18038.

Ducatelle, R., Goossens, E., De Meyer, F., Eeckhaut, V., Antonissen, G., Haesebrouck, F., & Van Immerseel, F. (2018). Biomarkers for monitoring intestinal health in poultry: Present status and future perspectives. **Veterinary Research**, 49(1), 43.

Elbaz, A. M., Ashmawy, E. S., Ali, S. A. M., Mourad, D. M., El-Samahy, H. S., Badri, F. B., & Thabet, H. A. (2023). Effectiveness of probiotics and clove essential oils in improving growth performance, immuno-antioxidant status, ileum morphometric, and microbial community structure for heat-stressed broilers. **Scientific Reports**, 13(1), 18846.

El-Maaty, A., Hayam, M. A., Rabie, M. H., & El-Khateeb, A. Y. (2014). Response of Heat-Stressed Broiler Chicks to Dietary Supplementation with Some Commercial Herbs. **Asian Journal of Animal and Veterinary Advances**, *9*(12), 743–755.

Fang, X., Nong, K., Qin, X., Liu, Z., Gao, F., Jing, Y., Fan, H., Wang, Z., Wang, X., & Zhang, H. (2023). Effect of purple sweet potato-derived anthocyanins on heat stress response in Wenchang chickens and preliminary mechanism study. **Poultry Science**, 102(9), 102861.

Farooqi, H. A. G., Khan, M. S., Khan, M. A., Rabbani, M., Pervez, K., & Khan, J. A. (n.d.). Evaluation of Betaine and Vitamin C in Alleviation of Heat Stress in Broilers. **International Journal of Biometeorology**, 65(7), 1231–1244.

Fatima, A., Chand, N., Naz, S., Saeed, M., Khan, N. U., & Khan, R. U. (2022). Coping heat stress by crushed fennel (Foeniculum vulgare) seeds in broilers: Growth, redox balance and humoral immune response. **Livestock Science**, 265 (11), 105082.

Georgiou-Siafis, S. K., & Tsiftsoglou, A. S. (2023). The Key Role of GSH in Keeping the Redox Balance in Mammalian Cells: Mechanisms and Significance of GSH in Detoxification via Formation of Conjugates. **Antioxidants**, 12(11), 1953.

Ghazi Harsini, S., Habibiyan, M., Moeini, M. M., & Abdolmohammadi, A. R. (2012). Effects of Dietary Selenium, Vitamin E, and Their Combination on Growth, Serum Metabolites, and Antioxidant Defense System in Skeletal Muscle of Broilers Under Heat Stress. **Biological Trace Element Research**, 148(3), 322–330.

Goncalves, R. L. S., Watson, M. A., Wong, H.-S., Orr, A. L., & Brand, M. D. (2020). The use of site-specific suppressors to measure the relative contributions of different mitochondrial sites to skeletal muscle superoxide and hydrogen peroxide production. **Redox Biology**, 28 (16), 101341.

Greene, E. S., Cauble, R., Kadhim, H., De Almeida Mallmann, B., Gu, I., Lee, S.-O., Orlowski, S., & Dridi, S. (2021). Protective effects of the phytogenic feed additive "comfort" on growth performance via modulation of hypothalamic feeding- and drinking-related neuropeptides in cyclic heat-stressed broilers. **Domestic Animal Endocrinology**, 74 (14), 106487.

Gu, X. H., Hao, Y., & Wang, X. L. (2012a). Overexpression of heat shock protein 70 and its relationship to intestine under acute heat stress in broilers: 2. Intestinal oxidative stress. **Poultry Science**, 91(4), 790–799.

Guo, Q., Zhao, B., Shen, S., Hou, J., Hu, J., & Xin, W. (1999). ESR study on the structure–antioxidant activity relationship of tea catechins and their epimers. **Biochimica et Biophysica Acta (BBA) - General Subjects**, 1427(1), 13–23.

Habashy, W. S., Milfort, M. C., Fuller, A. L., Attia, Y. A., Rekaya, R., & Aggrey, S. E. (2017). Effect of heat stress on protein utilization and nutrient transporters in meat-type chickens. **International Journal of Biometeorology**, 61(12), 2111–2118.

Habibian, M., Ghazi, S., Moeini, M. M., & Abdolmohammadi, A. (2014). Effects of dietary selenium and vitamin E on immune response and biological blood parameters of broilers reared under thermoneutral or heat stress conditions. **International Journal of Biometeorology**, 58(5), 741–752.

Habibian, M., Sadeghi, G., Ghazi, S., & Moeini, M. M. (2015). Selenium as a Feed Supplement for Heat-Stressed Poultry: A Review. **Biological Trace Element Research**, 165(2), 183–193.

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.

He, S., Yu, Q., He, Y., Hu, R., Xia, S., & He, J. (2019). Dietary resveratrol supplementation inhibits heat stress-induced high-activated innate immunity and inflammatory response in spleen of yellow-feather broilers. **Poultry Science**, 98(12), 6378–6387.

He, Y., Maltecca, C., & Tiezzi, F. (2021). Potential Use of Gut Microbiota Composition as a Biomarker of Heat Stress in Monogastric Species: A Review. **Animals**, 11(6), 1833.

Hidayat, D. F., Mahendra, M. Y. N., Kamaludeen, J., & Pertiwi, H. (2023b). Lycopene in Feed as Antioxidant and Immuno-Modulator Improves Broiler Chicken's Performance under Heat-Stress Conditions. **Veterinary Medicine International**, 2023(1), 5418081.

Hoek, J. B., & Pastorino, J. G. (2002). Ethanol, oxidative stress, and cytokine-induced liver cell injury. **Alcohol**, 27(1), 63–68.

Hong, D. S., Banerji, U., Tavana, B., George, G. C., Aaron, J., & Kurzrock, R. (2013). Targeting the molecular chaperone heat shock protein 90 (HSP90): Lessons learned and future directions. **Cancer Treatment Reviews**, 39(4), 375–387.

Hosseini-Vashan, S. J., Golian, A., & Yaghobfar, A. (2016). Growth, immune, antioxidant, and bone responses of heat stress-exposed broilers fed diets supplemented with tomato pomace. **International Journal of Biometeorology**, 60(8), 1183–1192.

Hosseinzadeh, S., & Hasanpur, K. (2023). Gene expression networks and functionally enriched pathways involved in the response of domestic chicken to acute heat stress. **Frontiers in Genetics**, 14(36), 1102136.

Hosseinzadeh, S., Shariatmadari, F., Karimi Torshizi, M. A., Ahmadi, H., & Scholey, D. (2023). *Plectranthus amboinicus* and rosemary (*Rosmarinus officinalis* L.) essential oils effects on performance, antioxidant activity, intestinal health, immune response, and plasma biochemistry in broiler chickens. **Food Science & Nutrition**, 11(7), 3939–3948.

Ibtisham, F., Nawab, A., Niu, Y., Wang, Z., Wu, J., Xiao, M., & An, L. (2019). The effect of ginger powder and Chinese herbal medicine on production performance, serum metabolites and antioxidant status of laying hens under heat-stress condition. **Journal of Thermal Biology**, 81(2), 20–24.

Jackson, S. E. (2012). Hsp90: Structure and Function. In S. Jackson (Ed.), **Molecular Chaperones** (Vol. 328, pp. 155–240). Springer Berlin Heidelberg.

Jendoubi, T. (2021). Approaches to Integrating Metabolomics and Multi-Omics Data: A Primer. **Metabolites**, 11(3), 184.

Jimoh, O. A., Ayedun, E. S., Oyelade, W. A., Oloruntola, O. D., Daramola, O. T., Ayodele, S. O., & Omoniyi, I. S. (2018). Protective effect of soursop (Annona muricata linn.) juice on oxidative stress in heat stressed rabbits. **Journal of Animal Science and Technology**, 60(1), 28.

Johnson, T. J., Youmans, B. P., Noll, S., Cardona, C., Evans, N. P., Karnezos, T. P., Ngunjiri, J. M., Abundo, M. C., & Lee, C.-W. (2018). A Consistent and Predictable Commercial Broiler Chicken Bacterial Microbiota in Antibiotic-Free Production Displays Strong Correlations with Performance. **Applied and Environmental Microbiology**, 84(12), e00362-18.

Karamać, M. (2009). Chelation of Cu (II), Zn (II), and Fe (II) by Tannin Constituents of Selected Edible Nuts. **International Journal of Molecular Sciences**, 10(12), 5485–5497.

Khalil, S. R., Elhakim, Y. A., Abd El-fattah, A. H., Ragab Farag, M., Abd El-Hameed, N. E., & EL-Murr, A. E. (2020). Dual immunological and oxidative responses in Oreochromis niloticus fish exposed to lambda cyhalothrin and concurrently fed with Thyme powder (Thymus vulgaris L.): Stress and immune encoding gene expression. **Fish & Shellfish Immunology**, 100 (9), 208–218.

Khan, R. U., Rahman, Z.-, Javed, I., & Muhammad, F. (2013). Supplementation of dietary vitamins, protein and probiotics on semen traits and immunohistochemical study of pituitary hormones in zinc-induced molted broiler breeders. **Acta Histochemica**, 115(7), 698–704.

Khoi, C.-S., Chen, J.-H., Lin, T.-Y., Chiang, C.-K., & Hung, K.-Y. (2021). Ochratoxin A-Induced Nephrotoxicity: Up-to-Date Evidence. **International Journal of Molecular Sciences**, 22(20), 11237.

Kikusato, M., & Toyomizu, M. (2013). Correction: Crucial Role of Membrane Potential in Heat Stress-Induced Overproduction of Reactive Oxygen Species in Avian Skeletal Muscle Mitochondria. **PLoS ONE**, 8(5). 61-81.

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.

Kleino, I., Frolovaitė, P., Suomi, T., & Elo, L. L. (2022). Computational solutions for spatial transcriptomics. **Computational and Structural Biotechnology Journal**, 20(8), 4870–4884.

Koch, F., Thom, U., Albrecht, E., Weikard, R., Nolte, W., Kuhla, B., & Kuehn, C. (2019). Heat stress directly impairs gut integrity and recruits distinct immune cell populations into the bovine intestine. **Proceedings of the National Academy of Sciences**, 116(21), 10333–10338.

Kumar, K. (2017). Effect of Ascorbic Acid on Some Biochemical Parameters during Heat Stress in Commercial Broilers. **International Journal of Current Microbiology and Applied Sciences**, 6(11), 5425–5434.

Kumar, M., Ratwan, P., Dahiya, S. P., & Nehra, A. K. (2021). Climate change and heat stress: Impact on production, reproduction and growth performance of poultry and its mitigation using genetic strategies. **Journal of Thermal Biology**, 97(21), 102867.

Kurutas, E. B. (2015). The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: Current state. **Nutrition Journal**, 15(1), 71.

Kuter, E., & Önol, A. G. (2021). Increased dietary methionine levels and supplemental L-carnitine do not prevent the development of white striping in broiler chickens. **Animal Feed Science and Technology**, 280(11), 115059.

Lara, L., & Rostagno, M. (2013). Impact of Heat Stress on Poultry Production. **Animals**, 3(2), 356–369.

Leandro, N. S. M., Gonzales, E., Ferro, J. A., Ferro, M. I. T., Givisiez, P. E. N., & Macari, M. (2004). Expression of heat shock protein in broiler embryo tissues after acute cold or heat stress. **Molecular Reproduction and Development**, 67(2), 172–177.

Lee, M. T., Lin, W. C., Yu, B., & Lee, T. T. (2016). Antioxidant capacity of phytochemicals and their potential effects on oxidative status in animals—A review. **Asian-Australasian Journal of Animal Sciences**, 30(3), 299–308.

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

Li, L., Cui, Z., Wang, H., Huang, B., & Ma, H. (2023). Dietary supplementation of dimethyl itaconate protects against chronic heat stress-induced growth performance impairment and lipid metabolism disorder in broiler chickens. **Journal of Animal Science**, 101(12), skad120.

Li, Z., Liu, A., Xu, J., & Zhang, C. (2023). Resveratrol Attenuates Heat-Stress-Impaired Immune and Inflammatory Responses of Broilers by Modulating Toll-Like Receptor-4 Signaling Pathway. **Brazilian Journal of Poultry Science**, 25(2), eRBCA-2022-1668.

Lian, P., Braber, S., Garssen, J., Wichers, H. J., Folkerts, G., Fink-Gremmels, J., & Varasteh, S. (2020). Beyond Heat Stress: Intestinal Integrity Disruption and Mechanism-Based Intervention Strategies. **Nutrients**, 12(3), 734.

Liochev, S. I., & Fridovich, I. (1999). Superoxide and Iron: Partners in Crime. **IUBMB Life**, 48(2), 157–161.

Liu, G., Zhu, H., Ma, T., Yan, Z., Zhang, Y., Geng, Y., Zhu, Y., & Shi, Y. (2020a). Effect of chronic cyclic heat stress on the intestinal morphology, oxidative status and cecal bacterial communities in broilers. **Journal of Thermal Biology**, 91(26), 102619.

Liu, L., Fu, C., Yan, M., Xie, H., Li, S., Yu, Q., He, S., & He, J. (2016). Resveratrol modulates intestinal morphology and HSP70/90, NF-κB and EGF expression in the jejunal mucosa of black-boned chickens on exposure to circular heat stress. **Food & Function**, 7(3), 1329–1338.

Liu, W. C., Pan, Z. Y., Zhao, Y., Guo, Y., Qiu, S. J., Balasubramanian, B., & Jha, R. (2022). Effects of Heat Stress on Production Performance, Redox Status, Intestinal Morphology and Barrier-Related Gene Expression, Cecal Microbiome, and Metabolome in Indigenous Broiler Chickens. **Frontiers in Physiology**, 13 (33), 890520.

Liu, X., Ma, Z., Wang, Y., Jia, H., Wang, Z., & Zhang, L. (2023). Heat stress exposure cause alterations in intestinal microbiota, transcriptome, and metabolome of broilers. **Frontiers in Microbiology**, 14 (33), 1244004.

Liu, Y., Li, C., Huang, X., Zhang, X., Deng, P., Jiang, G., & Dai, Q. (2022). Dietary rosemary extracts modulated gut microbiota and influenced the growth, meat quality, serum biochemistry, antioxidant, and immune capacities of broilers. **Frontiers in Microbiology**, 13 (89), 1024682.

Madkour, M., Alaqaly, A. M., Soliman, S. S., Ali, S. I., & Aboelazab, O. (2024). Growth performance, blood biochemistry, and mRNA expression of hepatic heat shock proteins of heat-stressed broilers in response to rosemary and oregano extracts. **Journal of Thermal Biology**, 119(20), 103791.

Mahasneh, Z. M. H., Abuajamieh, M., Abedal-Majed, M. A., Al-Qaisi, M., Abdelqader, A., & Al-Fataftah, A.-R. A. (2024). Effects of medical plants on alleviating the effects of heat stress on chickens. **Poultry Science**, 103(3), 103391.

Maini, S., Rastogi, S. K., Korde, J. P., Madan, A. K., & Shukla, S. K. (2007). Evaluation of Oxidative Stress and its Amelioration through Certain Antioxidants in Broilers during Summer. **The Journal of Poultry Science**, 44(3), 339–347.

Malila, Y., Thanatsang, K. V., Sanpinit, P., Arayamethakorn, S., Soglia, F., Zappaterra, M., Bordini, M., Sirri, F., Rungrassamee, W., Davoli, R., & Petracci, M. (2022). Differential expression patterns of genes associated with metabolisms, muscle growth and repair in Pectoralis major muscles of fast- and medium-growing chickens. **PLOS ONE**, 17(10), e0275160.

Messner, K. R., & Imlay, J. A. (1999). The Identification of Primary Sites of Superoxide and Hydrogen Peroxide Formation in the Aerobic Respiratory Chain and Sulfite Reductase Complex of Escherichia coli. **Journal of Biological Chemistry**, 274(15), 10119–10128.

Michalak, A. (2020). Phenolic Compounds and Their Antioxidant Activity in Plants Growing under Heavy Metal Stress. **European Journal of Integrative Medicine**, 8(4), 494–504.

Milarski, K. L., & Morimoto, R. I. (1989). Mutational analysis of the human HSP70 protein: Distinct domains for nucleolar localization and adenosine triphosphate binding. **The Journal of Cell Biology**, 109(5), 1947–1962.

Mirzaei, H., Ghorbani, M., Salari, S., & Mehrnia, M. (2023). Antioxidant properties of the fennel essential oil nanoemulsion: Effect on European production efficiency factor blood metabolites immune system and cecal microbial population of heat stressed broiler chickens. **Journal of Livestock Science and Technologies,** Online First. https://doi.org/10.22103/jlst.2023.21096.1462

Mishra, B., & Jha, R. (2019). Oxidative Stress in the Poultry Gut: Potential Challenges and Interventions. **Frontiers in Veterinary Science**, 6(12), 60.

Nagalakshmi, U., Wang, Z., Waern, K., Shou, C., Raha, D., Gerstein, M., & Snyder, M. (2008). The Transcriptional Landscape of the Yeast Genome Defined by RNA Sequencing. **Science**, 320(5881), 1344–1349.

Nagami, H., Yoshimoto, N., Umakoshi, H., Shimanouchi, T., & Kuboi, R. (2005). Liposome-assisted activity of superoxide dismutase under oxidative stress. **Journal of Bioscience and Bioengineering**, 99(4), 423–428.

Niu, Z., Liu, F., Yan, Q., & Li, L. (2009). Effects of different levels of selenium on growth performance and immunocompetence of broilers under heat stress. **Archives of Animal Nutrition**, 63(1), 56–65.

Obianwuna, U. E., Huang, L., Zhang, H., Wang, J., Qi, G., Qiu, K., & Wu, S. (2024). Fermented soybean meal improved laying performance and egg quality of laying hens by modulating cecal microbiota, nutrient digestibility, intestinal health, antioxidant and immunological functions. **Animal Nutrition**, S2405654524000581.

Papuc, C., Goran, G. V., Predescu, C. N., & Nicorescu, V. (2017). Mechanisms of Oxidative Processes in Meat and Toxicity Induced by Postprandial Degradation Products: A Review. **Comprehensive Reviews in Food Science and Food Safety**, 16(1), 96–123.

Patra, A. K. (2018). Interactions of plant bioactives with nutrient transport systems in gut of livestock. **Indian Journal of Animal Health**, 57(2), 125.

Patra, A. K., & Kar, I. (2021). Heat stress on microbiota composition, barrier integrity, and nutrient transport in gut, production performance, and its amelioration in farm animals. **Journal of Animal Science and Technology**, 63(2), 211–247.

Pearce, S. C., Sanz-Fernandez, M. V., Hollis, J. H., Baumgard, L. H., & Gabler, N. K. (2014). Short-term exposure to heat stress attenuates appetite and intestinal integrity in growing pigs1. **Journal of Animal Science**, 92(12), 5444–5454.

Qian, X., Ba, Y., Zhuang, Q., & Zhong, G. (2014). RNA-Seq Technology and Its Application in Fish Transcriptomics. **OMICS: A Journal of Integrative Biology**, 18(2), 98–110.

Qiao, N., Chen, H., Du, P., Kang, Z., Pang, C., Liu, B., Zeng, Q., Pan, J., Zhang, H., Mehmood, K., Tang, Z., & Li, Y. (2021). Acetyl-L-Carnitine Induces Autophagy to Promote Mouse Spermatogonia Cell Recovery after Heat Stress Damage. **BioMed Research International**, 2021(1), 8871328.

Quijano, C., Trujillo, M., Castro, L., & Trostchansky, A. (2016). Interplay between oxidant species and energy metabolism. **Redox Biology**, 8(16), 28–42.

Rehman, Z. U., Chand, N., & Khan, R. U. (2017). The effect of vitamin E, l-carnitine, and ginger on production traits, immune response, and antioxidant status in two broiler strains exposed to chronic heat stress. **Environmental Science and Pollution Research**, 24(34), 26851–26857.

Reis, J. H., Gebert, R. R., Barreta, M., Boiago, M. M., Souza, C. F., Baldissera, M. D., Santos, I. D., Wagner, R., Laporta, L. V., Stefani, L. M., & Da Silva, A. S. (2019). Addition of grape pomace flour in the diet on laying hens in heat stress: Impacts on health and performance as well as the fatty acid profile and total antioxidant capacity in the egg. **Journal of Thermal Biology**, 80(42), 141–149.

Reith, R. R., Sieck, R. L., Grijalva, P. C., Swanson, R. M., Fuller, A. M., Diaz, D. E., Schmidt, T. B., Yates, D. T., & Petersen, J. L. (2022). Transcriptome analyses indicate that heat stress-induced inflammation in white adipose tissue and oxidative stress in skeletal muscle is partially moderated by zilpaterol supplementation in beef cattle. **Journal of Animal Science**, 100(3), skac019.

Rostami, H., Seidavi, A., Dadashbeiki, M., Asadpour, Y., Simões, J., Shah, A. A., Laudadio, V., Losacco, C., Perillo, A., & Tufarelli, V. (2018). Supplementing dietary rosemary (Rosmarinus officinalis L.) powder and vitamin E in broiler chickens: Evaluation of humoral immune response, lymphoid organs, and blood proteins. **Environmental Science and Pollution Research**, 25(9), 8836–8842.

Roushdy, E. M., Zaglool, A. W., & El-Tarabany, M. S. (2018). Effects of chronic thermal stress on growth performance, carcass traits, antioxidant indices and the

expression of HSP70, growth hormone and superoxide dismutase genes in two broiler strains. **Journal of Thermal Biology**, 74(8), 337–343.

Sahin, K., Onderci, M., Sahin, N., Gulcu, F., Yıldız, N., Avcı, M., & Kucuk, O. (2006). Responses of quail to dietary Vitamin E and zinc picolinate at different environmental temperatures. **Animal Feed Science and Technology**, 129(1–2), 39–48.

Sahin, N., Onderci, M., Sahin, K., & Kucuk, O. (2008). Supplementation with Organic or Inorganic Selenium in Heat-distressed Quail. **Biological Trace Element Research**, 122(3), 229–237.

Salehi, B., Azzini, E., Zucca, P., Maria Varoni, E., V. Anil Kumar, N., Dini, L., Panzarini, E., Rajkovic, J., Valere Tsouh Fokou, P., Peluso, I., Prakash Mishra, A., Nigam, M., El Rayess, Y., El Beyrouthy, M., N. Setzer, W., Polito, L., Iriti, M., Sureda, A., Magdalena Quetglas-Llabrés, M., Sharifi-Rad, J. (2020). Plant-Derived Bioactives and Oxidative Stress-Related Disorders: A Key Trend towards Healthy Aging and Longevity Promotion. **Applied Sciences**, 10(3), 947.

Sandoval-Acuña, C., Ferreira, J., & Speisky, H. (2014). Polyphenols and mitochondria: An update on their increasingly emerging ROS-scavenging independent actions. **Archives of Biochemistry and Biophysics**, 559(14), 75–90.

Sanz Fernandez, M. V., Pearce, S. C., Mani, V., Gabler, N. K., Metzger, L., Patience, J. F., Rhoads, R. P., & Baumgard, L. H. (2014). Effects of dairy products on intestinal integrity in heat-stressed pigs. **Temperature**, 1(2), 128–134.

Saracila, M., Panaite, T. D., Papuc, C. P., & Criste, R. D. (2021). Heat Stress in Broiler Chickens and the Effect of Dietary Polyphenols, with Special Reference to Willow (Salix spp.) Bark Supplements—A Review. **Antioxidants**, 10(5), 686.

Saracila, M., Panaite, T. D., Predescu, N. C., Untea, A. E., & Vlaicu, P. A. (2023). Effect of Dietary Salicin Standardized Extract from Salix alba Bark on Oxidative Stress Biomarkers and Intestinal Microflora of Broiler Chickens Exposed to Heat Stress. **Agriculture**, 13(3), 698.

Seo, M., Lee, H.-J., Kim, K., Caetano-Anolles, K., Jeong, J. Y., Park, S., Oh, Y. K., Cho, S., & Kim, H. (2016). Characterizing Milk Production Related Genes in Holstein Using RNA-seq. **Asian-Australasian Journal of Animal Sciences**, 29(3), 343–351.

