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 (Li2CO3, 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 SEM	Contrasts^{2}		
	T1	T2	T3	T4		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

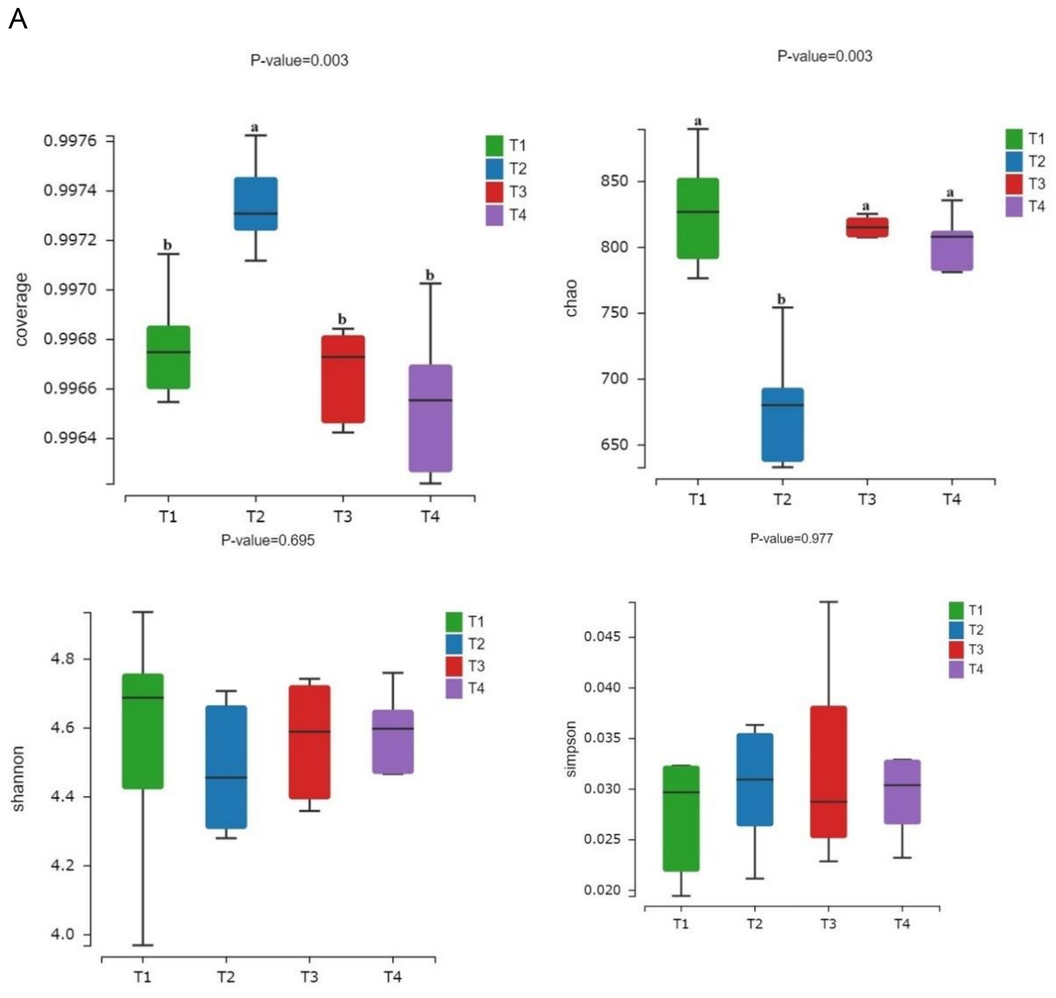
$^{a\text{-}c}$Means within each row with different superscripts are significantly different (P < 0 .05).

^{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).