Shakeri, M., Cottrell, J. J., Wilkinson, S., Zhao, W., Le, H. H., McQuade, R., Furness, J. B., & Dunshea, F. R. (2019). Dietary Betaine Improves Intestinal Barrier Function and Ameliorates the Impact of Heat Stress in Multiple Vital Organs as Measured by Evans Blue Dye in Broiler Chickens. **Animals**, 10(1), 38.

Shakeri, M., Oskoueian, E., Le, H., & Shakeri, M. (2020). Strategies to Combat Heat Stress in Broiler Chickens: Unveiling the Roles of Selenium, Vitamin E and Vitamin C. **Veterinary Sciences,** 7(2), 71.

Shendure, J., & Ji, H. (2008). Next-generation DNA sequencing. **Nature Biotechnology**, 26(10), 1135–1145.

Shi, D., Bai, L., Qu, Q., Zhou, S., Yang, M., Guo, S., Li, Q., & Liu, C. (2019). Impact of gut microbiota structure in heat-stressed broilers. **Poultry Science**, 98(6), 2405–2413.

Song, J., Lei, X., Luo, J., Everaert, N., Zhao, G., Wen, J., & Yang, Y. (2019). The effect of Epigallocatechin-3-gallate on small intestinal morphology, antioxidant capacity and anti-inflammatory effect in heat-stressed broilers. J**ournal of Animal Physiology and Animal Nutrition**, 103(4), 1030–1038.

Song, Z., Cheng, K., Zhang, L., & Wang, T. (2017). Dietary supplementation of enzymatically treated Artemisia annua could alleviate the intestinal inflammatory response in heat-stressed broilers. **Journal of Thermal Biology**, 69(7), 184–190.

Srikanth, K., Lee, E., Kwan, A., Lim, Y., Lee, J., Jang, G., & Chung, H. (2017). Transcriptome analysis and identification of significantly differentially expressed genes in Holstein calves subjected to severe thermal stress. **International Journal of Biometeorology**, 61(11), 1993–2008.

Su, L., Nalle, S. C., Shen, L., Turner, E. S., Singh, G., Breskin, L. A., Khramtsova, E. A., Khramtsova, G., Tsai, P., Fu, Y., Abraham, C., & Turner, J. R. (2013). TNFR2 Activates MLCK-Dependent Tight Junction Dysregulation to Cause Apoptosis-Mediated Barrier Loss and Experimental Colitis. **Gastroenterology**, 145(2), 407–415.

Surai, P. F., Kochish, I. I., Fisinin, V. I., & Kidd, M. T. (2019). Antioxidant Defence Systems and Oxidative Stress in Poultry Biology: An Update. **Antioxidants**, 8(7), 235.

Tajima, K., Nonaka, I., Higuchi, K., Takusari, N., Kurihara, M., Takenaka, A., Mitsumori, M., Kajikawa, H., & Aminov, R. I. (2007). Influence of high temperature and humidity on rumen bacterial diversity in Holstein heifers. **Anaerobe**, 13(2), 57–64.

Tomasello, G., Mazzola, M., Leone, A., Sinagra, E., Zummo, G., Farina, F., Damiani, P., Cappello, F., Gerges Geagea, A., Jurjus, A., Bou Assi, T., Messina, M., & Carini, F. (2016). Nutrition, oxidative stress and intestinal dysbiosis: Influence of diet on gut microbiota in inflammatory bowel diseases. **Biomedical Papers**, 160(4), 461–466.

Torki, M., Sedgh-Gooya, S., & Mohammadi, H. (2018). Effects of adding essential oils of rosemary, dill and chicory extract to diets on performance, egg quality and some blood parameters of laying hens subjected to heat stress. **Journal of Applied Animal Research**, 46(1), 1118–1126.

Tsimberidou, A. M., Fountzilas, E., Bleris, L., & Kurzrock, R. (2022). Transcriptomics and solid tumors: The next frontier in precision cancer medicine. **Seminars in Cancer Biology**, 84(20), 50–59.

Turner, J. R. (2009). Intestinal mucosal barrier function in health and disease. **Nature Reviews Immunology**, 9(11), 799–809.

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.

Walker, W. A. (2017). Dysbiosis. ***In The Microbiota in Gastrointestinal Pathophysiology*** (pp. 227–232). Elsevier.

Wallin, R. P. A., Lundqvist, A., Moré, S. H., Von Bonin, A., Kiessling, R., & Ljunggren, H.-G. (2002). Heat-shock proteins as activators of the innate immune system. **Trends in Immunology**, 23(3), 130–135.

Wan, X., Jiang, L., Zhong, H., Lu, Y., Zhang, L., & Wang, T. (2017). Effects of enzymatically treated *Artemisia annua* L. on growth performance and some blood parameters of broilers exposed to heat stress. **Animal Science Journal**, 88(8), 1239–1246.

Wan, Y. W., Al-Ouran, R., Mangleburg, C. G., Perumal, T. M., Lee, T. V., Allison, K., Swarup, V., Funk, C. C., Gaiteri, C., Allen, M., Wang, M., Neuner, S. M., Kaczorowski, C. C., Philip, V. M., Howell, G. R., Martini-Stoica, H., Zheng, H., Mei, H., Zhong, X., Logsdon, B. A. (2020). Meta-Analysis of the Alzheimer's Disease Human Brain

Transcriptome and Functional Dissection in Mouse Models. **Cell Reports**, 32(2), 107908.

Wang, X. J., Feng, J. H., Zhang, M. H., Li, X. M., Ma, D. D., & Chang, S. S. (2018). Effects of high ambient temperature on the community structure and composition of ileal microbiome of broilers. **Poultry Science**, 97(6), 2153–2158.

Wang, Y., Lupiani, B., Reddy, S. M., Lamont, S. J., & Zhou, H. (2014). RNA-seq analysis revealed novel genes and signaling pathway associated with disease resistance to avian influenza virus infection in chickens. **Poultry Science**, 93(2), 485–493.

Wang, Y. W., Ning, D., Peng, Y. Z., & Guo, Y. M. (2013). Effects of Dietary L-carnitine Supplementation on Growth Performance, Organ Weight, Biochemical Parameters and Ascites Susceptibility in Broilers Reared Under Low-temperature Environment. **Asian-Australasian Journal of Animal Sciences**, 26(2), 233–240.

Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: A revolutionary tool for transcriptomics. **Nature Reviews Genetics**, 10(1), 57–63.

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(22), 114053.

Wasti, S., Sah, N., & Mishra, B. (2020). Impact of Heat Stress on Poultry Health and Performances, and Potential Mitigation Strategies. **Animals**, 10(8), 1266.

Wu, P., Xia, S., Yu, H., Zhao, X., Zhang, G., & Wang, K. (2024). RNA-seq reveals changes in the transcriptome of the breast muscle of adult female chickens in response to heat stress. **BMC Genomics**, 25(1), 1158.

Xie, J., Tang, L., Lu, L., Zhang, L., Xi, L., Liu, H.-C., Odle, J., & Luo, X. (2014). Differential Expression of Heat Shock Transcription Factors and Heat Shock Proteins after Acute and Chronic Heat Stress in Laying Chickens (Gallus gallus). **PLoS ONE,** 9(7), e102204.

Xu, D., Li, W., Huang, Y., He, J., & Tian, Y. (2014). The Effect of Selenium and Polysaccharide of Atractylodes macrocephala Koidz. (PAMK) on Immune Response in Chicken Spleen Under Heat Stress. **Biological Trace Element Research**, 160(2), 232–237.

Yang, C., Luo, P., Chen, S., Deng, Z., Fu, X., Xu, D., Tian, Y., Huang, Y., & Liu, W. (2021). Resveratrol sustains intestinal barrier integrity, improves antioxidant capacity, and alleviates inflammation in the jejunum of ducks exposed to acute heat stress. **Poultry Science**, 100(11), 101459.

Yin, B., Lian, R., Li, Z., Liu, Y., Yang, S., Huang, Z., Zhao, Z., Li, Y., Sun, C., Lin, S., Wan, R., & Li, G. (2021). Tea Polyphenols Enhanced the Antioxidant Capacity and Induced Hsps to Relieve Heat Stress Injury. **Oxidative Medicine and Cellular Longevity**, 2021(1), 9615429.

Yousefi, J., Taherpour, K., Ghasemi, H. A., Akbari Gharaei, M., Mohammadi, Y., & Rostami, F. (2023). RETRACTED ARTICLE: Effects of emulsifier, betaine and L -carnitine on growth performance, immune response, gut morphology and nutrient digestibility in broiler chickens exposed to cyclic heat stress. **British Poultry Science**, 64(4), 3–12.

Yu, J., Yin, P., Liu, F., Cheng, G., Guo, K., Lu, A., Zhu, X., Luan, W., & Xu, J. (2010). Effect of heat stress on the porcine small intestine: A morphological and gene expression study. *Comparative Biochemistry and Physiology Part A:* **Molecular & Integrative Physiology**, 156(1), 119–128.

Zhang, X., Akhtar, M., Chen, Y., Ma, Z., Liang, Y., Shi, D., Cheng, R., Cui, L., Hu, Y., Nafady, A. A., Ansari, A. R., Abdel-Kafy, E.-S. M., & Liu, H. (2022). Chicken jejunal microbiota improves growth performance by mitigating intestinal inflammation. **Microbiome**, 10(1), 107.

Zhao, G., Wu, X., Chen, P., Zhang, L., Yang, C. S., & Zhang, J. (2018). Selenium nanoparticles are more efficient than sodium selenite in producing reactive oxygen species and hyper-accumulation of selenium nanoparticles in cancer cells generates potent therapeutic effects. **Free Radical Biology and Medicine**, 126(22), 55–66.

Zheng, C., Zhong, Y., Zhang, P., Guo, Q., Li, F., & Duan, Y. (2024). Dynamic transcriptome profiles of skeletal muscle growth and development in Shaziling and Yorkshire pigs using RNA-SEQUENCING. **Journal of the Science of Food and Agriculture**, 104(12), 7301–7314.

Zhou, H., Zhang, H., He, Y., Huang, B., Zhou, C., Yao, G., & Lai, B. (2021). Critical review of reductant-enhanced peroxide activation processes: Trade-off between

accelerated Fe3+/Fe2+ cycle and quenching reactions. **Applied Catalysis B: Environmental**, 286(6), 119900.

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.

Zilaee, M., Ferns, G. A. A., & Ghayour-Mobarhan, M. (2014). Heat Shock Proteins and Cardiovascular Disease. **In Advances in Clinical Chemistry** (Vol. 64, pp. 73–115). Elsevier.

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℃), and heat-treated group hens were subjected to acute HS (36℃ 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 ℃); or heat stress (36 ℃, 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.

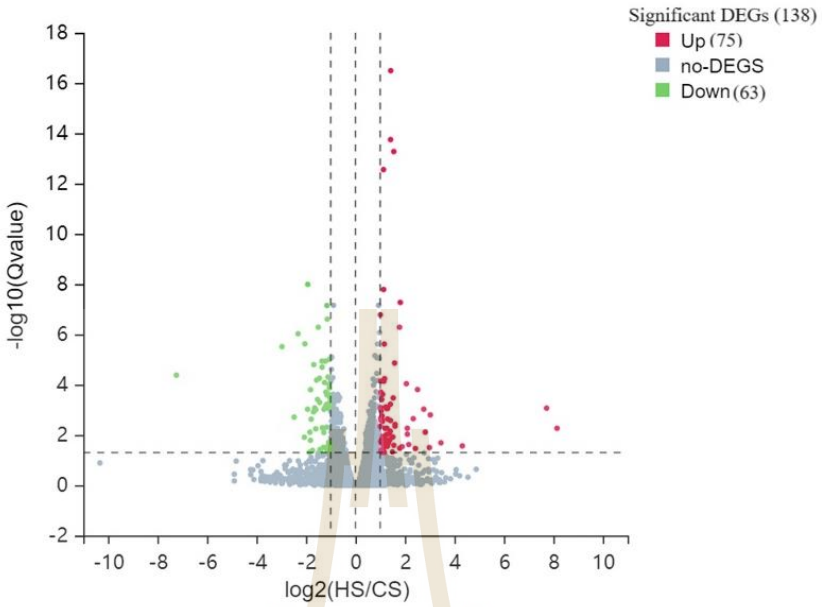


Figure 3.1 Volcano plot of differentially expressed genes in the jejunal mucosa in heat-stressed breeder hens. Genes meeting the conditions of adjusted P (q value) < 0.05 and |log2 FC| ≥ 1 are shown as significantly differentially expressed genes (DEGs), with red and green dots representing upregulated 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).

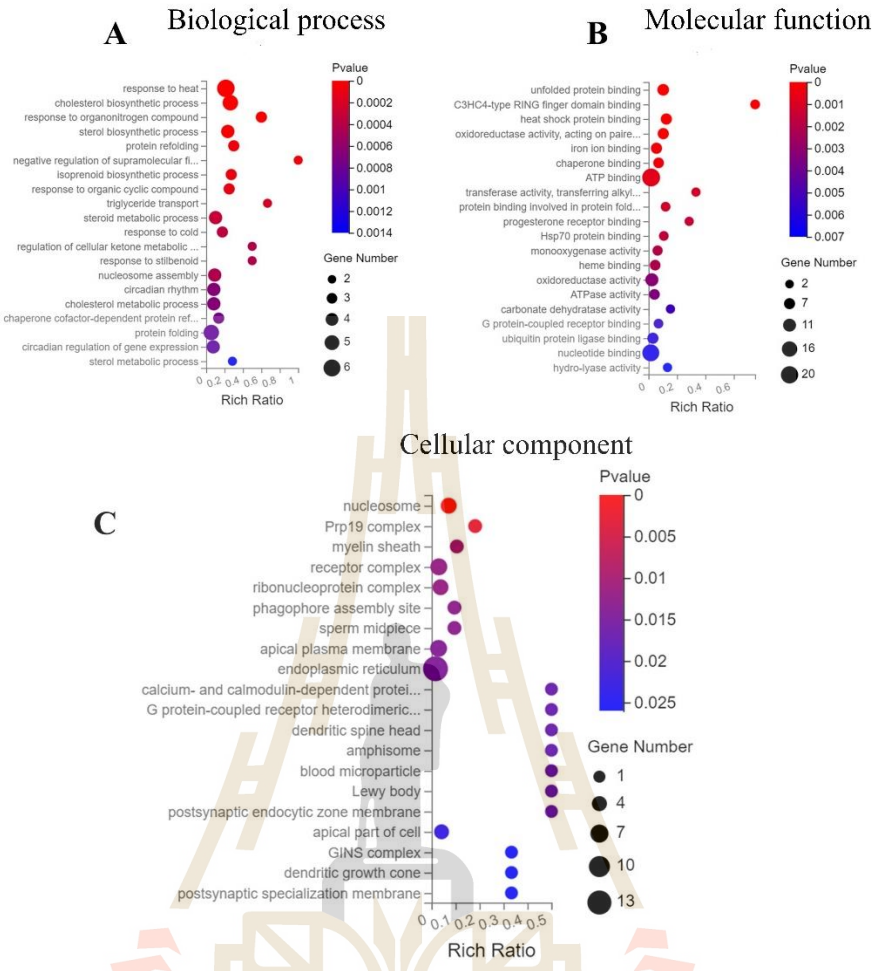


Figure 3.2 Top 20 enriched gene ontology terms of differentially expressed genes in jejunal mucosa in heat-stressed breeder hens. A: Biological process, B: Molecular function, and C: Cellular component. The circle size in each term corresponds to the number of genes. The circle's color goes from blue 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.

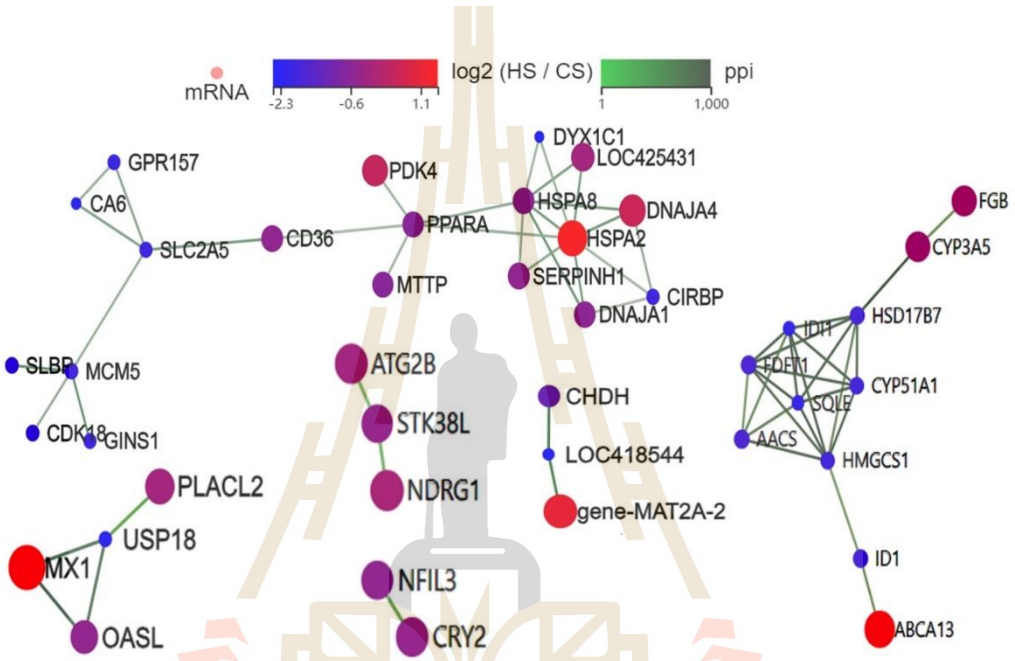


Figure 3.3 Protein–protein interaction network of differentially expressed genes in the jejunal mucosa in heat-stressed breeder hens. The color of the 'lines' becoming greener indicates a greater number of connected genes and associations with other genes. The size of the circle represents the values 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.

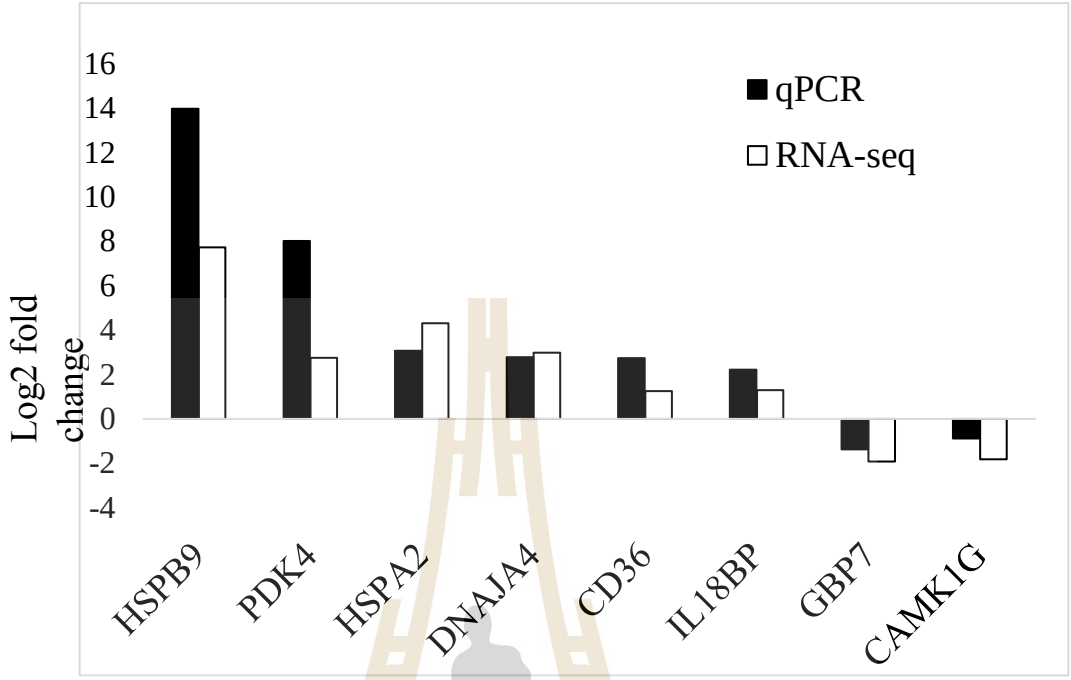


Figure 3.4 Quantitative polymerase chain reaction validation of 8 differentially expressed genes identified using RNA-sequencing. The x-axis represents the genes, and the y-axis represents their mRNA expression levels expressed in fold-change values. Expression levels determined via quantitative polymerase 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

Abdelli, N., A. Ramser, A., Greene, E. S., Beer, L., Tabler, T.W., Orlowski, S. K., Pérez, J. F., Solà-Oriol, D., Anthony, N. B., & Dridi. S. (2021). Effects of cyclic chronic heat stress on the expression of nutrient transporters in the jejunum of modern broilers and their ancestor, wild jungle fowl. **Frontiers in Physiology**, 12 (15), 733134.

Agarwal, A., Sengupta, P., & Durairajanayagam, D. (2018). Role of L-carnitine in female infertility. **Reproductive Biology and Endocrinology**, 16(1), 5.

Al-Zghoul, M. B., 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(4), 4113–4122.

Archana, P., Aleena, J., Pragna, P., Vidya, M., Niyas, A., Bagath, M., Krishnan, G., Manimaran, A., Beena, V., & Kurien, E. (2017). Role of heat shock proteins in livestock adaptation to heat stress. **Dairy Veterinary and Animal Research**, 5(12), 00127.

Arnal, M. E., & Lalles, J. P. (2016). Gut epithelial inducible heat-shock proteins and their modulation by diet and the microbiota. **Nutrition Reviews**, 74(1), 181–197.

Aviagen. 2016. Ross 308 Parent Stock: Nutrition Specification. **Aviagen Inc**., Huntsville, AL.

Barone, S., Fussell, S. L., Singh, A. K., Lucas, F., Xu, J., Kim, C., Wu, X., Yu, Y., Amlal, H., & Seidler, U. (2009). Slc2a5 (Glut5) is essential for the absorption of fructose in

the intestine and generation of fructose-induced hypertension. **Journal of Biological Chemistry**, 284(14), 5056–5066.

Barreto Sánchez, A. L., Wang, Q., Thiam, M., Wang, Z., Zhang, J., Zhang, Q., Zhang, N., Li, Q., Wen, J., & Zhao, G. (2022). Liver Transcriptome response to heat stress in Beijing You chickens and Guang Ming broilers. **Genes** 13(5), 416.

Bhat, S., Kumar, P., Kashyap, N., Deshmukh, B., Dige, M. S., Bhushan, B., Chauhan, A., Kumar, A., & Singh, G. (2016). Effect of heat shock protein 70 polymorphism on thermotolerance in Tharparkar cattle. Veterinary World, 9(4), 113–117.

Buchfink, B., Xie, C., & Huson, D. H. (2015). Fast and sensitive protein alignment using DIAMOND. **Nature Methods**, 12(11), 59–60.

Cai, J., Rimal, B., Jiang, C., Chiang, J. Y. L., & Patterson, A. D. (2022). Bile acid metabolism and signaling, the microbiota, and metabolic disease. **Pharmacology and Therapeutics**, 237(16), 108238.

Calik, A., Emami, N. K., Schyns, G., White, M. B., Walsh, M. C., Romero, L. F., & Dalloul, R. A. (2022). Influence of dietary vitamin E and selenium supplementation on broilers subjected to heat stress, Part II: Oxidative stress, immune response, gut integrity, and intestinal microbiota. **Poultry Science**, 101(6), 101858

Cândido, M. G. L., Tinôco, I. F. F., Albino, L. F. T., Freitas, L. C. S. R., Santos, T. C., Cecon, P. R., & Gates, R. S. (2020). Effects of heat stress on pullet cloacal and body temperature. **Poultry Science**, 99(5), 2469–2477.

Chang, Y., Wang, X. J., Feng, J. H., Zhang, M. H., Diao, H. J., Zhang, S. S., Peng, Q. Q., Zhou, Y., Li, M., & Li, X. (2018). Real-time variations in body temperature of laying hens with increasing ambient temperature at different relative humidity levels. **Poultry Science**, 97(9), 3119–3125.

Chen, B., Feder, M. E., & Kang, L. (2018). Evolution of heat‐shock protein expression underlying adaptive responses to environmental stress. **Molecular Ecology**, 27(15), 3040-3054.

Chen, M., Yang, Y., Braunstein, E., Georgeson, K. E., & Harmon, C. M. (2001). Gut expression and regulation of FAT/CD36: Possible role in fatty acid transport in rat enterocytes. **American Journal of Physiology-Endocrinology and Metabolism**, 281(5), E916–E923.

Chen, W., Xu, J., Wu, Y., Liang, B., Yan, M., Sun, C., Wang, D., Hu, X., Liu, L., Hu, W., Shao, Y., & Xing, D. (2023). The potential role and mechanism of circRNA/miRNA axis in cholesterol synthesis. **International Journal of Biological Sciences**, 19(9), 2879–2896.

Cock, P. J. A., Fields, C. J., Goto, N., Heuer, M. L., & Rice, P. M. (2010). The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. **Nucleic Acids Research**, 38(6), 1767–1771.

Chung, E. J., Jeong, Y. I., Lee, M.-R., Kim, Y. J., Lee, S. E., Cho, S. H., Lee, W. J., Park, M. Y., & Ju, J. W. (2017). Heat shock proteins 70 and 90 from Clonorchis sinensis induce Th1 response and stimulate antibody production. **Parasites & Vectors**, 10(1), 118.

Elnesr, S., & Abdel-Azim, A. (2023). The impact of heat stress on the gastrointestinal tract integrity of poultry. Labyrinth: **Fayoum Journal of Science and Interdisciplinary Studies**, 0(0), 0–0.

Forteza, M. J., Berg, M., Edsfeldt, A., Sun, J., Baumgartner, R., Kareinen, I., Casagrande, F. B., Hedin, U., Zhang, S., Vuckovic, I., Dzeja, P. P., Polyzos, K. A., Gisterå, A., Trauelsen, M., Schwartz, T. W., Dib, L., Herrmann, J., Monaco, C., Matic, L., … Ketelhuth, D. F. J. (2023). Pyruvate dehydrogenase kinase regulates vascular inflammation in atherosclerosis and increases cardiovascular risk. **Cardiovascular Research**, 119(7), 1524–1536.

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.

Girisa, S., Hegde, M., & Kunnumakkara, A. B. (2024). Molecular Mechanism of Spices and Their Active Constituents for the Prevention and Treatment of Diseases. In P. N. Ravindran, K. Sivaraman, S. Devasahayam, & K. N. Babu (Eds.), **Handbook of Spices in India**: 75 Years of Research and Development (pp. 695–753). Springer Nature Singapore.

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.

Grüning, N.-M., Rinnerthaler, M., Bluemlein, K., Mülleder, M., Wamelink, M. M. C., Lehrach, H., Jakobs, C., Breitenbach, M., & Ralser, M. (2011). Pyruvate Kinase Triggers a Metabolic Feedback Loop that Controls Redox Metabolism in Respiring Cells. **Cell Metabolism**, 14(3), 415–427.

Gu, X. H., Hao, Y., & Wang, X. L. (2012). Overexpression of heat shock protein 70 and its relationship to intestine under acute heat stress in broilers: 2. Intestinal oxidative stress. **Poultry Science**, 91(4), 790–799.

Haas, B. J., & Zody, M. C. (2010). Advancing RNA-Seq analysis. **Nature Biotechnology**, 28(5), 421–423.

Hageman, J., Rujano, M. A., Van Waarde, M. A. W. H., Kakkar, V., Dirks, R. P., Govorukhina, N., Oosterveld-Hut, H. M. J., Lubsen, N. H., & Kampinga, H. H. (2010). A DNAJB Chaperone Subfamily with HDAC-Dependent Activities Suppresses Toxic Protein Aggregation. **Molecular Cell**, 37(3), 355–369.

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.

Honda, K., Takagi, S., Kurachi, K., Sugimoto, H., Saneyasu, T., & Kamisoyama, H. (2017). Fasting and Glucagon Stimulate Gene Expression of Pyruvate Dehydrogenase Kinase 4 in Chickens. **The Journal of Poultry Science**, 54(4), 292–295.

Hwang, Y. S., Ko, M. H., Kim, Y. M., Park, Y. H., Ono, T., & Han, J. Y. (2016). The avian-specific small heat shock protein HSP25 is a constitutive protector against environmental stresses during blastoderm dormancy. **Scientific Reports**, 6(1), 36704.

Ihim, S. A., Abubakar, S. D., Zian, Z., Sasaki, T., Saffarioun, M., Maleknia, S., & Azizi, G. (2022). Interleukin-18 cytokine in immunity, inflammation, and autoimmunity: Biological role in induction, regulation, and treatment. **Frontiers in Immunology**, 13, 919973.

Jee, H. (2016). Size-dependent classification of heat shock proteins: A mini-review. **Journal of Exercise Rehabilitation**, 12(4), 255–259.

Jia, W., Wei, M., Rajani, C., & Zheng, X. (2021). Targeting the alternative bile acid synthetic pathway for metabolic diseases. **Protein & Cell**, 12(5), 411–425.

Kahl, S., Elsasser, T. H., Rhoads, R. P., Collier, R. J., & Baumgard, L. H. (2015). Environmental heat stress modulates thyroid status and its response to repeated endotoxin challenge in steers. **Domestic Animal Endocrinology**, 52, 43–50.

Khan, M. Z., Khan, A., Chen, W., Chai, W., & Wang, C. (2024). Advancements in Genetic Biomarkers and Exogenous Antioxidant Supplementation for Safeguarding Mammalian Cells against Heat-Induced Oxidative Stress and Apoptosis. **Antioxidants**, 13(3), 258.

Kim, D. Y., Han, G. P., Lim, C., Kim, J.-M., & Kil, D. Y. (2023). Effect of dietary betaine supplementation on the liver transcriptome profile in broiler chickens under heat stress conditions. **Animal Bioscience**, 36(11), 1632–1646.

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.

Kumbhar, S., Khan, A. Z., Parveen, F., Nizamani, Z. A., Siyal, F. A., El-Hack, M. E. A., Gan, F., Liu, Y., Hamid, M., Nido, S. A., & Huang, K. (2018). Impacts of selenium and vitamin E supplementation on mRNA of heat shock proteins, selenoproteins and antioxidants in broilers exposed to high temperature. **AMB Express**, 8(1), 112.

Lan, R., Wang, Y., Wei, L., Wu, F., & Yin, F. (2022). Heat stress exposure changed liver lipid metabolism and abdominal fat deposition in broilers. **Italian Journal of Animal Science**, 21(1), 1326–1333.

Li, B., & Dewey, C. N. (2011). RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. **BMC Bioinformatics**, 12(1), 323.

Li, N., Liu, D., Wang, C., Yan, G., Zhang, S., Jiang, Y., Shen, M., Jia, B., Xu, L., Huang, B., Zhu, R., & Wei, K. (2023). Comparison study of protective effects of porcine bile acids and sheep bile acids against heat stress in chickens. **Journal of the Science of Food and Agriculture**, 103(12), 5687–5696.

Li, X., Abdel-Moneim, A.-M. E., Mesalam, N. M., & Yang, B. (2022). Effects of Lysophosphatidylcholine on Jejuna Morphology and Its Potential Mechanism. **Frontiers in Veterinary Science**, 9, 911496.

Li, X., Zhao, X., Yu, M., Zhang, M., & Feng, J. (2024). Effects of Heat Stress on Breast Muscle Metabolomics and Lipid Metabolism Related Genes in Growing Broilers. **Animals**, 14(3), 430.

Liu, H., Dicksved, J., Lundh, T., & Lindberg, J. (2014). Heat Shock Proteins: Intestinal Gatekeepers that Are Influenced by Dietary Components and the Gut Microbiota. **Pathogens**, 3(1), 187–210.

Liu, L., Fu, C., Yan, M., Xie, H., Li, S., Yu, Q., He, S., & He, J. (2016). Resveratrol modulates intestinal morphology and HSP70/90, NF-κB, and EGF expression in the jejunal mucosa of black-boned chickens on exposure to circular heat stress. **Food & Function**, 7(3), 1329–1338.

Liu, M., Lu, Y., Gao, P., Xie, X., Li, D., Yu, D., & Yu, M. (2020). Effect of curcumin on laying performance, egg quality, endocrine hormones, and immune activity in heat-stressed hens. **Poultry Science**, 99(4), 2196–2202.

Liu, W.-C., Ou, B. H., Liang, Z. L., Zhang, R., & Zhao, Z. H. (2021). Algae-derived polysaccharides supplementation ameliorates heat stress-induced impairment of the bursa of Fabricius via modulating NF-κB signaling pathway in broilers. **Poultry Science**, 100(8), 101139.

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. **Genome Biology**, 15(12), 550.

Lu, Z., He, X., Ma, B., Zhang, L., Li, J., Jiang, Y., Zhou, G., & Gao, F. (2017). Chronic Heat Stress Impairs the Quality of Breast-Muscle Meat in Broilers by Affecting Redox Status and Energy-Substance Metabolism. **Journal of Agricultural and Food Chemistry**, 65(51), 11251–11258.

Ma, B., Zhang, L., Li, J., Xing, T., Jiang, Y., & Gao, F. (2021). Heat stress alters muscle protein and amino acid metabolism and accelerates liver gluconeogenesis for energy supply in broilers. **Poultry Science**, 100(1), 215–223.

Mackei, M., Mátis, G., Molnár, A., Sebők, C., Vörösházi, J., Pál, L., Dublecz, K., Husvéth, F., & Neogrády, Z. (2021). The relationship between small heat shock proteins and redox homeostasis during acute heat stress in chickens. **Journal of Thermal Biology**, 100, 103040.

Makar, A. B., McMartin, K. E., Palese, M., & Tephly, T. R. (1975). Formate assay in body fluids: Application in methanol poisoning. **Biochemical Medicine**, 13(2), 117–126.

Meng, Q., Zhang, Y., Li, J., Shi, B., Ma, Q., & Shan, A. (2022). Lycopene Affects Intestinal Barrier Function and the Gut Microbiota in Weaned Piglets via Antioxidant Signaling Regulation. **The Journal of Nutrition**, 152(11), 2396–2408.

Meyer, P., Prodromou, C., Hu, B., Vaughan, C., Roe, S. M., Panaretou, B., Piper, P. W., & Pearl, L. H. (2003). Structural and Functional Analysis of the Middle Segment of Hsp90: Implications for ATP Hydrolysis and Client Protein and Cochaperone Interactions. **Molecular Cell**, 11(3), 647–658.

Miyata, M., Yamakawa, H., Hamatsu, M., Kuribayashi, H., Takamatsu, Y., & Yamazoe, Y. (2011). Enterobacteria Modulate Intestinal Bile Acid Transport and Homeostasis through Apical Sodium-Dependent Bile Acid Transporter (SLC10A2) Expression. **The Journal of Pharmacology and Experimental Therapeutics**, 336(1), 188–196.

Mountzouris, K. C., Paraskeuas, V. V., & Fegeros, K. (2020). Priming of intestinal cytoprotective genes and antioxidant capacity by dietary phytogenic inclusion in broilers. **Animal Nutrition**, 6(3), 305–312.

Nakao, N., Kaneda, H., Tsushima, N., Ohta, Y., & Tanaka, M. (2015). Characterization of primary structure and tissue expression profile of the chicken apical sodium-dependent bile acid transporter mRNA. **Poultry Science**, 94(4), 722–727.

National Research Council. 1994. Nutrient Requirements of Poultry: Ninth Revised Edition, **National Academies Press**, Washington, DC.

Nguyen, P., Leray, V., Diez, M., Serisier, S., Bloc'h, J. L., Siliart, B., & Dumon, H. (2008). Liver lipid metabolism. **Journal of Animal Physiology and Animal Nutrition**, 92(3), 272–283.

Ni, H.-Y., Yu, L., Zhao, X.-L., Wang, L., Zhao, C., Huang, H., Zhu, H.-L., Efferth, T., Gu, C.-B., & Fu, Y.-J. (2022). Seed oil of Rosa roxburghii Tratt against non-alcoholic fatty liver disease in vivo and in vitro through PPARα/PGC-1α-mediated mitochondrial oxidative metabolism. **Phytomedicine**, 98, 153919.

Pasri, P., Mermillod, P., & Khempaka, S. (2023). Antioxidant properties and cytotoxic effects of selected edible plants in Southeast Asia for further use as phytogenic antioxidant additives. **Saudi Journal of Biological Sciences**, 30(5), 103631.

Pasri, P., Rakngam, S., Gérard, N., Mermillod, P., & Khempaka, S. (2024). Synthetic and phytogenic antioxidants improve productive performance, antioxidant activity,

gene expression, and offspring quality in breeder hens subjected to heat stress. **Poultry Science**, 103(3), 103390.

Oluwagbenga, E. M., & Fraley, G. S. (2023). Heat stress and poultry production: A comprehensive review. **Poultry Science**, 102(12), 103141.

Oluwagbenga, E. M., Tetel, V., Schober, J., & Fraley, G. S. (2022). Chronic heat stress part 1: Decrease in egg quality, increase in cortisol levels in egg albumen, and reduction in fertility of breeder pekin ducks. **Frontiers in Physiology**, 13, 1019741.

Oyake, J., Otaka, M., Matsuhashi, T., Jin, M., Odashima, M., Komatsu, K., Wada, I., Horikawa, Y., Ohba, R., Hatakeyama, N., Itoh, H., & Watanabe, S. (2006). Over-expression of 70-kDa heat shock protein confers protection against monochloramine-induced gastric mucosal cell injury. **Life Sciences**, 79(3), 300–305.

Saeed, M., Abbas, G., Alagawany, M., Kamboh, A. A., Abd El-Hack, M. E., Khafaga, A. F., & Chao, S. (2019). Heat stress management in poultry farms: A comprehensive overview. **Journal of Thermal Biology**, 84, 414–425.

Salcedo, J., & McCormick, K. (2020). SPSS statistics for dummies. **John Wiley & Sons**.

Saracila, M., Panaite, T. D., Predescu, N. C., Untea, A. E., & Vlaicu, P. A. (2023). Effect of Dietary Salicin Standardized Extract from Salix alba Bark on Oxidative Stress Biomarkers and Intestinal Microflora of Broiler Chickens Exposed to Heat Stress. **Agriculture**, 13(3), 698.

Shi, K.-P., Dong, S.-L., Zhou, Y.-G., Li, Y., Gao, Q.-F., & Sun, D.-J. (2019). RNA-seq reveals temporal differences in the transcriptome response to acute heat stress in the Atlantic salmon (Salmo salar). **Comparative Biochemistry and Physiology Part D: Genomics and Proteomics**, 30, 169–178.

Shneider, B. L. (2001). Intestinal bile acid transport: biology, physiology, and pathophysiology. J. Pediatr. Gastroenterol. **Nutrition**, 32(14), 407–417.

Song, Z. H., Cheng, K., Zheng, X. C., Ahmad, H., Zhang, L. L., & Wang, T. (2018). Effects of dietary supplementation with enzymatically treated Artemisia annua on growth performance, intestinal morphology, digestive enzyme activities, immunity, and antioxidant capacity of heat-stressed broilers. **Poultry Science**, 97(2), 430–437.

Stamm, O., Latscha, U., Janecek, P., & Campana, A. (1976). Development of a special electrode for continuous subcutaneous pH measurement in the infant scalp. **American Journal of Obstetrics and Gynecology**, 124(2), 193–195.

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.

Surai, P. F., Kochish, I. I., & Fisinin, V. I. (2018). Glutathione peroxidases in poultry biology: Part 1. Classification and mechanisms of action. **World's Poultry Science Journal**, 74(2), 185–198.

Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M., Doncheva, N. T., Morris, J. H., Bork, P., Jensen, L. J., & Mering, C. von. (2019). STRING v11: Protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. **Nucleic Acids Research**, 47(D1), D607–D613.

Tellez, G. (2017). Evaluation of A Lactic Acid Based Probiotic on Leaky Gut and Microbiome Associated with Salmonella Enteritidis Infection and Feed Restriction in Broiler Chickens. **Approaches in Poultry, Dairy & Veterinary Sciences**, 1(1).

Tokutake, Y., Takanashi, R., Kikusato, M., Toyomizu, M., & Sato, K. (2022). Effect of Dietary 4-Phenylbuthyric Acid Supplementation on Acute Heat-Stress-Induced Hyperthermia in Broiler Chickens. **Animals**, 12(16), 2056.

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.

Vital, A. C. P., Croge, C., Da Silva, D. F., Araújo, P. J., Gallina, M. Z., & Matumoto-Pintro, P. T. (2018). Okara residue as source of antioxidants against lipid oxidation in milk enriched with omega-3 and bioavailability of bioactive compounds after in vitro gastrointestinal digestion. **Journal of Food Science and Technology**, 55(4), 1518–1524.C

Wahli, W., & Michalik, L. (2012). PPARs at the crossroads of lipid signaling and inflammation. **Trends in Endocrinology & Metabolism**, 23(7), 351–363.

Wen, Y., Hu, J., Wang, J., Liu, X., Li, S., & Luo, Y. (2021). Effect of glycolysis and heat shock proteins on hypoxia adaptation of Tibetan sheep at different altitude. **Gene**, 803, 145893.

Yahav, S. (2009). Alleviating heat stress in domestic fowl: Different strategies. **World's Poultry Science Journal**, 65(4), 719–732.

Yin, C., Tang, S., Liu, L., Cao, A., Xie, J., & Zhang, H. (2021). Effects of Bile Acids on Growth Performance and Lipid Metabolism during Chronic Heat Stress in Broiler Chickens. **Animals**, 11(3), 630.

Yin, C., Xia, B., Tang, S., Cao, A., Liu, L., Zhong, R., Chen, L., & Zhang, H. (2021). The Effect of Exogenous Bile Acids on Antioxidant Status and Gut Microbiota in Heat-Stressed Broiler Chickens. **Frontiers in Nutrition**, 8, 747136.

Yin, C., Zhou, C., Shi, Y., Ge, Y., Gao, X., Wu, C., Xu, Z., Huang, C., Hu, G., Liu, P., & Guo, X. (2023). Effects and potential mechanism of dietary vitamin C supplementation on hepatic lipid metabolism in growing laying hens under chronic heat stress. **Journal of Animal Science**, 101, skad308.

Zhang, Y., Chen, H., Cong, W., Zhang, K., Jia, Y., & Wu, L. (2023). Chronic Heat Stress Affects the Bile Acid Profile and Gut Microbiota in Broilers. **International Journal of Molecular Sciences**, 24(12), 10238.

Zhang, Z. Y., Jia, G. Q., Zuo, J. J., Zhang, Y., Lei, J., Ren, L., & Feng, D. Y. (2012). Effects of constant and cyclic heat stress on muscle metabolism and meat quality of broiler breast fillet and thigh meat. **Poultry Science**, 91(11), 2931–2937.

Zhong, X., Wang, T., Zhang, X., & Li, W. (2010). Heat shock protein 70 is upregulated in the intestine of intrauterine growth retardation piglets. **Cell Stress and Chaperones**, 15(3), 335–342.

Zuo, D., Subjeck, J., & Wang, X.-Y. (2016). Unfolding the Role of Heat Shock Proteins: New Insights and Therapeutic Implications. **Frontiers in Immunology**, 7.

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°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. 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

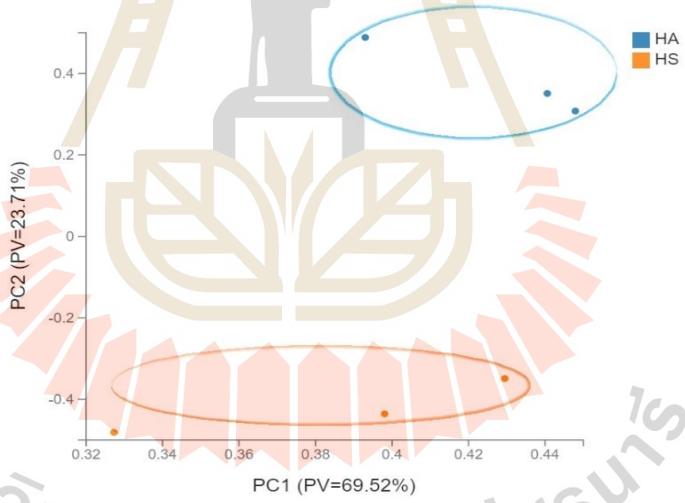
[2]Q20 and Q30 indicate the percentage of bases with P value ≥ 20 and 30, respectively.

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



B

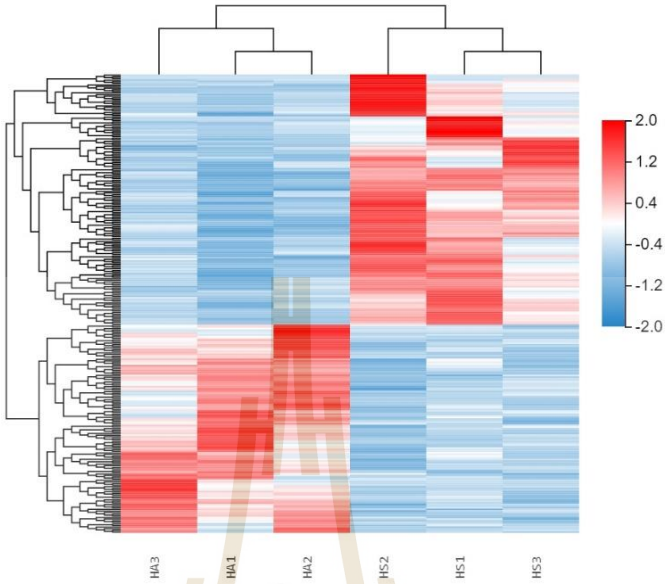
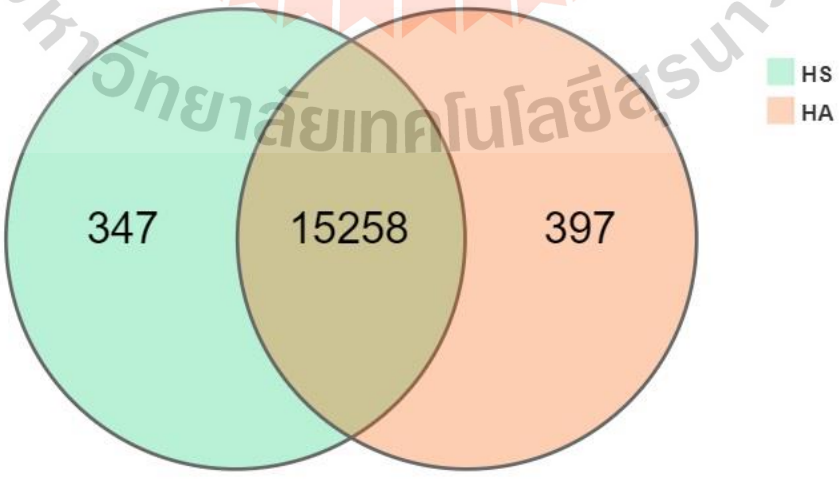


Figure 4.1 DEGs analysis in jejunal mucosa between HA and HS breeder hens under heat stress. (A) PCA of DEGs. Blue and orange nodes represent individuals from HA and HS breeder hens, respectively. (B) Hierarchical clustering heatmap of DEGs. Each row represents DEGs, and the column represents the sample 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

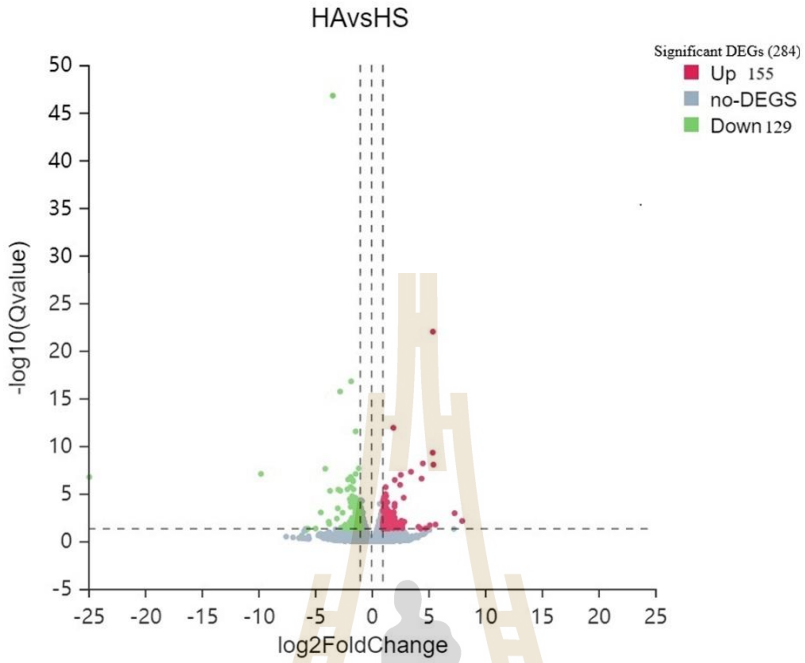


Figure 4.2 DEGs analysis was performed on jejunal mucosa tissues between HA and HS breeder hens under heat stress. (A) Venn diagram illustrating the distribution of DEGs identified in the jejunal tissues. (B) Volcano plot displaying the DEGs between the HA and HS groups. Red and green dots represent 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.

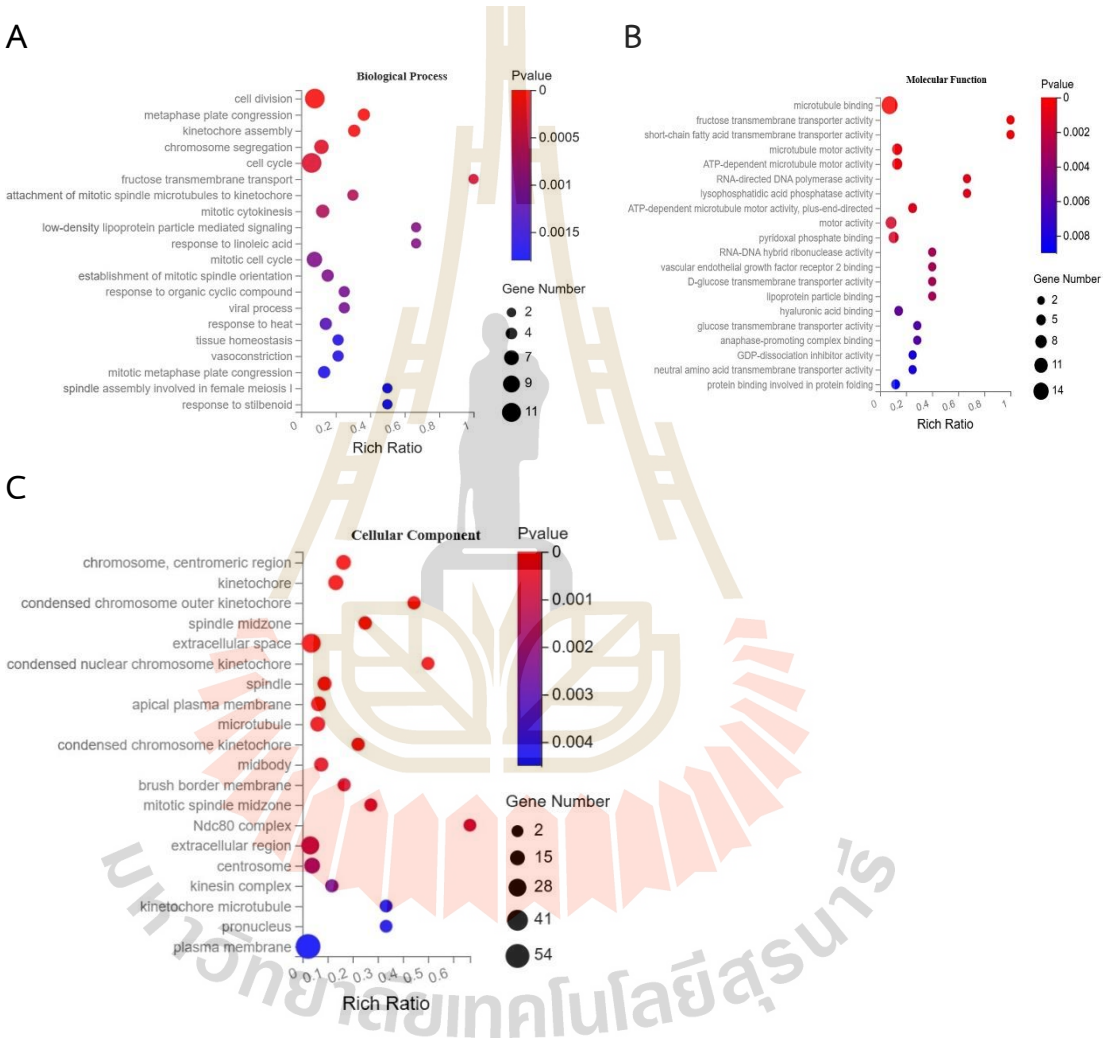


Figure 4.3 Functional enrichment analysis of identified DEGs in jejunal mucosa between HA and HS breeder hens. The top 20 enriched GO terms of DEGs are shown for (A) biological process, (B) molecular function, and (C) cellular component categories. The x-axis represents the richness ratio, and the y-axis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

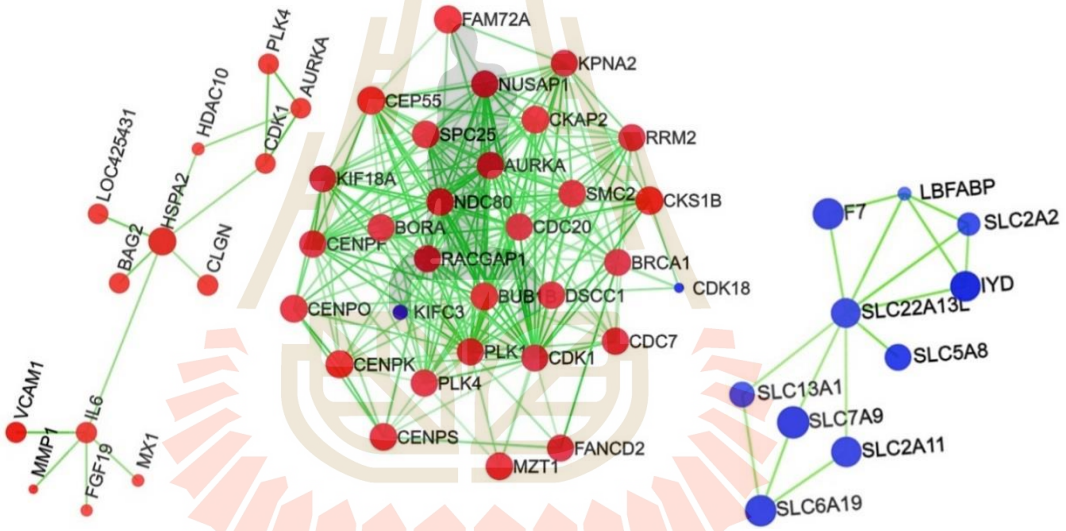


Figure 4.4 PPI network of DEGs in jejunal mucosa between HA and HS breeder hens under heat stress. The size of the circle represents the values of log2 fold change. Red and blue nodes indicate the upregulated and downregulated genes, respectively.

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.

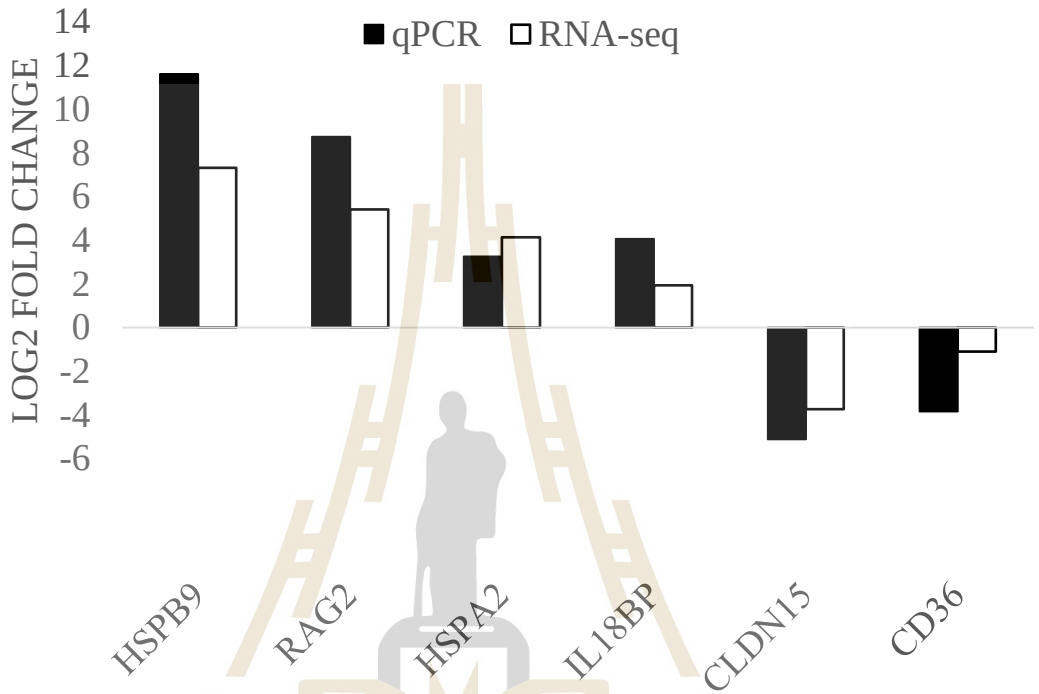


Figure 4.5 Expression of 6 DEGs was detected using either RNA-seq or RT-qPCR. The x-axis represents the genes, and the y-axis represents their mRNA expression levels expressed in fold-change values. Expression levels determined via qPCR and RNA-seq are represented by black and white fill columns, respectively. 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-**K**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.

CHAPTER V

DIETARY SYNTHETIC AND PHYTOGENIC ANTIOXIDANTS MODULATE JEJUNAL MUCOSA GENE EXPRESSION, CECAL SHORT-CHAIN FATTY ACIDS CONCENTRATION, AMMONIA PRODUCTION, AND MICROBIOTA IN HEAT-STRESSED BREEDER HENS

5.1 Abstract

This study aimed to investigate the efficacy of synthetic antioxidants (a combination of vitamin E, vitamin C, selenium, and L-carnitine) and phytogenic antioxidants (a combination of clove, green tea pomace, and Vietnamese coriander) on the expression of the genes related to antioxidant capacity, immunity, and heat shock proteins (HSPs), cecal short-chain fatty acids (SCFAs), ammonia productions, and microbiota of heat-stressed (HS) breeder hens. One hundred hens were randomly assigned to either a thermoneutral (TN; 23 °C) or an HS room (HS; 36°C, 4 h/d from week 38 to 52). All hens were randomly allotted to four groups (25 hens each): T1) basal diet in TN zone; T2) basal diet under HS; T3) basal diet supplemented with synthetic antioxidants under HS; and T4) basal diet supplemented with phytogenic antioxidants under HS. Compared to heat-stressed hens, both synthetic and phytogenic antioxidant sources increased jejunum antioxidant (SOD and GSH-Px), tight-junction protein (CLDN1), and anti-inflammatory cytokine (IL-10) gene expression, and cecal concentrations of acetate, propionate, butyrate, isobutyrate, isovalerate, and total SCFAs, while decreasing the expression of HSPs (HSP70 and HSP90), immunity-related genes (IL-6, TNF-α, NF-κB, and TLR4), and ammonia production ($P < 0.05$). The abundance of SCFA-producing bacteria, including *Firmicutes*, *Lachnospiraceae*, *Ruminococcaceae*, and *Megamonas*, increased in the HS group receiving synthetic and photogenic antioxidants compared to the HS group without supplementation. PICRUSt2 analysis revealed enriched metabolic pathways of bacterial chemotaxis, thiamine metabolism, and lysine biosynthesis in HS hens receiving both antioxidant sources. Spearman correlation analysis showed that the abundances of *Lachnospiraceae*,

Ruminococcaceae, and *Megamonas*, were shown to be positively correlated with the expression of SOD and IL-10, the concentration of butyrate, isobutyrate, and total SCFA, whereas negatively correlated with the expression of HSP70 in heat-stressed breeder hens. In conclusion, either synthetic or photogenic antioxidants effectively alleviated HS in breeder hens by enhancing antioxidant capacity, regulating immune responses, increasing SCFA concentrations, reducing ammonia levels, and modulating cecal microbiota composition, offering potential strategies to mitigate HS effects in poultry.

Keywords: Antioxidant, Breeder hen, Heat stress, Immunity, Microbiota.

5.2 Introduction

Heat stress (HS) severely affects chicken health, welfare, and productivity, particularly in tropical and subtropical regions (Khan et al., 2023). Elevated body temperature during HS causes intestinal ischemia and epithelial damage, compromising intestinal barrier integrity, leading to increased permeability, triggering inflammation, and disrupting microbiota composition, ultimately impairing intestinal health and immune function (Zhao et al., 2023). HS also induces excessive reactive oxygen species (ROS) generation, creating oxidative stress (OS) that damages cellular components and disrupts redox homeostasis (Reith et al., 2022; Zhao et al., 2023).

Various strategies-nutritional, managerial, and genetic-have been proposed to alleviate HS effects, with dietary intervention emerging as a particularly cost-effective approach (Saeed et al., 2019). Antioxidant supplementation combines vitamins E and C, minerals such as selenium (Se), manganese, and zinc, as well as phytogenic bioactive compounds, demonstrating synergistic efficacy in enhancing antioxidant activity, reducing OS, strengthening immune function, and gut dysbiosis regulation (Ghazi Harsini et al., 2012; Kumbhar et al., 2018), and mitigating HS and lipid peroxidation in poultry (Leskovec et al., 2019). In addition, L-carnitine, a potent antioxidant, plays a crucial role in scavenging free radicals and protecting tissues from ROS-induced oxidative damage (Agarwal et al., 2018). Studies have shown that L-carnitine supplementation improves antioxidant activity (Çetin and Güçlü, 2020), enhances intestinal histology, modulates gut microbiota, reduces harmful bacteria populations, and promotes Lactobacilli growth in both laying hens and broilers under high stocking density (Eskandani et al.,

2022). Phytogenic compounds, rich in bioactive chemicals like polyphenols and flavonoids, are gaining attention for their stress-reducing properties (Shehata et al., 2022) and their ability to improve antioxidant enzyme (superoxide dismutase [SOD], glutathione peroxidase [GPx], and catalase [CAT]) activity by inhibiting NF-κB activation and reducing ROS production, and balance intestinal microbiota (Abd El-Hack et al., 2020). Among the herbs of interest, Camellia sinensis (green tea) with its primary antioxidant catechins (particularly epigallocatechin gallate), Syzygium aromatum (clove) rich in eugenol, and Persicaria odorata (Vietnamese coriander) containing gallic acid, quercetin have shown potential as feed additives to mitigate HS, offering several beneficial functions (Erener et al., 2011; Hosseinzadeh et al., 2014; El-Maati et al., 2016; Arif et al., 2022; Aziz-Aliabadi et al., 2023; Saracila et al., 2023). Pasri et al. (2023) reported that bioactive compounds from clove, green tea pomace, and Vietnamese coriander rich in phenolics and total flavonoid content (including eugenol, gallic acid, catechin, ellagic acid, quercetin, and kaempferol) demonstrated synergistic antioxidant activity in vitro by effectively scavenging free radicals without cytotoxicity. 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) enhanced free radical scavenging, upregulated SOD, CAT, and GSH-Px mRNA and downregulated NF-κB, HSP70, and HSP90 mRNA expressions in the liver of breeder hens under HS (Pasri et al., 2024).

This study focuses on these two antioxidant sources in relation to gut health, as the gut is the primary organ affected by HS, which subsequently influences other physiological systems. The jejunum serves as the most heat-sensitive section, whereas HS modulates the expression of heat shock proteins (HSPs), i.e., HSP70 and HSP90, along with inflammatory markers including nuclear factor kappa B (NF-kB), interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), toll-like receptor 4 (TLR4), and tight junction (TJ) proteins (claudin-1 [CLDN1], and zonula occludens-1 [ZO1]) in the jejunum (Santos et al., 2019). In addition, HS affects the abundance and composition of cecal microbiota. In chickens, Firmicutes, Bacteroidetes, and Actinobacteria dominate the cecal microflora (Wen et al., 2021). Previous studies have reported that HS decreased the relative abundance of Firmicutes and Ruminococcus, whereas the relative

abundance of Bacteroidetes was reduced in the cecum of broilers (Fang et al., 2023; Oetomiloye et al., 2024). Furthermore, modifications in the composition of gut microbiota typically result in changes to intestinal SCFA levels (D'Alessandro et al., 2024). The impact of HS on immune responses, antioxidant properties, intestinal barrier function, and cecal microbiota in chickens has been well documented; however, little is known about the supplementation with synthetic and phytogenic antioxidants on mechanisms of molecular regulation in the jejunum and changes in the cecal intestinal microbiota. Therefore, this study aimed to investigate the efficacy of two sources of antioxidants-synthetic (a combination of vitamin E, vitamin C, Se, and L-carnitine) and phytogenic (a combination of clove, green tea pomace, and Vietnamese coriander) -in breeder hens' diets on gut health parameters, including expression of antioxidants, HSPs, immunity, and TJ protein genes, cecal short-chain fatty acids (SCFAs) concentration, ammonia production, and cecal microbiota under HS conditions. Moreover, our results provide a new perspective on the adverse effects of HS in chickens.

5.3 Materials and methods

5.3.1 Ethics statement

The experiment was 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).

5.3.2 Housing, birds, and experimental diets

A total of one hundred 33-week-old SUT breeder hens, a synthesized line developed as commercial female breeders, were raised at the SUT farm and used in this study. Prior to the start of the experiment, the hens were individually housed in wire cages with a size of 40 × 45 × 40 cm^3 (length × width × height) and acclimated to a controlled temperature of 23 ± 1°C for 5 weeks (33-38 week of age). After the acclimation period, the hens were equally divided into four treatments (T1, T2, T3, and T4), each consisting of 25 hens, using a completely randomized design at 38 weeks of age. Throughout the experimental period from 38 to 52 weeks of age, the control group (T1) remained in the thermoneutral (TN) conditions at 23 ± 1°C with a relative humidity of 40–70%, regulated by an air conditioner system. Conversely, the T2, T3, and T4 groups were relocated to an HS room, where they were subjected to an elevated

temperature of 36 ± 1°C with approximately 40% relative humidity for 4 consecutive hours daily (1 pm – 5 pm), in accordance with established temperature and humidity stress index (Mirzaie et al., 2018; Roushdy et al., 2018). The HS room was maintained using a gas heater (liquefied petroleum gas) with thermostat-controlled equipment to regulate temperature. After completing heat treatment each day, the hens were returned to their original conditions for the same as the TN. The experimental diets were as follows: T1) basal diet under TN, T2) basal diet under HS, T3) basal diet supplemented with synthetic antioxidant combination (200 mg vitamin C/kg, 150 mg vitamin E/kg, 0.30 mg Se yeast/kg, and 150 mg L-carnitine/kg) under HS, as recommended by Ross 308 parent standards (Aviagen, 2021) and previous studies (Çetin and Güçlü, 2020; Shakeri et al., 2020), and T4) basal diet supplemented with 1% phytogenic antioxidant (a mixture of cloves, green tea pomace, and Vietnamese coriander powder in a ratio of 1:1:1/v: v: v) under HS (Pasri et al., 2023). Diets were formulated to meet nutritional requirements according to the recommendations of the National Research Council (1994) and Ross 308 parent stock standard recommendations (Aviagen, 2021), containing 15% CP and 2800 kcal ME/kg with calcium levels of 3.51%. The ingredient composition and calculations were previously reported by Pasri et al. (2024) (Table 5.1). All hens received 140 g daily feed (~20-21 g/hen/day of CP and ~392 kcal ME /hen/day) and a 16 h light cycle, with water provided ad libitum. At the end of 52 weeks of age, 6 breeder hens in each group were randomly selected and euthanized, and jejunal mucosal tissues were collected, immediately frozen in liquid nitrogen, and stored at −80°C for subsequent gene expression analysis. Cecal digesta from both sides of the breeder hens was aseptically collected: the right for microbial analysis and the left for SCFAs and ammonia analysis, then frozen at −20°C until analysis.

Table 5.1 Ingredients and chemical composition of the basal diet.

	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	-	-
Premix	0.52[1]	0.52[1]
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
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

[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.

5.3.3 Jejunal mucosa gene expression

Total RNA was extracted from jejunal mucosal tissue, using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and purified using a QIAamp spin column (Qiagen, Hilden, Germany). RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and quality assessed via 1% agarose gel electrophoresis in 0.5×TAE buffer at 100 V for 25 min. One microgram of total RNA was used for cDNA synthesis with QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) and random primers (Promega, Madison, WI, USA), following the manufacturer's protocol. Real-time polymerase chain reaction (PCR) was performed using the QuantiNova SYBR Green PCR kit (Qiagen, Hilden, Germany) and analyzed in triplicate as described by Humam et al. (2019). For real-time PCR, the master mix for each reaction (8 µL) included 5 µL of SYBR Green, 0.4 µL of forward primer, 0.4 µL of reverse primer, and 2.2 µL of nuclease-free water, with 2 µL of cDNA samples added to a 96-well microplate. The mRNA abundance of oxidative stress-related genes (superoxide dismutase [SOD], glutathione peroxidase [GSH-Px]), heat shock protein ([HSP]70 and HSP90), immune-related genes (interleukin [IL]-6, IL-10, tumor necrosis factor-alpha [TNFα], nuclear factor-κB [NF-κB], Toll-like receptor [TLR]4), and TJ proteins (claudin-1[CLDN1], zona occludens [ZO]-1) was determined by real-time quantitative PCR (RT-qPCR) using a CFX96 real-time PCR system (BioRad, Hercules, CA). The primer sequences for these genes are presented in Table 5.2. The RT-qPCR program, 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. Relative gene expression was calculated using a comparative method $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001) with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control.

Table 5.2 Primer sequences used for real-time PCR.

Gene	Primer sequences[1]	Accession No.
SOD	F-5'-CACTGCATCATTGGCCGTACCA-3'	NM_205064.1
	R-5'-GCTTGCACACGGAAGAGCAAGT-3'	
GSH-Px	F-5'-GCTGTTGCCTTCCTGAGAG-3'	NM_001277853.1
	R-5'-GTTCCAGGAGACGTCGTTGC-3'	
HSP70	F-5'-GATCTGGGCACCACGTATTCT-3'	FJ217667.1
	R-5'-GGTTCATTGCCACTTGGTTCTT-3'	
HSP90	F-5'-ACACATGCCAACCGCATTTA-3'	NM_001109785.1
	R-5'-CCTCCTCAGCAGCAGTATCA-3'	
IL-6	F-5'-CAAGGTGACGGAGGAGGAC-3'	AJ309540
	R-5'-TGGCGAGGAGGGATTTCT-3'	
IL-10	F-5'-GGAGCTGAGGGTGAAGTTTTGA-3'	NM_001004414.2
	R-5'-GACACAGACTGGCAGCCAAA-3'	
TNF-α	F-5'-CCCCTACCCTGTCCCACAA-3'	NM_204267.1
	R-5'-TGAGTACTGCGGAGGGTTCAT-3'	
TLR4	F-5'-CCCACACACCTGCCTACATGAA-3'	NM_001030693
	R-5'-GGATGGCAAGAGGACATATCAAA-3'	
NF-kB	F-5'-GAAGGAATCGTACCGGGAACA-3'	NM_205134
	R-5'--CTCAGAGGGCCTTGTGACAGTAA-3'	
ZO-1	F-5'-GGAGTACGAGCAGTCAACATAC-3'	XM_413773
	R-5'-GAGGCGCACGATCTTCATAA-3'	
CLDN1	F-5'-GATCCAGTGCAAGGTGTACGA-3'	NM_001013611
	R-5'-AAAGACAGCCATCCGCATCT-3'	
GAPDH	F-5'-GGTGGTGCTAAGCGTGTTAT-3'	K01458
	R-5'-ACCTCTGCCATCTCTCCACA-3'	

5.3.4 Short-chain fatty acids (SCFAs) and ammonia analysis

The concentrations of SCFAs (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid) were analyzed using a modified procedure (Mookiah et al., 2014). The cecal digesta were treated with 24% metaphosphoric acid in 1.5 M H2SO4 (the sample-to-solution ratio was 1:1), and

vortexed to ensure thorough mixing. The samples were left at room temperature overnight, then centrifuged at 10,000 × g at 4°C for 20 min, the supernatant was collected for analysis. The supernatants were analyzed by gas chromatography (Agilent 7890B; Agilent Technologies, Santa Clara, CA) using flame ionization detection (FID) with nitrogen as the carrier gas. A fused silica capillary column (0.32 mm × 25 m; CP-Sil 5 CB, J&W GC Column, Agilent Technologies, Santa Clara, CA) was used for the analysis. SCFAs were analyzed using 4-methylvaleric acid (Alfa Aesar, Heysham, UK) as an internal standard. The external standards for SCFA peak identification included a volatile acid mixture (C1–C7, 10 mM each in water, Supelco, Bellefonte, PA).

The ammonia content of cecal digesta was determined using a modified procedure (Willis et al., 1996). A total of 175 mg of sample was added to a polypropylene test tube, followed by the addition of 25 mL of 5% lithium carbonate (Li_2CO_3, Sigma-Aldrich, St Louis, MO). After vortexing, the mixture was centrifuged at 10,000 × g at 4°C for 15 min. 500 µL of supernatant was transferred to a 15 mL tube, then mixed with 4 mL of salicylate reagent and 1 mL of hypochlorite reagent by brief vortexing. The mixture was incubated at room temperature for 30 min, then absorbance was measured at 685 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA), and compared to a standard ammonia calibration curve.

5.3.5 DNA extraction and microbiome analysis

DNA was extracted from cecal digesta using MagPure Stool DNA KF Kit B (MAGEN, Guangzhou, China) following the manufacturer's instructions. DNA purity was verified by 0.8% agarose gel electrophoresis and quantified using a Qubit 2.0 Fluorometer (Toyobo, Osaka, Japan). Library preparation was performed using the 2 × Phanta Max Master Mix kit (VAZYME, Guangzhou, China), and sequencing was conducted on a DNBSEQ-G400 platform at BGI Genomics Co., Ltd. (Shenzhen, China) targeting the V3-V4 region of the 16S rRNA gene. The amplification primer sequences were 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Liu et al., 2022). High-quality clean reads are generated by filtering raw data to remove low-quality bases, adapter contamination, ambiguous bases, low-complexity reads, and reads reduced to less than 75% of their original length (He et al., 2013). Sequence splicing was conducted with FLASH software (v1.2.11) by assembling paired-end reads into single sequences based on the overlap, generating tags in highly variable

regions (Magoč and Salzberg, 2011). The spliced tags were clustered into operational taxonomic units (OTUs) using USEARCH (v7.0.1090), with clustering at a 97% similar threshold via UPARSE (Edgar, 2013). Chimeras were filtered using UCHIME (v4.2.40) (Edgar et al., 2011). All tags were aligned to representative OTU sequences using USEARCH's global method to obtain sample abundance statistics. Representative out sequences were aligned with a reference database using RDP Classifier (v2.2) (Wang et al., 2007) for species annotation, with sequence identity set to 0.6. Alpha and beta diversity were evaluated using mothur software (v.1.31.2) (Schloss et al., 2009) and QIIME (v1.80) (Lozupone et al., 2011), respectively. Distinctive taxa among treatment groups were identified using linear discriminant analysis effect size (LEfSe), on the Galaxy/Hutlab workflow platform (Segata et al., 2011). Phylogenetic investigation of communities by reconstruction of unobserved states 2 (PICRUSt2) (v2.3.0-b) (Douglas et al., 2019) was used to predict microbial community functional abundance based on marker gene sequencing profiles, using a threshold of relative abundance > 1% and $P < 0.05$.

5.3.6 Statistical analysis

Data for gene expression, SCFAs, and ammonia analysis were analyzed using SPSS software (version 27.0). All data values are presented as mean ± SEM. Orthogonal contrasts compared: 1) TN vs. HS conditions; 2) non-supplement vs. supplement; and 3) synthetic vs. phytogenic antioxidants. Additionally, Tukey's multiple comparison test was used to assess significant differences among treatments. Values were considered statistically different at $P < 0.05$. Alpha diversity analysis was calculated based on the Coverage, Chao1 index, Shannon index, and Simpson index. Significant differences in alpha diversity among different groups were calculated based on Kruskal-Wallis's test, where a significant difference level was set at $P < 0.05$. Beta diversity was calculated using unweighted UniFrac distance, with statistical comparisons among groups performed by permutational multivariate ANOVA. Figures were generated in GraphPad Prism (Graph Pad Software Inc., San Diego, CA). Predicted KEGG pathways' functional differences were analyzed by the Wilcoxon signed-rank test using R (v3.4.1) software. Spearman rank correlation analysis assessed the relationship between microbiota and other parameters.

5.4 Results

5.4.1 Jejunal mucosa gene expression

The effect of dietary synthetic and phytogenic antioxidant supplementation in breeder hen under HS conditions on gene expression related to antioxidant enzymes, HSPs, inflammatory mediators, and TJ proteins is shown in Table 5.3. Based on orthogonal contrasts, the HS challenge significantly altered the mRNA expression in the jejunal mucosa of genes related to antioxidant enzymes (SOD, GSH-Px), HSPs (HSP70, HSP90), immunity (IL-6, IL-10), and TJ proteins (ZO-1, CLDN1) compared to the TN group ($P < 0.05$). Either synthetic or phytogenic antioxidants supplementation significantly increased the expression of SOD, GSH-Px, IL-10, and CLDN1, while decreasing HSP70, HSP90, IL-6, TNF- α, NF-κB, and TLR4 compared to the HS group without supplementation ($P < 0.05$). Phytogenic antioxidant supplementation significantly increased the expression of GSH-Px compared to synthetic antioxidant supplementation under HS ($P < 0.05$). Interestingly, Tukey's multiple comparison tests indicated that the expression levels of SOD, GSH-Px, and IL-10 were significantly higher in both supplemented groups compared to the TN group ($P < 0.05$).

5.4.2 Cecal short-chain fatty acids (SCFAs) concentrations and ammonia production in cecal digesta

The effect of dietary antioxidant supplementation on the concentrations of cecal SCFAs and ammonia production in breeder hen exposed to HS conditions is presented in Table 5.4. Orthogonal contrasts revealed that under HS, dietary supplementation with either synthetic or phytogenic antioxidants significantly increased the concentrations of acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, and total SCFAs while decreasing ammonia production compared to the HS group without supplementation ($P < 0.05$). No significant differences in SCFA profiles were observed between the antioxidant sources ($P > 0.05$). Tukey's multiple comparison tests indicated that phytogenic antioxidant supplementation significantly increased acetic acid, butyric acid, and total SCFA concentrations while decreasing ammonia production, whereas synthetic antioxidant supplementation significantly increased isovaleric acid concentrations compared to the TN group ($P < 0.05$).

Table 5.3 Effect of antioxidant supplementation in breeder hen diets under heat stress conditions on gene expression related to the antioxidant enzymes, heat shock proteins, immunity, and tight junction proteins.

Items	Treatments[1]				Pooled	Contrasts[2]		
	T1	T2	T3	T4	SEM	1	2	3
Antioxidant enzymes								
SOD	0.85^{b}	0.64^{c}	1.53^{a}	1.58^{a}	0.117	0.009	<0.001	0.814
GSH-Px	1.05^{c}	0.75^{c}	1.51^{b}	1.85^{a}	0.117	0.014	<0.001	0.025
Heat shock proteins								
HSP70	1.43^{b}	2.48^{a}	0.81^{c}	0.70^{c}	0.184	0.037	<0.001	0.066
HSP90	1.08^{b}	1.94^{a}	1.14^{b}	1.07^{b}	0.107	0.041	<0.001	0.647
Inflammatory								
IL-10	1.22^{b}	1.51^{b}	1.97^{a}	2.08^{a}	0.098	<0.001	<0.001	0.410
IL-6	1.54^{b}	2.67^{a}	1.58^{b}	1.59^{b}	0.138	0.028	<0.001	0.932
TNF-α	1.29^{b}	1.99^{a}	1.22^{b}	1.11^{b}	0.161	0.281	<0.001	0.555
NF-κB	1.16^{b}	1.84^{a}	0.96^{b}	1.02^{b}	0.113	0.514	<0.001	0.797
TLR4	0.95^{b}	1.75^{a}	0.96^{b}	0.82^{b}	0.121	0.253	0.001	0.555
Tight junction proteins								
ZO-1	1.81^{a}	1.26^{b}	1.63^{ab}	1.54^{ab}	0.076	0.048	0.059	0.599
CLDN1	1.80^{a}	0.90^{b}	1.50^{a}	1.68^{a}	0.108	0.016	0.001	0.379

$^{a-c}$Means within each row with different superscripts are significantly different (P < 0 .05).

[1]T1, thermoneutral zone (23±1°C) + basal diet; T2, heat stress (36±1°C, 4 h/day) + basal diet; T3, heat stress (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress (36±1°C, 4 h/day) + basal diets with phytogenic.

[2]Orthogonal contrasts: 1, thermoneutral (T1) vs. heat stress conditions (T2, T3, T4); 2, non-supplement (T2) vs. supplement (T3, T4); 3, synthetic (T3) vs. phytogenic antioxidants (T4).

Table 5.4 Effect of antioxidant supplementation in breeder hen diets under heat stress conditions on cecal short-chain fatty acids and ammonia concentrations

Items	Treatments[1]				Pooled	Contrasts[2]		
	T1	T2	T3	T4	SEM	1	2	3
Short-chain fatty acids (μmol/g of cecal content)								
Acetate	25.38^{b}	17.25^{c}	30.74^{ab}	33.36^{a}	1.444	0.301	<0.001	0.207
Propionate	9.51^{a}	4.18^{b}	12.55^{a}	12.15^{a}	0.864	0.928	<0.001	0.800
Butyrate	3.80^{bc}	2.67^{c}	4.42^{ab}	5.31^{a}	0.253	0.395	<0.001	0.074
Isobutyrate	1.23^{a}	0.84^{b}	1.41^{a}	1.38^{a}	0.055	0.829	0.037	0.087
Valerate	1.16^{ab}	1.08^{b}	1.28^{a}	1.23^{a}	0.028	0.479	0.010	0.477
Isovalerate	1.14^{bc}	1.05^{c}	1.43^{a}	1.40^{ab}	0.458	0.061	<0.001	0.757
Total SCFA	42.2^{b}	27.07^{c}	51.82^{ab}	54.82^{a}	2.533	0.417	<0.001	0.402
Ammonia production (mg/g of cecal digesta)								
	1.02^{ab}	1.07^{a}	0.84^{bc}	0.79^{c}	0.034	0.079	0.002	0.534

$^{a-c}$Means within each row with different superscripts are significantly different (P < 0 .05).

[1]T1, thermoneutral zone (23±1°C) + basal diet; T2, heat stress (36±1°C, 4 h/day) + basal diet; T3, heat stress (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress (36±1°C, 4 h/day) + basal diets with phytogenic.

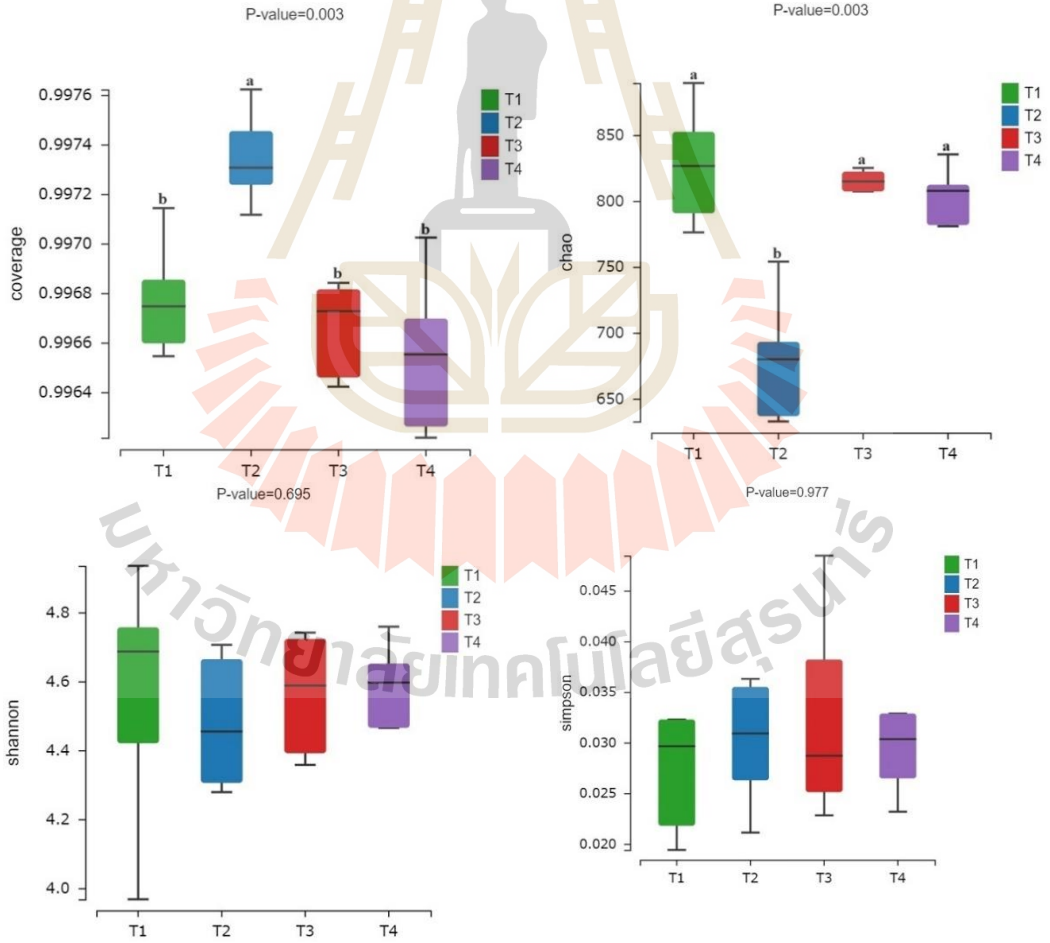
[2]Orthogonal contrasts: 1, thermoneutral (T1) vs. heat stress conditions (T2, T3, T4); 2, non-supplement (T2) vs. supplement (T3, T4); 3, synthetic (T3) vs. phytogenic antioxidants (T4).

5.4.3 Microbial alpha and beta diversity analysis

High-throughput 16S rRNA gene sequencing was performed to determine the effect of dietary synthetic and phytogenic antioxidants on the cecal microbiome of breeder hens under HS. In this study, Kruskal-Wallis's test revealed that the HS group without supplementation exhibited significantly higher Coverage indices compared to the TN group (P = 0.003), while supplementation with either synthetic or phytogenic antioxidant sources significantly lowered the Coverage indices compared to the HS group without supplementation (P = 0.003) (Figure 5.1A). Based on the Kruskal-Wallis's test showed that breeder hens exposed to the HS group without supplementation had

a significantly lower Chao1 richness index compared to the TN group (P = 0.003), however, dietary supplementation with either synthetic or phytogenic antioxidants restored the Chao1 index to levels similar to the TN group (P > 0.05) (Figure 5.1A). No significant differences were observed in Shannon diversity and Simpson index among all treatment groups (P > 0.05) (Figure 5.1A). In addition, the beta diversity of the cecal microbial composition among treatment groups was visualized using a principal coordinate analysis plot based on the unweighted UniFrac distances (Figure 5.1B). Permutational multivariate ANOVA showed that samples in the HS group without supplementation were separated from the TN group and the HS group receiving synthetic and phytogenic antioxidants (P = 0.0001).

A



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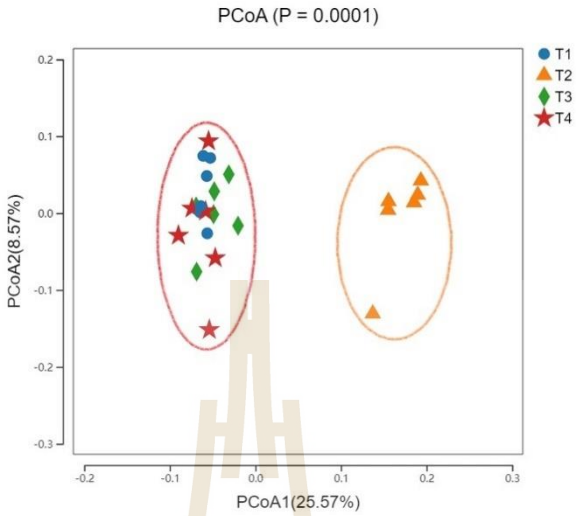


Figure 5.1 Effects of antioxidant supplementation in heat-stressed breeder hen diets under heat stress on microbial alpha diversity metrics (Coverage, Chao 1, Shannon entropy, and Simpson's index) (**A**), and principal coordinate analysis in heat-stressed hens (**B**).

[a-b] Means the effect of treatment was statistically different at $P < 0.05$.

T1, thermoneutral zone (23±1°C) + basal diet; T2, heat stress (36±1°C, 4 h/day) + basal diet; T3, heat stress (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress (36±1°C, 4 h/day) + basal diets with phytogenic.

5.4.4 Cecal microbial enrichments

Differential abundance analysis was performed using MetaStat to determine significant differences in microbial composition among treatment groups. The relative abundance of cecal microbiota at the phylum, family, and genus levels among the treatment groups is presented in Figure 5.2. At the phylum level, *Firmicutes* and *Bacteroidetes* were identified as the predominant phyla across all treatment groups (Figure 5.2A). In the TN, *Firmicutes* and *Bacteroidetes* comprised 62.89% and 27.60% of the total abundance, respectively. HS altered these proportions to 54.83% *Firmicutes* and 34.57% *Bacteroidetes*. However, supplementation with synthetic antioxidants increased *Firmicutes* to 69.37% while reducing *Bacteroidetes* to 23.75%. Similarly, phytogenic antioxidant supplementation resulted in 73.10% and 18.67%,

respectively. Among the most abundant phyla, Tukey's multiple comparison tests revealed significant differences in three major groups. Compared to the HS group without supplementation, dietary supplementation with either synthetic or phytogenic antioxidants significantly increased the relative abundance of *Firmicutes* (P = 0.003) while decreasing *Bacteroidetes* (P = 0.004), reaching levels similar to the TN group (P > 0.05). In addition, either synthetic or phytogenic antioxidants showed a significantly lower relative abundance of *Spirochaetes* compared to the TN group (P = 0.016).

The cecal bacterial communities at family level were dominated by *Lachnospiraceae*, *Bacteroidaceae*, and *Ruminococcaceae*, with distinct proportions observed across treatment groups: TN (25.21%, 12.15%, and 9.12%, respectively), HS (31.48%, 13.70%, and 6.45%, respectively), HS+ synthetic antioxidants (36.48%, 11.94%, and 11.43%, respectively), and HS+ phytogenic antioxidants (37.89%, 8.51%, and 11.13%, respectively) groups (Figure 5.2B). Among the top ten most abundant bacterial families, taxonomic analysis using ANOVA showed that under HS, either synthetic or phytogenic antioxidant supplementation significantly increased the relative abundance of *Lachnospiraceae* (P = 0.016) and *Ruminococcaceae* (P = 0.006), and decreased the relative abundance of *Coriobacteriaceae* (P = 0.037).

At the genus level, the taxon-based analysis revealed that the cecal bacterial communities were predominantly composed of B*acteroides*, *Ruminococcus2*, and *Romboutsia*, with distinct proportions observed across the treatments group: TN (12.15%, 7.75%, and 10.35%, respectively), HS (13.69%, 8.81%, and 4.35%, respectively), HS+ synthetic antioxidants (11.94%, 9.88%, and 4.99%, respectively), and HS+ phytogenic antioxidants (8.51%, 11.63%, and 4.07%, respectively) groups (Figure 5.2C). Among the top ten most abundant bacterial genera, taxonomic analysis using ANOVA showed that under HS, either synthetic or phytogenic antioxidant supplementation significantly increased the relative abundance of *Megamonas*, with higher abundance observed in the TN group compared to the HS group without supplementation (P = 0.036).

Linear discriminant analysis effect size (LEfSe) analysis with a threshold LDA score > 3.0 was performed to identify differential taxonomic biomarkers in the cecal microbiota across treatment groups. The analysis revealed that breeder hens raised under TN conditions exhibited a significantly higher relative abundance of several

bacterial taxa, including *p_Spirochaetota*, *c_Spirochaetia*, *o_Spirochaetales*, *f_Spirochaetaceae*. In addition, the HS group without supplementation increased the relative abundance of *o_Coriobacteriales*, *f_Coriobacteriaceae*, *g_Odoribacter*, *g_Rheinheimera*, *f_Chromatiaceae*, and *f_Leuconostocaceae*. Among the HS group, synthetic antioxidant treatment enhanced the relative abundances of *f_Ruminococcaceae*, *g_Pseudoflavonifractor*, and *g_Clostridium_XVIII*. Similarly, phytogenic antioxidants increased the relative abundances of *g_Ruminococcus2* and *o_Aeromonadales* (Figure 5.3).

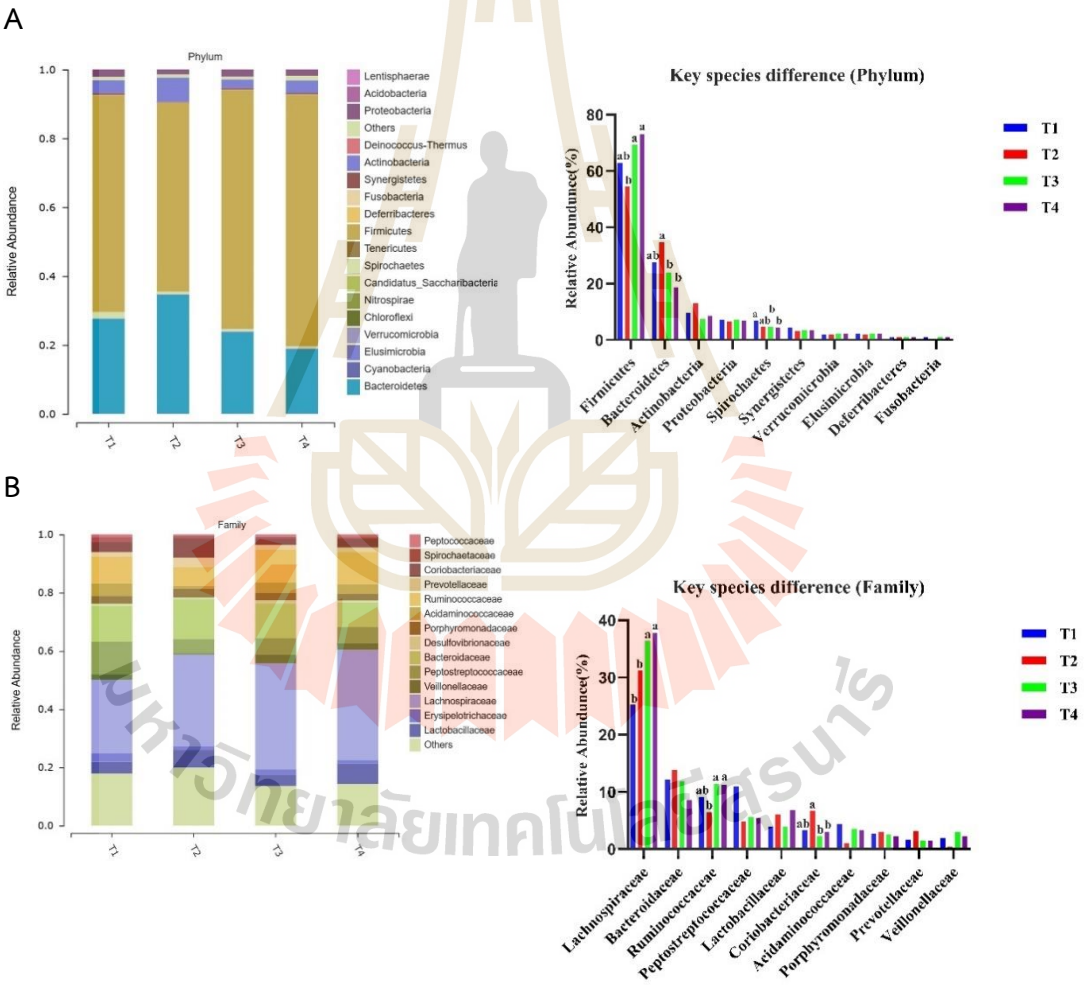


Figure 5.2 Effect of antioxidant supplementation in heat-stressed breeder hen diets on the relative abundance of microbiota and top 10 taxa in terms of phylum (**A**), family (**B**), and genus level (**C**).

[a-b] Means the effect of treatment was statistically different at $P < 0.05$.

T1, thermoneutral zone (23±1°C) + basal diet; T2, heat stress (36±1°C, 4 h/day) + basal diet; T3, heat stress (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress (36±1°C, 4 h/day) + basal diets with phytogenic.

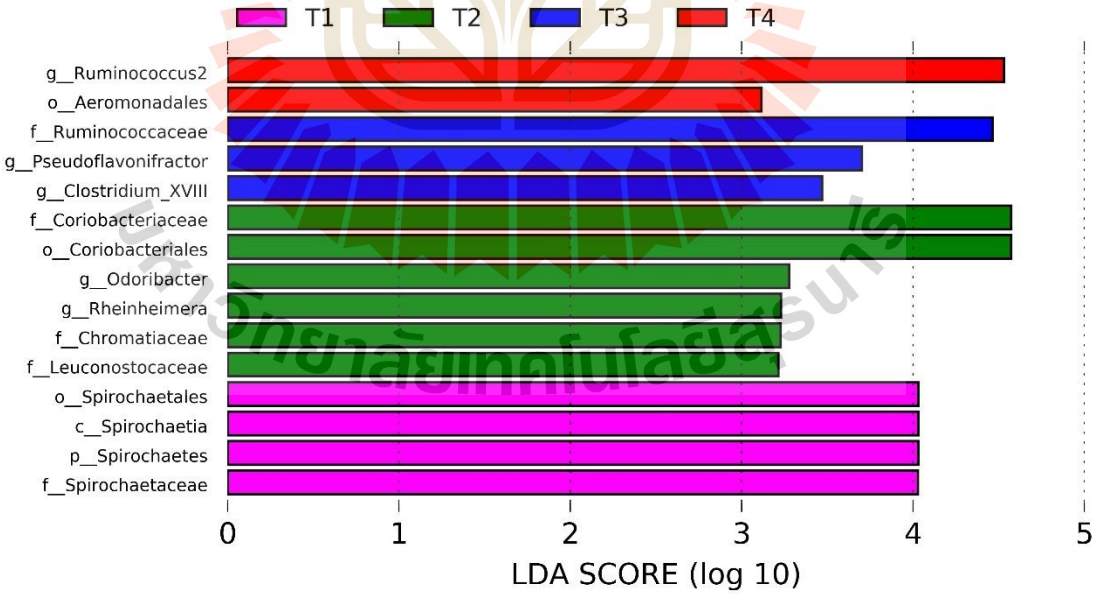


Figure 5.3 Effect of antioxidant supplementation in heat-stressed breeder hen diets on gut microbiota.

Abbreviation: p, phylum; o, order; g, genus; c, class; f, family.

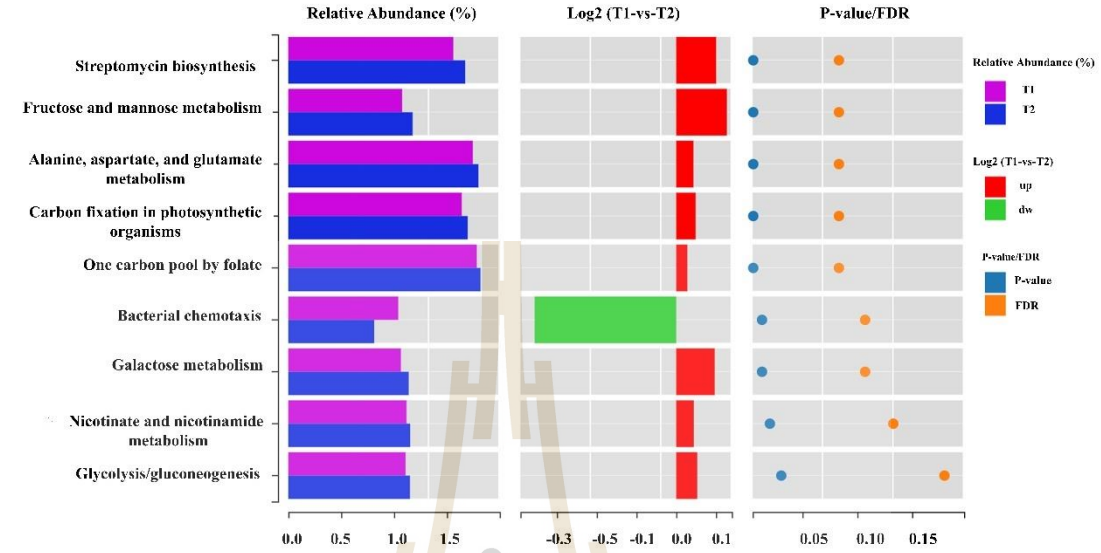
[a-b] Means the effect of treatment was statistically different at $P < 0.05$.

T1, thermoneutral zone (23±1°C) + basal diet; T2, heat stress (36±1°C, 4 h/day) + basal diet; T3, heat stress (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress (36±1°C, 4 h/day) + basal diets with phytogenic.

5.4.5 Functional prediction of cecal microbiota

A phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) analysis was performed to examine level 3 KEGG pathways and predict functional alterations in the cecal microbiota. HS group without supplementation significantly upregulated several metabolic pathways, including carbohydrate metabolism (fructose and mannose metabolism, galactose metabolism, and glycolysis/gluconeogenesis), amino acid metabolism (alanine, aspartate, and glutamate metabolism), energy metabolism (carbon fixation in photosynthetic organisms), metabolism of cofactors and vitamins (one carbon pool by folate, nicotinate and nicotinamide metabolism), and biosynthesis of secondary metabolites (streptomycin biosynthesis) ($P < 0.05$) (Figure 5.4A). Conversely, the HS group without supplementation significantly downregulated bacterial chemotaxis ($P < 0.05$). Dietary supplementation with either synthetic or phytogenic antioxidants under HS conditions, the altered nicotinate and nicotinamide metabolism, streptomycin biosynthesis, carbon fixation in photosynthetic organisms, and one carbon pool by folate functions (Figure 5.4B and 5.4C). Most notably, both antioxidant treatments significantly upregulated bacterial chemotaxis, thiamine metabolism, and lysine biosynthesis pathways and downregulated biosynthesis of vancomycin group antibiotics and folate biosynthesis ($P < 0.05$).

A



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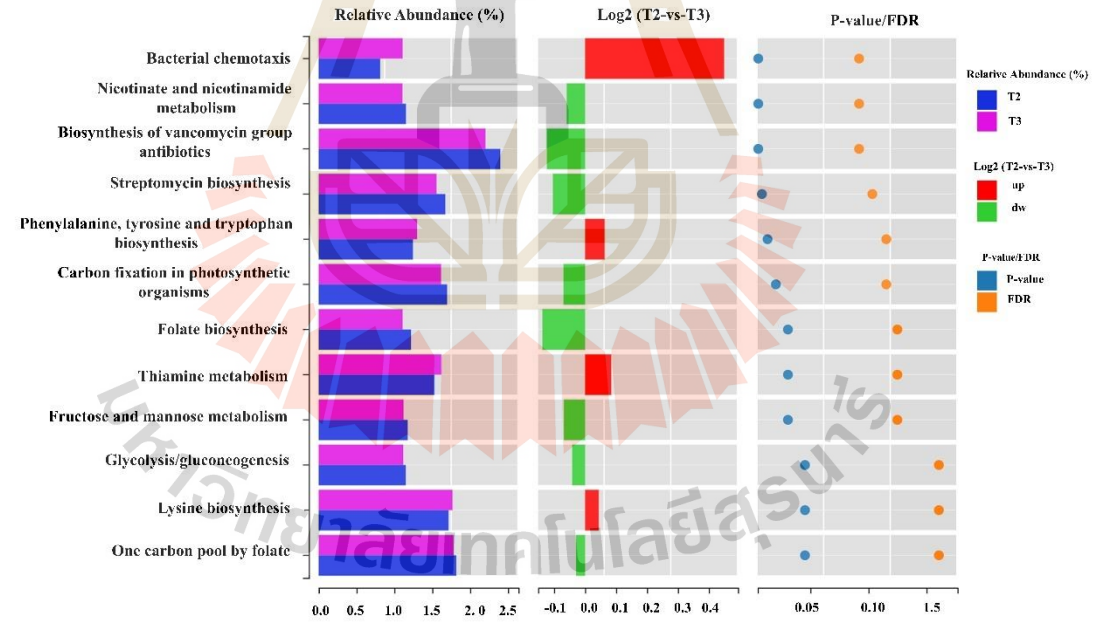

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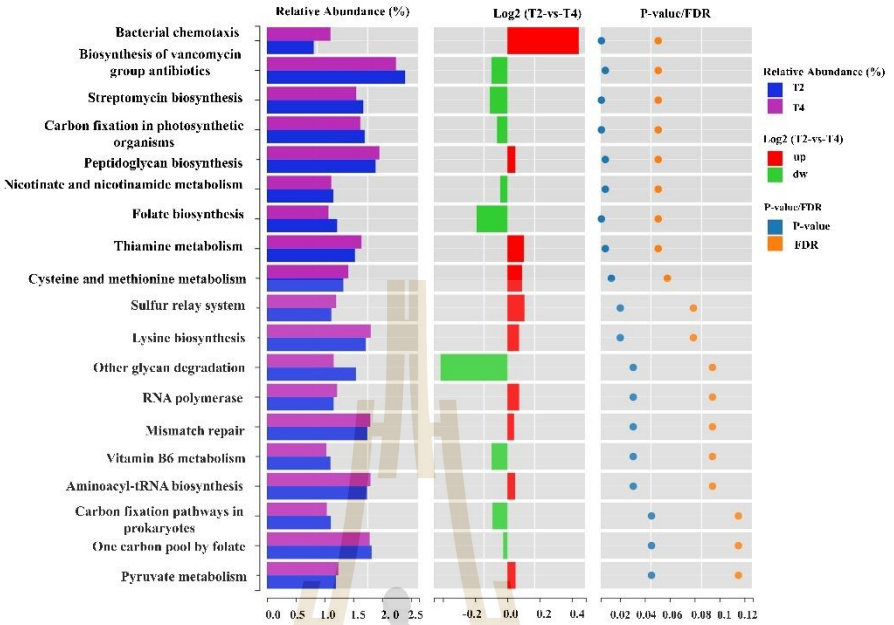

Figure 5.4 Predicted functions of cecal microbiota in heat-stressed breeder hens receiving dietary antioxidants at KEGG level 3 among the different groups: differentially regulated metabolic pathways in T1 vs. T2 (A), T2 vs. T3 (B), T2 vs. T4 (C).

T1, thermoneutral zone (23±1°C) + basal diet; T2, heat stress (36±1°C, 4 h/day) + basal diet; T3, heat stress (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress (36±1°C, 4 h/day) + basal diets with phytogenic.

5.4.6 Correlations between microbiota and measurement parameters

According to Spearman's correlation heat map analysis, the differentially enriched family *Lachnospiraceae* and *Ruminococcaceae* were positively correlated with butyric acid, isobutyric acid, and total SCFA concentrations ($P < 0.05$) (Figure 5.5A). Moreover, *Lachnospiraceae* and *Ruminococcaceae* were positively correlated with the expression of antioxidant-related genes SOD and GPx, and IL-10, whereas they negatively correlated with the HSP genes HSP70 ($P < 0.05$). Conversely, the differentially enriched family *Coriobacteriaceae* was positively correlated with the expression of inflammation-related genes (HSP70, HSP90, IL-6, TNF-α, NF-κB, and TLR4), whereas it negatively correlated with propionic acid, isobutyric acid, valeric acid, and total SCFA

concentrations, and the expression of antioxidant-related genes SOD and GPx ($P < 0.05$) (Figure 5.5A). In addition, the differentially enriched genus *Megamonas* exhibited a positive correlation with the concentration of acetic acid, butyric acid, isobutyric acid, isovaleric acid, and total SCFA and the expression of antioxidant-related genes SOD and anti-inflammatory cytokines (IL-10) ($P < 0.05$) (Figure 5.5B). In contrast, *Megamonas* had a negative correlation with the expression of pro-inflammatory-related genes (TNF-α and NF-κB) and heat stress-related genes (HSP70) in the jejunum ($P < 0.05$).

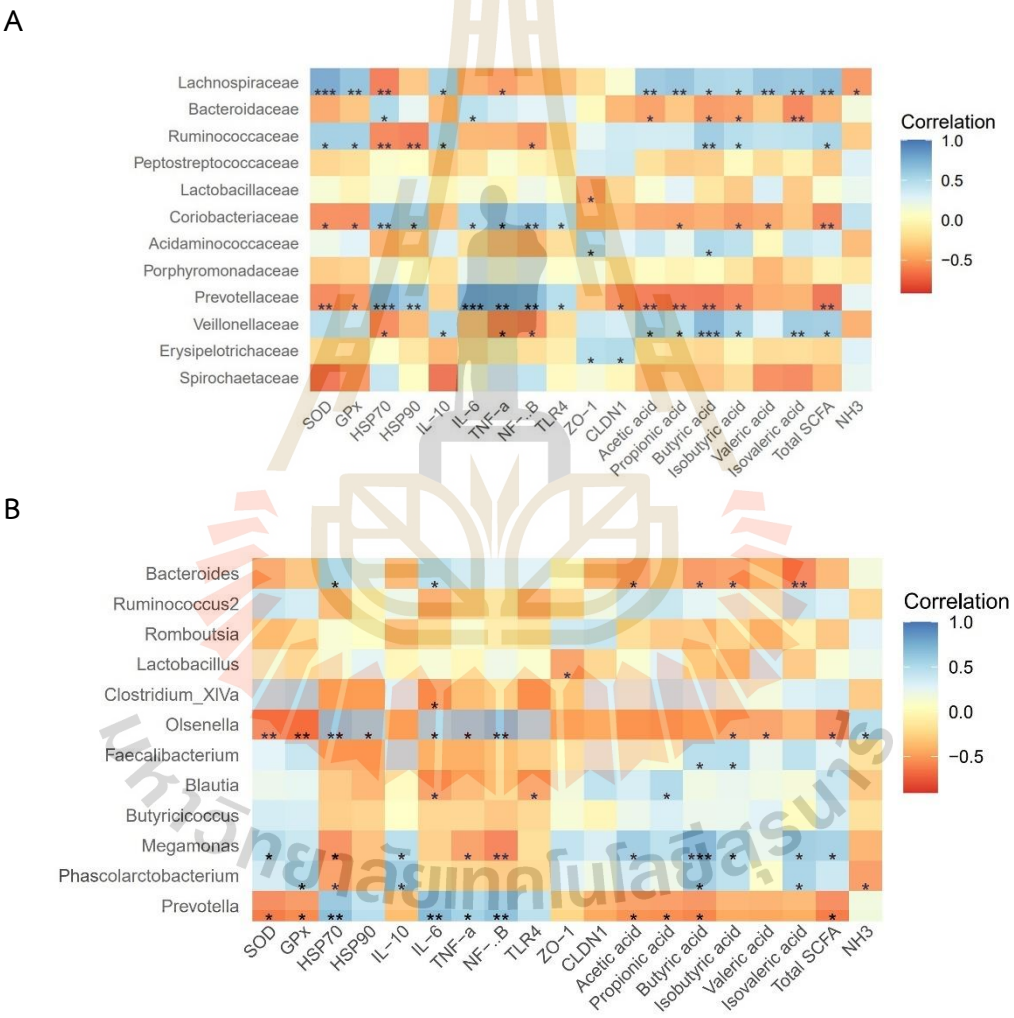

Figure 5.5 Spearman correlation analysis between different parameters and cecal microbial composition at the genus levels in heat-stressed breeder hens among the treatment groups. The row names represent the genera, and the column names represent the different parameters. The red and blue

squares represent the positive and negative correlation, respectively, with the shade of color indicating the level of correlation. Family (A), Genus (B). * P < 0.05, ** P < 0.01, *** P < 0.001.

5.5 Discussion

HS causes excessive ROS production, which ultimately disturbs the balance of the oxidative state and induces OS, compromises intestinal barrier integrity, impairs immune responses, and damages cells and tissues, leading to decreased performance, compromised welfare, and increased mortality and pathogen susceptibility in poultry (Chen et al., 2018; Humam et al., 2019; Emami et al., 2020; Lian et al., 2020; Calik et al., 2022). Therefore, it is crucial to mitigate HS in broilers through sustainable strategies. This study found that the HS group without supplementation challenge significantly altered the expressions of antioxidants enzymes (SOD), HSPs (HSP70 and HSP90), immune-related genes (IL-6, TNF- α, NF-κB, and TLR4), and TJ proteins (ZO-1 and CLDN1) in jejunal mucosa in breeder hens. However, either synthetic or phytogenic antioxidants supplementation significantly increased the expression of antioxidant enzymes (SOD and GSP-Px), immune-related (IL-10), and TJ proteins (CLDN1), whereas significantly decreased HSPs (HSP70 and HSP90) and pro-inflammatory cytokines (IL-6, TNF-α, NF-κB, and TLR4). Further, both types of dietary antioxidants increase cecal acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, and total SCFA concentrations, while also decreasing ammonia production in breeder hens under HS. This study demonstrates that both synthetic and phytogenic antioxidants could be beneficial supplements in breeder hens for combating the negative effects of HS.

In this study, we found that the HS group without supplementation had lower SOD and higher HSP70 levels compared to the TN conditions. HS-induced signaling pathways result in the upregulation of HSP70 and SOD through the HS-associated increase in ROS level (Banerjee Mustafi et al., 2009). Dietary supplementation with synthetic and phytogenic antioxidants had higher SOD and lower HSP70 levels compared to the HS group without supplementation and the TN condition in our study. Previous studies reported that either synthetic or phytogenic antioxidant supplementation exhibited higher antioxidant capacity, and upregulated the expression of SOD and GSP-Px

while downregulated HSP70 expression in the liver of breeder hens under HS, as evidenced by increased 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activities, and lower malondialdehyde (MDA) level in the liver compared to the HS group without supplementation (Pasri et al., 2023). Moreover, ROS levels are difficult to directly measure with high accuracy and precision because of their short half-life and quick reactivity with components that regulate redox states. A practical substitute method for determining OS in clinical samples is the indirect measurement of ROS by looking at the oxidative damage these radicals cause to the lipids, proteins, and nucleic acids of the cells (Gáspár, 2011). Synthetic antioxidants vitamins C and E have strong reactivity as electron (vitamin C) or hydrogen (vitamin E) donors to free radical oxidants, which avert oxidative damage to cells and tissues (Zwolak, 2020). The highly conjugated system, numerous hydroxyl groups, and aromatic structural features of phytogenic antioxidants (polyphenols) enable them to neutralize ROS or inhibit cellular OS and prevent oxidative damage to biomolecules (proteins, lipids, and DNA) (Checa and Aran, 2020). These findings suggest that both types of antioxidants may effectively eliminate excess ROS to prevent oxidative damage. Interestingly, in the present study, no significant differences were observed in the expression of pro-inflammatory-related genes (IL-6, TNF-α, NF-kB, and TLR4) and intestinal barrier-related genes (ZO-1 and CLDN1) between the HS groups receiving antioxidant supplementation and the TN condition, indicating that the ROS levels in the HS group with supplementation were comparable to those in the TN condition. Similarly, other studies have reported that supplementation with dietary vitamins E, C, and Se (Calik et al., 2022), microalgae (Chaudhary et al., 2023), dried plum (Wasti et al., 2021), and both synthetic and phytogenic sources (Pasri et al., 2024) did not result in significant differences in the mRNA abundance of IL-6, TNF-α, NF-kB, TLR4, ZO-1, and CLDN1 in several tissues in heat-stressed chickens compared to the TN condition. This may be attributed to the reduction of ROS by antioxidant sources in the HS group with supplementation. Although the current study did not measure ROS production in tissues, both synthetic and phytogenic antioxidant sources increased radical scavenging activities (DPPH, ABTS, and FRAP) and decreased MDA levels in the yolk and liver (Pasri et al., 2023). Thus, further measurements of ROS production in tissues to directly

correlate antioxidant supplementation with the modulation of gene expression, to better understand the underlying mechanisms of HS mitigation.

Our study also demonstrated that phytogenic antioxidants more effectively upregulate GSH-Px gene expression than synthetic antioxidants. This may be due to synthetic antioxidants containing only one type of Se, the key precursor for GSH-Px synthesis, while phytogenic antioxidants in cloves, green tea pomace, and Vietnamese coriander include a range of polyphenols with antioxidant properties. Although studies on the combined use of vitamin E, vitamin C, Se, and L-carnitine are limited, several studies have reported the synergistic effects of vitamin E, vitamin C, and Se (Shakeri et al., 2020; Calik et al., 2022), which enhance the effectiveness of the antioxidant defense system. In the body's antioxidant defense system, vitamin E safeguards cells and membranes from OS (Gao et al., 2010), supported by vitamin C which restores vitamin E and combats HS (Sahin et al., 2003). In addition, Se plays a vital role by enabling GSH-Px activity (Habibian et al., 2015). SOD and GSH-Px work synergistically to provide a robust defense against OS; SOD converts harmful superoxide radicals (O2-) into less harmful hydrogen peroxide, which GSH-Px then neutralizes (Surai et al., 2018). L-carnitine complements this system by scavenging free radicals, enhancing fatty acid metabolism, and regulating antioxidant enzyme activity, thereby improving overall health and performance under OS (Jia et al., 2014). Therefore, the combined use of vitamin E, vitamin C, Se, and L-carnitine exhibits antioxidant activity against ROS, potentially through hydrogen atom transfer and single electron transfer mechanisms. In addition, phytogenic antioxidants, particularly polyphenols, effectively reduce OS by regulating antioxidant gene expression through indirect mechanisms such as chelating transition metals and promoting the dissociation of the Kelch-like ECH-associated protein 1(Keap1)-Nrf2 complex leading to increased expression of key antioxidant enzymes like SOD, CAT, and GSH-Px (Di Meo et al., 2013; Lee et al., 2016). Pasri et al. (2023) reported that cloves, green tea, and Vietnamese coriander are rich sources of antioxidant polyphenols, including eugenol, gallic acid, catechins, ellagic acid, quercetin, and kaempferol. Hence, phytogenic antioxidants may be rich in bioactive compounds such as polyphenols, which can inhibit the production of ROS to alleviate OS in heat-stressed breeder hens, ultimately strengthening their antioxidant defense system. As expected, we observed significantly higher HSP90 levels in the HS group

without supplementation, whereas dietary supplementation with synthetic and phytogenic antioxidants significantly reduced HSP90 levels and increased IL-10 levels compared to the HS group without supplementation. HS triggers ROS production in chickens, activating the p38 mitogen-activated protein kinase pathway and inducing heat shock gene transcription, increasing HSP production in response to stress (Banerjee Mustafi et al., 2009). Previous studies have reported that dietary antioxidant supplementation with vitamin E, Se, and betaine significantly downregulated HSP70 and HSP90 mRNA levels in the liver and jejunal tissues of heat-stressed broilers (Alhotan et al. 2021; Calik et al., 2022). Moreover, the upregulation of IL-10 can protect cells from the damaging effects of OS by promoting the production of anti-inflammatory cytokines, aiding innate immunity in clearing pathogens, and providing cellular protection (Fayed et al., 2024). These findings imply that both antioxidant sources can mitigate HS-induced OS, inflammation, and intestinal barrier disruption by enhancing the endogenous antioxidant system, reducing ROS production, and regulating IL-10 expression.

In this study, we found that the concentrations of acetic acid, propionic acid, isobutyric acid, and total SCFA were significantly decreased in the HS group without supplementation compared to the TN group. However, among the HS group, dietary supplementation with either synthetic or phytogenic antioxidants increased the concentrations of acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, and total SCFAs. Previous studies have shown HS reduced cecal propionic acid and total SCFA concentrations in broilers, whereas supplementation with dried plum significantly increased acetic acid, propionic acid, and total SCFA levels (Wasti et al., 2021). SCFAs are crucial for maintaining intestinal health in birds, by contributing to epithelial integrity, immune regulation, energy metabolism, pH balance, and pathogen defense (Ojo et al., 2021). These metabolites primarily generate energy through gluconeogenesis and glycolysis, providing vital energy to intestine epithelial cells and promoting epithelial repair and regeneration (Jha et al., 2019). Among SCFAs, acetic acid, and butyric acid concentrations were significantly higher in the HS groups receiving phytogenic antioxidants than in TN conditions and HS conditions without supplementations. Acetic acid suppresses intestinal apoptosis and promotes mucin production (Liu et al., 2017), inflammasome activation (Macia et al., 2015), and

intestinal barrier integrity (Nowarski et al., 2015). Butyric acid is particularly significant for inhibiting intestinal pathogen colonization, maintaining balanced intestinal flora (Borda-Molina et al., 2021), and enhancing intestinal barrier function (Mathewson et al., 2016). Therefore, our findings suggest that either synthetic or phytogenic antioxidants increased the production of SCFAs, and alleviated gut inflammation, thereby promoting intestinal health in heat-stressed breeder hens.

In addition, ammonia emissions in poultry primarily result from the fermentation of nitrogen-containing substances by cecal microorganisms and the metabolism of urea and uric acid (Singer, 2003). In this study, we found that under HS, either synthetic or phytogenic antioxidant supplementation significantly decreases the ammonia production in the cecal digesta of breeder hens. Polyphenols from phytogenic antioxidants directly inhibit microbial urease activity, reducing the hydrolysis of urea to ammonia in the ceca (Yu et al., 2021). It has been reported that urease could hydrolyze urea into CO32- and NH4+, and NH4+ could release NH3 (Wei et al., 2022). Okrathok et al. (2023) have reported that dietary fiber from cassava pulp reduced the production of ammonia in the caecum of broilers by inhibiting uric acid-degrading bacterial enzymes. In addition, the ammonia content is related to the digestibility of nutrients (Yan et al., 2011; Jeong and Kim, 2014), because the increase in digestibility may reduce the substrate for microbial fermentation in the large intestine, which consequently decreases the ammonia content. Moreover, studies have shown that synthetic antioxidants and polyphenols may regulate the microbiota composition, and higher SCFA-producing bacterial populations like *Lachnospiraceae* and *Ruminococcaceae*, result in decreasing cecal pH, creating an environment less favorable for ammonia production and enhancing the absorption of nitrogenous compounds into the bloodstream (Yang et al., 2020; Elling Staats et al., 2022). The data indicate that synthetic and phytogenic antioxidants could reduce ammonia production by inhibiting urease activity and enhancing SCFA-producing bacteria (lower cecal pH). However, further tests, such as measuring urea, uric acid, urease activity, pH, and nutrient digestibility are needed to clarify the mechanism behind the changes in cecal ammonia concentrations in breeder hens under HS, future research could explore the specific mechanisms by which synthetic and phytogenic antioxidants modulate microbial urease activity.

The unique anatomy of the poultry gut, characterized by a shorter intestinal tract and rapid digesta transit, distinctly influences microbial diversity (Pan and Yu, 2014). In this study, *Firmicutes* and *Bacteroidetes* were the dominant phyla across different treatments in the cecum of breeder hens. This finding aligns with the previous study on the cecal content microbiota of laying hens (Xing et al., 2019). Moreover, our study revealed that either synthetic or phytogenic antioxidant supplementation significantly increased *Firmicutes* abundance and decreased *Bacteroidetes* abundance under HS. *Firmicutes*, which include many beneficial genera for gut health, are major contributors to SCFA production, which reduces gut pH. This acidic environment benefits *Firmicutes* but may hinder certain *Bacteroidetes*, resulting in their decreased abundance (Xiao et al., 2017; Qin et al., 2023), this may explain why the HS groups receiving antioxidant sources reduced the *Bacteroidetes* abundance in this study. In addition, *Firmicutes* decompose polysaccharides that cannot be digested by the host in the intestinal tract, promoting the digestion and absorption of nutrients by the body (Medinger et al., 2010; Lozupone et al., 2012; Johnson et al., 2015). *Bacteroidetes* play a key role in carbohydrate metabolism by breaking down sugars into SCFA, which are subsequently absorbed and utilized by the gut (Johnson et al., 2015). *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* are the 3 major phyla that inhabit the human large intestine, and these bacteria possess a fascinating array of enzymes that can degrade complex dietary substrates (Scott et al., 2013). In humans, the ratio of *Firmicutes* to *Bacteroidetes* (F/B) is known to be correlated with obesity. Obese children reportedly have a higher F/B ratio (Bervoets et al., 2013). Huang et al. (2021) found that the high feed efficiency group had a higher cecal F/B ratio than the low feed efficiency group in commercial yellow broilers, which may propose that the changes in the relative abundance of *Firmicutes* and *Bacteroidetes* may be linked to feed efficiency. In this study, the F/B ratios of chicken under TN conditions and HS without supplementation are 2.27 and 1.56, respectively. The increased F/B ratios were observed in the chickens supplemented with synthetic (2.92) and phytogenic (3.92) antioxidants under HS. This suggests that both antioxidant sources may increase feed efficiency in heat-stressed breeder hens. At the family level, either synthetic or phytogenic antioxidant supplementation increased the relative abundance of *Ruminococcaceae* and *Lachnospiraceae*, while decreasing the relative abundance of *Coriobacteriaceae*

compared to the HS group without supplementation in this study, consistent with previous findings (Calik et al., 2022; Fang et al., 2023; Oretomiloye and Adewole, 2024). *Ruminococcaceae* represent major butyrate producers. The majority of *Ruminococcaceae* produce butyrate by carbohydrate fermentation via the conversion of two acetyl-CoA molecules into crotonyl-CoA (Eeckhaut et al., 2016; Esquivel-Elizondo et al., 2017; Medvecky et al., 2018). Although some *Lachnospiraceae*, e.g., *Eubacterium hallii*, *Clostridium lactatifermentans*, *Clostridium saccharolyticum*, *Clostridium clostridioforme* or *Roseburia hominis* can produce butyrate from acetyl-CoA, representatives of this family do represent the most important butyrate producers (Medvecky et al., 2018). Since vegetative cells of *Ruminococcaceae* and *Lachnospiraceae* are highly sensitive to oxygen, these bacteria are among the first ones to disappear from gut microbiota during inflammatory diseases due to the production of reactive oxygen species by macrophages and granulocytes (Winter et al., 2010; Thiennimitr et al., 2011). In most cases, the decrease of *Ruminococcaceae* and *Lachnospiraceae* is therefore not the cause of the inflammation but its consequence (Medvecky et al., 2018). *Coriobacteriaceae* is involved in periodontitis and other zoonotic diseases (Pandit et al., 2018). At the genus level, either synthetic or phytogenic antioxidant supplementation increased the relative abundance of *Megamonas* compared to the HS group without supplementation. *Megamonas* have also been shown to utilize amino acids or carbohydrates to produce acetic acid, which plays a crucial role in intestinal energy supply, maintenance of the intestinal mucosal barrier, and regulation of intestinal motility (Biasato et al., 2020; Feng et al., 2023). Moreover, *Megamonas* effectively colonizes the broiler intestine and inhibits Salmonella growth in vitro (Yadav et al., 2021; Poudel et al., 2022), which warrants further investigation of its role in this genus in the chicken gastrointestinal tract. In healthy conditions, the gut lumen maintains an anoxic environment, however, under HS, ROS overproduction can result in injury to membrane permeability with the invasion of facultative anaerobic bacteria (Tomasello et al., 2016; Dam et al., 2019). Whereas both synthetic and phytogenic antioxidants have been shown to have ROS-scavenging properties that mitigate OS, maintain the hypoxic environment of the intestinal lumen, and are beneficial in promoting symbiotic gut microbiome communities (Sahin et al., 2012; Wang et al., 2020; Erener et al., 2011; El-Saber Batiha et al., 2020; Basit et al., 2020).

LEfSe analysis revealed that *Coriobacteriaceae* (at the family level) and *Coriobacteriales* (at the order level) were enriched in the HS group without supplementation. Dietary synthetic and phytogenic antioxidant supplementation significantly enriched *Ruminococcaceae* (at the family level) and Ruminococcus2 (at the genus level), respectively, in the heat-stressed breeder hens. The results of the LEFSe analysis were in agreement with the results of gut microbial abundance, indicating these bacteria can be microbiota biomarkers. Therefore, the increase in the abundance of SCFA-producing bacteria, including *Firmicutes*, *Lachnospiraceae*, *Ruminococcaceae*, and *Megamonas* by both antioxidant sources despite the HS challenge indicates that both synthetic and phytogenic antioxidants can mitigate HS-impaired ceca microbiota balance and contribute to gut health and function in heat-stressed chickens. These combined results may explain the significant increase in SCFA levels. In this study, the abundance of *Lachnospiraceae*, *Ruminococcaceae*, *Megamonas*, and *Coriobacteriaceae* in the cecum was found to be either positively or negatively correlated with the expression of SOD, HSP70, and the concentrations of isobutyric acid and total SCFAs, suggesting that these microbiota may serve as potential indicators for heat-stressed chickens. However, further research is needed to explore the cause of the trend between these microbiota and SOD, HSP70 expression, isobutyric acid, and total SCFA concentrations.

KEGG was used to predict the metabolic function changes in the microbial community. In this study, PICRUSt 2 analysis revealed that the HS group without supplementation significantly suppressed bacterial chemotaxis compared to TN conditions. Previous studies demonstrated that HS significantly downregulated bacterial chemotaxis compared to TN condition, whereas purple sweet potato anthocyanins and vitamin E, Se significantly increased bacterial chemotaxis subjected to HS (Calik et al., 2022; Fang et al.,2023), which agrees with our finding. Additionally, either synthetic or phytogenic antioxidants significantly upregulated bacterial chemotaxis, lysine biosynthesis, and thiamine metabolism in heat-stressed breeder hens compared to non-supplemented HS conditions. In butyrate synthesis, the glutamate, succinate, and lysine pathways have been identified (Bui et al., 2015). Based on the distribution of genes in intestinal metagenome libraries, the acetyl-CoA pathway was found to be the most prevalent, followed by the lysine pathway (Vital et al., 2014).

In addition, previous studies reported some gut commensal bacteria (including members of Eubacterium) can produce butyrate from lysine, although no gut microbe is known to contain the complete pathway (Bui et al., 2015). Rychlik, (2020) indicated that *Flavonifractor* and *Pseudoflavinofractor* can produce butyrate also by lysine fermentation or by reduction of succinate. In this study, LEfSe analysis revealed that supplementation with synthetic antioxidants enhanced the relative abundances of *Pseudoflavonifractor*, and at the KEGG module level, higher enrichment of lysine biosynthesis pathways of the HS group receiving synthetic antioxidants. Therefore, synthetic antioxidants may enhance butyrate production by increasing the abundance of *Pseudoflavonifractor* and upregulating the lysine biosynthesis pathway, which is crucial in reducing inflammation and maintaining gut epithelial health. Furthermore, thiamine is involved in functions of multiple enzymes necessary for the metabolism of carbohydrates, fatty acids, and amino acids (Rudzki et al., 2021). Magnusdottir et al. (2015) determined that *Firmicutes* cannot synthesize thiamine monophosphate, whereas thiamine biosynthesis is predominantly observed in *Bacteroidetes* and *Fusobacteria*. A previous study reported that certain propionate-producing bacteria synthesize thiamine or its precursors, and the primary pathway for propionate production is the succinate pathway, which is utilized by *Bacteroidetes* to convert carbohydrates into propionate (Louis et al., 2014). Taken together, these findings suggest that the altered thiamine metabolism by both antioxidant sources may be related to the decreasing relative abundance of *Bacteroidetes* in the cecum and may influence propionate production.

5.6 Conclusion

HS challenge induced multiple detrimental effects, including upregulated mRNA levels of HSPs and pro-inflammatory cytokines, downregulated antioxidant enzymes and barrier-related genes, decreased cecal SCFA concentrations, altered cecal microbiota composition, and reduced ammonia production. However, dietary supplementation with either synthetic antioxidants (vitamins C and E, Se, and L-carnitine) or phytogenic antioxidants (clove, green tea pomace, and Vietnamese coriander) effectively mitigated these negative effects by enhancing antioxidant status,

modulating immune-related and TJ genes (IL-10, IL-6, TNF-α, NF-κB, TLR4, and CLDN1) expression, increasing cecal SCFA concentrations, reducing ammonia production, and enhanced beneficial bacteria of cecal microbiota. These findings suggest that either synthetic or phytogenic antioxidants could potentially be utilized as a natural and effective dietary supplement to alleviate HS-induced adverse effects on the gut health of breeder hens. The findings of this study have to be seen in light of one limitation: HS is thought to impair mitochondrial metabolic capacity, leading to excessive ROS production, which disrupts the oxidative balance and triggers OS. This excess ROS further contributes to cellular and tissue damage. However, ROS production and OS damage were not measured in this study. Therefore, the indicators of OS, such as protein carbonyl content and thiobarbituric acid reactive substances measurements, are required in future experiments.

5.7 References

Abd El-Hack, M. E., Abdelnour, S. A., Taha, A. E., Khafaga, A. F., Arif, M., Ayasan, T., Swelum, A. A., Abukhalil, M. H., Alkahtani, S., Aleya, L., & Abdel-Daim, M. M. (2020). Herbs as thermoregulatory agents in poultry: An overview. **Science of The Total Environment**, 703, 134399.

Agarwal, A., Sengupta, P., & Durairajanayagam, D. (2018). Role of L-carnitine in female infertility. **Reproductive Biology and Endocrinology**, 16(1), 5.

Alhotan, R. A., Al Sulaiman, A. R., Alharthi, A. S., & Abudabos, A. M. (2021). Protective influence of betaine on intestinal health by regulating inflammation and improving barrier function in broilers under heat stress. **Poultry Science**, 100(9), 101337.

Arif, M., Rehman, A. U., Naseer, K., Abdel-Hafez, S. H., Alminderej, F. M., El-Saadony, M. T., Abd El-Hack, M. E., Taha, A. E., Elnesr, S. S., Salem, H. M., & Alagawany, M. (2022). Effect of Aloe vera and clove powder supplementation on growth performance, carcass and blood chemistry of Japanese quails. **Poultry Science**, 101(4), 101702.

Aziz - Aliabadi, F., Noruzi, H., & Hassanabadi, A. (2023). Effect of different levels of green tea (Camellia sinensis) and mulberry (Morus alba) leaves powder on

performance, carcass characteristics, immune response and intestinal morphology of broiler chickens. V**eterinary Medicine and Science**, 9(3), 1281–1291.

Banerjee Mustafi, S., Chakraborty, P. K., Dey, R. S., & Raha, S. (2009). Heat stress upregulates chaperone heat shock protein 70 and antioxidant manganese superoxide dismutase through reactive oxygen species (ROS), p38MAPK, and Akt. **Cell Stress and Chaperones**, 14(6), 579–589.

Bervoets, L., Van Hoorenbeeck, K., Kortleven, I., Van Noten, C., Hens, N., Vael, C., Goossens, H., Desager, K. N., & Vankerckhoven, V. (2013). Differences in gut microbiota composition between obese and lean children: A cross-sectional study. **Gut Pathogens**, 5(1), 10.

Biasato, I., Ferrocino, I., Grego, E., Dabbou, S., Gai, F., Gasco, L., Cocolin, L., Capucchio, M. T., & Schiavone, A. (2020). Yellow Mealworm Inclusion in Diets for Heavy-Size Broiler Chickens: Implications for Intestinal Microbiota and Mucin Dynamics. **Animals**, 10(10), 1909.

Borda-Molina, D., Mátis, G., Mackei, M., Neogrády, Z., Huber, K., Seifert, J., & Camarinha-Silva, A. (2021). Caeca Microbial Variation in Broiler Chickens as a Result of Dietary Combinations Using Two Cereal Types, Supplementation of Crude Protein and Sodium Butyrate. **Frontiers in Microbiology**, 11, 617800.

Bui, T. P. N., Ritari, J., Boeren, S., De Waard, P., Plugge, C. M., & De Vos, W. M. (2015). Production of butyrate from lysine and the Amadori product fructoselysine by a human gut commensal. **Nature Communications**, 6(1), 10062.

Calik, A., Emami, N. K., Schyns, G., White, M. B., Walsh, M. C., Romero, L. F., & Dalloul, R. A. (2022). Influence of dietary vitamin E and selenium supplementation on broilers subjected to heat stress, Part II: Oxidative stress, immune response, gut integrity, and intestinal microbiota. **Poultry Science**, 101(6), 101858.

Calik, A., & Ergün, A. (2015). Effect of lactulose supplementation on growth performance, intestinal histomorphology, cecal microbial population, and short-chain fatty acid composition of broiler chickens. **Poultry Science**, 94(9), 2173–2182.

Çetin, E., & Güçlü, B. K. (2020). Effect of dietary L - carnitine supplementation and energy level on oxidant/antioxidant balance in laying hens subjected to high

stocking density. **Journal of Animal Physiology and Animal Nutrition**, 104(1), 136 – 143.

Chaudhary, A., Mishra, P., Amaz, S. A., Mahato, P. L., Das, R., Jha, R., & Mishra, B. (2023). Dietary supplementation of microalgae mitigates the negative effects of heat stress in broilers. **Poultry Science**, 102(10), 102958.

Checa, J., & Aran, J. M. (2020). Reactive Oxygen Species: Drivers of Physiological and Pathological Processes. **Journal of Inflammation Research**, 13, 1057–1073.

D'Alessandro, A. G., Desantis, S., Fracchiolla, G., Porrelli, R., Dibenedetto, R. S., Di Luca, A., & Martemucci, G. (2024). Response of laying hens fed diet supplemented with a mixture of olive, laurel, and rosemary leaf powders: Metabolic profile, oxidative status, intestinal histomorphology, and egg quality. **Research in Veterinary Science**, 174, 105294.

Dam, B., Misra, A., & Banerjee, S. (2019). Role of Gut Microbiota in Combating Oxidative Stress. In S. Chakraborti, T. Chakraborti, D. Chattopadhyay, & C. Shaha (Eds.), Oxidative Stress in Microbial Diseases (pp. 43–82). **Springer Singapore**.

Di Meo, F., Lemaur, V., Cornil, J., Lazzaroni, R., Duroux, J.-L., Olivier, Y., & Trouillas, P. (2013). Free Radical Scavenging by Natural Polyphenols: Atom versus Electron Transfer. **The Journal of Physical Chemistry A**, 117(10), 2082–2092.

Dokladny, K., Ye, D., Kennedy, J. C., Moseley, P. L., & Ma, T. Y. (2008). Cellular and Molecular Mechanisms of Heat Stress-Induced Up-Regulation of Occludin Protein Expression. **The American Journal of Pathology**, 172(3), 659–670.

Du, E., Wang, W., Gan, L., Li, Z., Guo, S., & Guo, Y. (2016). Effects of thymol and carvacrol supplementation on intestinal integrity and immune responses of broiler chickens challenged with Clostridium perfringens. **Journal of Animal Science and Biotechnology**, 7(1), 19.

Duncan, S. H., Barcenilla, A., Stewart, C. S., Pryde, S. E., & Flint, H. J. (2002). Acetate Utilization and Butyryl Coenzyme A (CoA): Acetate-CoA Transferase in Butyrate-Producing Bacteria from the Human Large Intestine. **Applied and Environmental Microbiology**, 68(10), 5186–5190.

Edgar, R.C. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads. **Nature Methods**, 10, 996–998.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., & Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. **Bioinformatics**, 27(16), 2194–2200.

Eeckhaut, V., Wang, J., Van Parys, A., Haesebrouck, F., Joossens, M., Falony, G., Raes, J., Ducatelle, R., & Van Immerseel, F. (2016). The Probiotic Butyricicoccus pullicaecorum Reduces Feed Conversion and Protects from Potentially Harmful Intestinal Microorganisms and Necrotic Enteritis in Broilers. **Frontiers in Microbiology,** 2, 7.

El-Maati, M. F. A., Mahgoub, S. A., Labib, S. M., Al-Gaby, A. M. A., & Ramadan, M. F. (2016). Phenolic extracts of clove (Syzygium aromaticum) with novel antioxidant and antibacterial activities. **European Journal of Integrative Medicine**, 8(4), 494–504.

El-Saber Batiha, G., Alkazmi, L. M., Wasef, L. G., Beshbishy, A. M., Nadwa, E. H., & Rashwan, E. K. (2020). Syzygium aromaticum L. (Myrtaceae): Traditional Uses, Bioactive Chemical Constituents, **Pharmacological and Toxicological Activities. Biomolecules**, 10(2), 202.

Emami, N. K., Jung, U., Voy, B., & Dridi, S. (2020). Radical Response: Effects of Heat Stress-Induced Oxidative Stress on Lipid Metabolism in the Avian Liver. **Antioxidants**, 10(1), 35.

Erener, G., Ocak, N., Altop, A., Cankaya, S., Aksoy, H. M., & Ozturk, E. (2011). Growth Performance, Meat Quality and Caecal Coliform Bacteria Count of Broiler Chicks Fed Diet with Green Tea Extract. **Asian-Australasian Journal of Animal Sciences**, 24(8), 1128–1135.

Eskandani, M., Navidshad, B., Eskandani, M., Vandghanooni, S., Aghjehgheshlagh, F. M., Nobakht, A., & Shahbazfar, A. A. (2022). The effects of L-carnitine-loaded solid lipid nanoparticles on performance, antioxidant parameters, and expression of genes associated with cholesterol metabolism in laying hens. **Poultry Science**, 101(12), 102162.

Esquivel-Elizondo, S., Ilhan, Z. E., Garcia-Peña, E. I., & Krajmalnik-Brown, R. (2017). Insights into Butyrate Production in a Controlled Fermentation System via Gene Predictions. **mSystems**, 2(4), e00051-17.

Fang, X., Nong, K., Qin, X., Liu, Z., Gao, F., Jing, Y., Fan, H., Wang, Z., Wang, X., & Zhang, H. (2023). Effect of purple sweet potato-derived anthocyanins on heat stress

response in Wenchang chickens and preliminary mechanism study. **Poultry Science**, 102(9), 102861.

Fayed, R. H., Ali, S. E., Yassin, A. M., Madian, K., & Bawish, B. M. (2024). Terminalia bellirica and Andrographis paniculata dietary supplementation in mitigating heat stress-induced behavioral, metabolic and genetic alterations in broiler chickens. **BMC Veterinary Research**, 20(1), 388.

Feng, J., Li, Z., Ma, H., Yue, Y., Hao, K., Li, J., Xiang, Y., & Min, Y. (2023). Quercetin alleviates intestinal inflammation and improves intestinal functions via modulating gut microbiota composition in LPS-challenged laying hens. **Poultry Science**, 102(3), 102433.

Ferat-Osorio, E., Sánchez-Anaya, A., Gutiérrez-Mendoza, M., Boscó-Gárate, I., Wong-Baeza, I., Pastelin-Palacios, R., Pedraza-Alva, G., Bonifaz, L. C., Cortés-Reynosa, P., Pérez-Salazar, E., Arriaga-Pizano, L., López-Macías, C., Rosenstein, Y., & Isibasi, A. (2014). Heat shock protein 70 down-regulates the production of toll-like receptor-induced pro-inflammatory cytokines by a heat shock factor-1/constitutive heat shock element-binding factor-dependent mechanism. **Journal of Inflammation**, 11(1), 19.

Gao, J., Lin, H., Wang, X. J., Song, Z. G., & Jiao, H. C. (2010). Vitamin E supplementation alleviates the oxidative stress induced by dexamethasone treatment and improves meat quality in broiler chickens. **Poultry Science**, 89(2), 318–327.

Ghazi Harsini, S., Habibiyan, M., Moeini, M. M., & Abdolmohammadi, A. R. (2012). Effects of Dietary Selenium, Vitamin E, and Their Combination on Growth, Serum Metabolites, and Antioxidant Defense System in Skeletal Muscle of Broilers Under Heat Stress. **Biological Trace Element Research**, 148(3), 322–330.

Habibian, M., Sadeghi, G., Ghazi, S., & Moeini, M. M. (2015). Selenium as a Feed Supplement for Heat-Stressed Poultry: A Review. **Biological Trace Element Research**, 165(2), 183–193.

He, W., Zhao, S., & Liu, X. (2013). ReSeqTools: an integrated toolkit for large large-scale next-generation sequencing-based resequencing analysis. **Genetics and Molecular Research**, 12, 6275-6283.

Hosseinzadeh, H., Alaw Qotbi, A. A., Seidavi, A., Norris, D., & Brown, D. (2014). Effects of Different Levels of Coriander (Coriandrum sativum) Seed Powder and Extract on

Serum Biochemical Parameters, Microbiota, and Immunity in Broiler Chicks. **The Scientific World Journal**, 2014, 1–11.

Huang, Y., Lv, H., Song, Y., Sun, C., Zhang, Z., & Chen, S. (2021). Community composition of cecal microbiota in commercial yellow broilers with high and low feed efficiencies. **Poultry Science**, 100(4), 100996.

Humam, A. M., Loh, T. C., Foo, H. L., Samsudin, A. A., Mustapha, N. M., Zulkifli, I., & Izuddin, W. I. (2019). Effects of Feeding Different Postbiotics Produced by Lactobacillus plantarum on Growth Performance, Carcass Yield, Intestinal Morphology, Gut Microbiota Composition, Immune Status, and Growth Gene Expression in Broilers under Heat Stress. **Animals**, 9(9), 644.

Jeong, J. S., & Kim, I. H. (2014). Effect of Bacillus subtilis C-3102 spores as a probiotic feed supplement on growth performance, noxious gas emission, and intestinal microflora in broilers. **Poultry Science**, 93(12), 3097–3103.

Jha, R., Fouhse, J. M., Tiwari, U. P., Li, L., & Willing, B. P. (2019). Dietary Fiber and Intestinal Health of Monogastric Animals. **Frontiers in Veterinary Science**, 6, 48.

Johnson, D. R., Lee, T. K., Park, J., Fenner, K., & Helbling, D. E. (2015). The functional and taxonomic richness of wastewater treatment plant microbial communities are associated with each other and with ambient nitrogen and carbon availability. **Environmental Microbiology**, 17(12), 4851–4860.

Khan, R. U., Naz, S., Ullah, H., Ullah, Q., Laudadio, V., Qudratullah, Bozzo, G., & Tufarelli, V. (2023). Physiological dynamics in broiler chickens under heat stress and possible mitigation strategies. **Animal Biotechnology**, 34(2), 438–447.

Kumbhar, S., Khan, A. Z., Parveen, F., Nizamani, Z. A., Siyal, F. A., El-Hack, M. E. A., Gan, F., Liu, Y., Hamid, M., Nido, S. A., & Huang, K. (2018). Impacts of selenium and vitamin E supplementation on mRNA of heat shock proteins, selenoproteins and antioxidants in broilers exposed to high temperature. **AMB Express**, 8(1), 112.

Lee, M. T., Lin, W. C., Yu, B., & Lee, T. T. (2016). Antioxidant capacity of phytochemicals and their potential effects on oxidative status in animals—A review. **Asian-Australasian Journal of Animal Sciences**, 30(3), 299–308.

Leskovec, J., Levart, A., Perić, L., Đukić Stojčić, M., Tomović, V., Pirman, T., Salobir, J., & Rezar, V. (2019). Antioxidative effects of supplementing linseed oil-enriched diets with α-tocopherol, ascorbic acid, selenium, or their combination on carcass and meat quality in broilers. **Poultry Science**, 98(12), 6733–6741.

Liu, J., Wang, J., Shi, Y., Su, W., Chen, J., Zhang, Z., Wang, G., & Wang, F. (2017). Short Chain Fatty Acid Acetate Protects against Ethanol-Induced Acute Gastric Mucosal Lesion in Mice. **Biological & Pharmaceutical Bulletin**, 40(9), 1439–1446.

Liu, W.-C., Pan, Z.-Y., Zhao, Y., Guo, Y., Qiu, S.-J., Balasubramanian, B., & Jha, R. (2022). Effects of Heat Stress on Production Performance, Redox Status, Intestinal Morphology and Barrier-Related Gene Expression, Cecal Microbiome, and Metabolome in Indigenous Broiler Chickens. **Frontiers in Physiology**, 13, 890520.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. **Methods**, 25(4), 402–408.

Louis, P., Hold, G. L., & Flint, H. J. (2014). The gut microbiota, bacterial metabolites and colorectal cancer. **Nature Reviews Microbiology**, 12(10), 661–672.

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., & Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. **Nature**, 489(7415), 220–230.

Lozupone, C., Lladser, M. E., Knights, D., Stombaugh, J., & Knight, R. (2011). UniFrac: An effective distance metric for microbial community comparison. **The ISME Journal**, 5(2), 169–172.

Macia, L., Tan, J., Vieira, A. T., Leach, K., Stanley, D., Luong, S., Maruya, M., Ian McKenzie, C., Hijikata, A., Wong, C., Binge, L., Thorburn, A. N., Chevalier, N., Ang, C., Marino, E., Robert, R., Offermanns, S., Teixeira, M. M., Moore, R. J., … Mackay, C. R. (2015). Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. **Nature Communications**, 6(1), 6734.

Magnúsdóttir, S., Ravcheev, D., De Crécy-Lagard, V., & Thiele, I. (2015). Systematic genome assessment of B-vita**min biosynthesis suggests co-operation among gut microbes.** Frontiers in Genetics, 1, 6.

Magoč, T., & Salzberg, S. L. (2011). FLASH: Fast length adjustment of short reads to improve genome assemblies. **Bioinformatics**, 27(21), 2957–2963.

Mathewson, N. D., Jenq, R., Mathew, A. V., Koenigsknecht, M., Hanash, A., Toubai, T., Oravecz-Wilson, K., Wu, S.-R., Sun, Y., Rossi, C., Fujiwara, H., Byun, J., Shono, Y., Lindemans, C., Calafiore, M., Schmidt, T. M., Honda, K., Young, V. B., Pennathur, S., … Reddy, P. (2016). Gut microbiome–derived metabolites modulate intestinal epithelial cell damage and mitigate graft-versus-host disease. **Nature Immunology**, 17(5), 505–513.

Medinger, R., Nolte, V., Pandey, R. V., Jost, S., Ottenwälder, B., Schlötterer, C., & Boenigk, J. (2010). Diversity in a hidden world: Potential and limitation of next-generation sequencing for surveys of molecular diversity of eukaryotic microorganisms. **Molecular Ecology**, 19(s1), 32–40.

Medvecky, M., Cejkova, D., Polansky, O., Karasova, D., Kubasova, T., Cizek, A., & Rychlik, I. (2018). Whole genome sequencing and function prediction of 133 gut anaerobes isolated from chicken caecum in pure cultures. **BMC Genomics**, 19(1), 561.

Mirzaie, S., Zirak-Khattab, F., Hosseini, S. A., & Donyaei-Darian, H. (2018). Effects of dietary Spirulina on antioxidant status, lipid profile, immune response and performance characteristics of broiler chickens reared under high ambient temperature. **Asian-Australasian Journal of Animal Sciences**, 31(4), 556–563.

Mookiah, S., Sieo, C. C., Ramasamy, K., Abdullah, N., & Ho, Y. W. (2014). Effects of dietary prebiotics, probiotic and synbiotics on performance, caecal bacterial populations and caecal fermentation concentrations of broiler chickens: Effects of dietary prebiotics, probiotic and synbiotics on performance. **Journal of the Science of Food and Agriculture**, 94(2), 341–348.

Nowarski, R., Jackson, R., Gagliani, N., de Zoete, M. R., Palm, N. W., Bailis, W., Low, J. S., Harman, C. C. D., Graham, M., Elinav, E., & Flavell, R. A. (2015). Epithelial IL-18 Equilibrium Controls Barrier Function in Colitis. **Cell**, 163(6), 1444–1456.

Nutrient requirements of poultry. (1994). 9th rev. ed. **National Academy Press, Washington**, D.C.

Ojo, B. A., Lu, P., Alake, S. E., Keirns, B., Anderson, K., Gallucci, G., Hart, M. D., El-Rassi, G. D., Ritchey, J. W., Chowanadisai, W., Lin, D., Clarke, S., Smith, B. J., & Lucas, E.

A. (2021). Pinto beans modulate the gut microbiome, augment MHC II protein, and antimicrobial peptide gene expression in mice fed a normal or western-style diet. **The Journal of Nutritional Biochemistry**, 88, 108543.

Oke, O. E., Akosile, O. A., Oni, A. I., Opowoye, I. O., Ishola, C. A., Adebiyi, J. O., Odeyemi, A. J., Adjei-Mensah, B., Uyanga, V. A., & Abioja, M. O. (2024). Oxidative stress in poultry production. **Poultry Science**, 103(9), 104003.

Oretomiloye, F., & Adewole, D. (2024). Exploring the modulatory effects of brown seaweed meal and extracts on intestinal microbiota and morphology of broiler chickens challenged with heat stress. **Poultry Science**, 103(4), 103562.

Pan, D., & Yu, Z. (2014). Intestinal microbiome of poultry and its interaction with host and diet. **Gut Microbes**, 5(1), 108–119.

Pandit, R. J., Hinsu, A. T., Patel, N. V., Koringa, P. G., Jakhesara, S. J., Thakkar, J. R., Shah, T. M., Limon, G., Psifidi, A., Guitian, J., Hume, D. A., Tomley, F. M., Rank, D. N., Raman, M., Tirumurugaan, K. G., Blake, D. P., & Joshi, C. G. (2018). Microbial diversity and community composition of caecal microbiota in commercial and indigenous Indian chickens determined using 16s rDNA amplicon sequencing. **Microbiome**, 6(1), 115.

Pasri, P., Mermillod, P., & Khempaka, S. (2023). Antioxidant properties and cytotoxic effects of selected edible plants in Southeast Asia for further use as phytogenic antioxidant additives. **Saudi Journal of Biological Sciences**, 30(5), 103631.

Pasri, P., Rakngam, S., Gérard, N., Mermillod, P., & Khempaka, S. (2024). Synthetic and phytogenic antioxidants improve productive performance, antioxidant activity, gene expression, and offspring quality in breeder hens subjected to heat stress. **Poultry Science**, 103(3), 103390.

Poudel, B., Shterzer, N., Sbehat, Y., Ben-Porat, N., Rakover, M., Tovy-Sharon, R., Wolicki, D., Rahamim, S., Bar-Shira, E., & Mills, E. (2022). Characterizing the chicken gut colonization ability of a diverse group of bacteria. **Poultry Science**, 101(11), 102136.

Qin, Q., Li, Z., Zhang, M., Dai, Y., Li, S., Wu, H., Zhang, Z., & Chen, P. (2023). Effects of melittin on production performance, antioxidant function, immune function, heat shock protein, intestinal morphology, and cecal microbiota in heat-stressed quails. **Poultry Science**, 102(10), 102713.

Reith, R. R., Sieck, R. L., Grijalva, P. C., Swanson, R. M., Fuller, A. M., Diaz, D. E., Schmidt, T. B., Yates, D. T., & Petersen, J. L. (2022). Transcriptome analyses indicate that heat stress-induced inflammation in white adipose tissue and oxidative stress in skeletal muscle is partially moderated by zilpaterol supplementation in beef cattle. **Journal of Animal Science**, 100(3), skac019.

Roushdy, E. M., Zaglool, A. W., & El-Tarabany, M. S. (2018). Effects of chronic thermal stress on growth performance, carcass traits, antioxidant indices and the expression of HSP70, growth hormone and superoxide dismutase genes in two broiler strains. **Journal of Thermal Biology**, 74, 337–343.

Rudzki, L., Stone, T. W., Maes, M., Misiak, B., Samochowiec, J., & Szulc, A. (2021). Gut microbiota-derived vitamins – underrated powers of a multipotent ally in psychiatric health and disease. **Progress in Neuro-Psychopharmacology and Biological Psychiatry**, 107, 110240.

Rychlik, I. (2020). Composition and Function of Chicken Gut Microbiota. **Animals**, 10(1), 103.

Sahin, K., Orhan, C., Akdemir, F., Tuzcu, M., Iben, C., & Sahin, N. (2012). Resveratrol protects quail hepatocytes against heat stress: Modulation of the Nrf2 transcription factor and heat shock proteins. **Journal of Animal Physiology and Animal Nutrition**, 96(1), 66–74.

Sahin, K., Sahin, N., & Kucuk, O. (2003). Effects of chromium, and ascorbic acid supplementation on growth, carcass traits, serum metabolites, and antioxidant status of broiler chickens reared at a high ambient temperature (32°C). **Nutrition Research**, 23(2), 225–238.

Santos, R. R., Awati, A., Roubos-van Den Hil, P. J., Van Kempen, T. A. T. G., Tersteeg-Zijderveld, M. H. G., Koolmees, P. A., Smits, C., & Fink-Gremmels, J. (2019). Effects of a feed additive blend on broilers challenged with heat stress**. Avian Pathology**, 48(6), 582–601.

Saracila, M., Panaite, T. D., Predescu, N. C., Untea, A. E., & Vlaicu, P. A. (2023). Effect of Dietary Salicin Standardized Extract from Salix alba Bark on Oxidative Stress Biomarkers and Intestinal Microflora of Broiler Chickens Exposed to Heat Stress. **Agriculture**, 13(3), 698.

Schloss, P. D., Westcott, S. L., & Ryabin, T. (2009). Introducing mothur: open open-source, platform platform-independent, community community-supported software for describing and comparing microbial communities. **Applied and Environmental Microbiology**, 15, 398.

Scott, K. P., Gratz, S. W., Sheridan, P. O., Flint, H. J., & Duncan, S. H. (2013). The influence of diet on the gut microbiota. **Pharmacological Research**, 69(1), 52–60.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. **Genome Biology**, 12(6), R60.

Shakeri, M., Oskoueian, E., Le, H., & Shakeri, M. (2020). Strategies to Combat Heat Stress in Broiler Chickens: Unveiling the Roles of Selenium, Vitamin E and Vitamin C. **Veterinary Sciences**, 7(2), 71.

Shehata, A. A., Yalçın, S., Latorre, J. D., Basiouni, S., Attia, Y. A., Abd El-Wahab, A., Visscher, C., El-Seedi, H. R., Huber, C., Hafez, H. M., Eisenreich, W., & Tellez-Isaias, G. (2022). Probiotics, Prebiotics, and Phytogenic Substances for Optimizing Gut Health in Poultry. **Microorganisms**, 10(2), 395.

Singer, M. A. (2003). Do mammals, birds, reptiles and fish have similar nitrogen conserving systems? Comparative Biochemistry and Physiology Part B: **Biochemistry and Molecular Biology**, 134(4), 543–558.

Surai, P. F., Kochish, I. I., & Fisinin, V. I. (2018). Glutathione peroxidases in poultry biology: Part 1. Classification and mechanisms of action. **World's Poultry Science Journal**, 74(2), 185–198.

Thiennimitr, P., Winter, S. E., Winter, M. G., Xavier, M. N., Tolstikov, V., Huseby, D. L., Sterzenbach, T., Tsolis, R. M., Roth, J. R., & Bäumler, A. J. (2011). Intestinal inflammation allows Salmonella to use ethanolamine to compete with the microbiota. **Proceedings of the National Academy of Sciences**, 108(42), 17480–17485.

Tomasello, G., Mazzola, M., Leone, A., Sinagra, E., Zummo, G., Farina, F., Damiani, P., Cappello, F., Gerges Geagea, A., Jurjus, A., Bou Assi, T., Messina, M., & Carini, F. (2016). Nutrition, oxidative stress, and intestinal dysbiosis: Influence of diet on gut microbiota in inflammatory bowel diseases. Biomed. **Biomedical Papers of**

the Medical Faculty of the University Palacky, Olomouc, Czech Republic, 160, 461–466.

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.

Vital, M., Howe, A. C., & Tiedje, J. M. (2014). Revealing the Bacterial Butyrate Synthesis Pathways by Analyzing (Meta)genomic Data. **mBio**, 5(2), e00889-14.

Wang, R. X., Lee, J. S., Campbell, E. L., & Colgan, S. P. (2020). Microbiota-derived butyrate dynamically regulates intestinal homeostasis through regulation of actin-associated protein synaptopodin. **Proceedings of the National Academy of Sciences**, 117(21), 11648–11657.

Wasti, S., Sah, N., Singh, A. K., Lee, C. N., Jha, R., & Mishra, B. (2021). Dietary supplementation of dried plum: A novel strategy to mitigate heat stress in broiler chickens. **Journal of Animal Science and Biotechnology**, 12(1), 58.

Wei, T., Yashir, N., An, F., Imtiaz, S. A., Li, X., & Li, H. (2022). Study on the performance of carbonate-mineralized bacteria combined with eggshell for immobilizing Pb and Cd in water and soil. **Environmental Science and Pollution Research**, 29(2), 2924–2935.

Wen, C., Yan, W., Mai, C., Duan, Z., Zheng, J., Sun, C., & Yang, N. (2021). Joint contributions of the gut microbiota and host genetics to feed efficiency in chickens. **Microbiome**, 9(1), 126.

Willis, R. B., Montgomery, M. E., & Allen, P. R. (1996). Improved Method for Manual, Colorimetric Determination of Total Kjeldahl Nitrogen Using Salicylate. **Journal of Agricultural and Food Chemistry**, 44(7), 1804–1807.

Winter, S. E., Thiennimitr, P., Winter, M. G., Butler, B. P., Huseby, D. L., Crawford, R. W., Russell, J. M., Bevins, C. L., Adams, L. G., Tsolis, R. M., Roth, J. R., & Bäumler, A. J. (2010). Gut inflammation provides a respiratory electron acceptor for Salmonella. **Nature**, 467(7314), 426–429.

Xiao, Y., Xiang, Y., Zhou, W., Chen, J., Li, K., & Yang, H. (2017). Microbial community mapping in intestinal tract of broiler chicken. **Poultry Science**, 96(5), 1387–1393.

Xing, S., Wang, X., Diao, H., Zhang, M., Zhou, Y., & Feng, J. (2019). Changes in the cecal microbiota of laying hens during heat stress is mainly associated with reduced feed intake. **Poultry Science**, 98(11), 5257–5264.

Yadav, S., Caliboso, K. D., Nanquil, J. E., Zhang, J., Kae, H., Neupane, K., Mishra, B., & Jha, R. (2021). Cecal microbiome profile of Hawaiian feral chickens and pasture-raised broiler (commercial) chickens determined using 16S rRNA amplicon sequencing. **Poultry Science**, 100(7), 101181.

Yan, L., Meng, Q. W., & Kim, I. H. (2011). The effect of an herb extract mixture on growth performance, nutrient digestibility, blood characteristics and fecal noxious gas content in growing pigs. **Livestock Science**, 141(2–3), 143–147.

Yang, Q., Liang, Q., Balakrishnan, B., Belobrajdic, D. P., Feng, Q.-J., & Zhang, W. (2020). Role of Dietary Nutrients in the Modulation of Gut Microbiota: A Narrative Review. **Nutrients**, 12(2), 381.

Yu, K., Choi, I., & Yun, C.-H. (2021). Immunosecurity: Immunomodulants enhance immune responses in chickens. **Animal Bioscience**, 34(3), 321–337.

Zhao, L., Liu, M., Sun, H., Yang, J.-C., Huang, Y.-X., Huang, J.-Q., Lei, X., & Sun, L.-H. (2023). Selenium deficiency-induced multiple tissue damage with dysregulation of immune and redox homeostasis in broiler chicks under heat stress. **Science China Life Sciences**, 66(9), 2056–2069.

Zhou, Y., Zhang, M., Liu, Q., & Feng, J. (2021). The alterations of tracheal microbiota and inflammation caused by different levels of ammonia exposure in broiler chickens. **Poultry Science**, 100(2), 685–696.

Zwolak, I. (2020). Protective Effects of Dietary Antioxidants against Vanadium-Induced Toxicity: A Review. **Oxidative Medicine and Cellular Longevity**, 2020, 1–14.

CHAPTER VI

OVERALL CONCLUSION AND IMPLICATION

6.1 Overall conclusion

Heat stress (HS) in poultry causes significant economic losses and threatens poultry health and welfare globally. This study used transcriptomic techniques to identify mechanisms and gene markers in jejunal mucosal tissue of heat-adapted and heat-sensitive breeder hens to enhance chicken thermotolerance. Moreover, the study evaluated the effectiveness of either synthetic (a combination of vitamin E, vitamin C, Se, and L-carnitine) or phytogenic (a combination of clove, green tea pomace, and Vietnamese coriander) antioxidants in heat-sensitive breeder hens to determine their potential in alleviating HS. The main results are summarized as follows:

6.1.1 In heat-sensitive breeder hens under acute HS, 138 DEGs were identified, enriched in pathways including steroid biosynthesis, protein processing in endoplasmic reticulum, PPAR signaling, and adipocytokine signaling. Acute HS affected energy metabolism, fat metabolism, and glucose transport in the jejunal mucosa. The different expressions of HSPB9, HSPA2, IL-18BP, and CD36 genes have the potential to serve as gene markers indicative of HS effects in the jejunal mucosal tissue of heat-sensitive breeder hens. Furthermore, supplementation with either synthetic or phytogenic antioxidants induced upregulation of CD36 and downregulation of HSPB9, HSPA2, and IL18BP, which improved intestinal health by enhancing immune response, lipid and energy metabolism in breeder hens, thereby repairing HS damage in breeder hens.

6.1.2 Comparative analysis between heat-adapted and heat-sensitive breeds revealed 284 differentially expressed genes (DEGs) associated with response to heat, cell division, and transport of glucose and amino acids. Key pathways included VEGF signaling, MAPK signaling, steroid biosynthesis, cell adhesion molecules, neuroactive ligand-receptor interaction, and cell cycle. KEGG pathway and PPI analyses showed that acute HS may affect the cell cycle (CDK1, PLK1, CDC7, and CDC20), immunity (HSPA2, IL6), and organic acid (SLC22A13L), glucose, and fatty acids transport (LBFABP,

SLC2A2) in the jejunal mucosa of breeder hens. Heat-stressed hens increase the expression of HSPs as a protective mechanism for their cells. These changes might indicate that jejunal mucosal tissue was more damaged by HS in heat-sensitive breeder hens than in heat-adapted breeder hens. 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 HS.

6.1.3 Antioxidant supplementation effectively alleviated HS negative effects by enhancing antioxidant status (SOD, GSH-Px), modulating the expression of immune-related and tight junction genes (IL-10, IL-6, TNF-α, NF-κB, TLR4, and CLDN1), increasing cecal SCFAs concentrations, reducing ammonia production, and enhancing beneficial bacteria of cecal microbiota. In addition, both antioxidant sources also changed the expression of marker genes (HSPB9, HSPA2, RAG2, CLDN15, IL-18BP, and CD36) identified in heat-sensitive breeder hens, contributing to improving intestinal integrity and function under thermal challenge.

6.2 Implication

6.2.1 The identified gene markers (HSPB9, HSPA2, IL-18BP, and CD36) provide molecular targets for selective breeding programs focused on heat tolerance. However, validation of these markers across different commercial breeds and production systems is required to confirm their universal applicability.

6.2.2 Both synthetic and phytogenic antioxidants effectively mitigate HS, presenting viable dietary strategies for the commercial poultry industry. Future research should focus on developing practical on-farm tests for assessing HS susceptibility based on identified biomarkers. Additionally, large-scale field trials are necessary to validate the effectiveness of these strategies across diverse management systems and environmental conditions.

APPENDIX

Supplementary Table 3.1 Differentially expressed genes (DEGs) in the jejunal mucosal tissue in heat-sensitive breeder hens under heat stress.

Supplementary Table 3.2 Gene Ontology (GO) terms for the differentially expressed genes (DEGs) in the jejunal mucosal tissue in heat-sensitive breeder hens under heat stress.

Supplementary Table 4.1 Differentially expressed genes (DEGs) in the jejunal mucosal tissue between heat-adapted and heat-sensitive breeder hens under heat stress.

Supplementary Table 4.2 Gene Ontology (GO) terms for the differentially expressed genes (DEGs) in the jejunal mucosal tissue between heat-adapted and heat-sensitive breeder hens under heat stress.

QR code for Supplementary files

BIOGRAPHY

Yongcai Zhu was born in Guizhou province, China. In 2018, he obtained his Bachelor of Veterinary Medicine, College of Animal Science, Guizhou University. In 2021, he received his Master of Animal Husbandry, Institute of Animal Science, Guizhou University. In 2021, he was awarded a scholarship by Suranaree University of Technology for their financial support through the One Research One Graduate (OROG) program for his Doctor of Philosophy (Ph.D.) study in Animal Production Technology at the School of Animal Technology and Innovation, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima.

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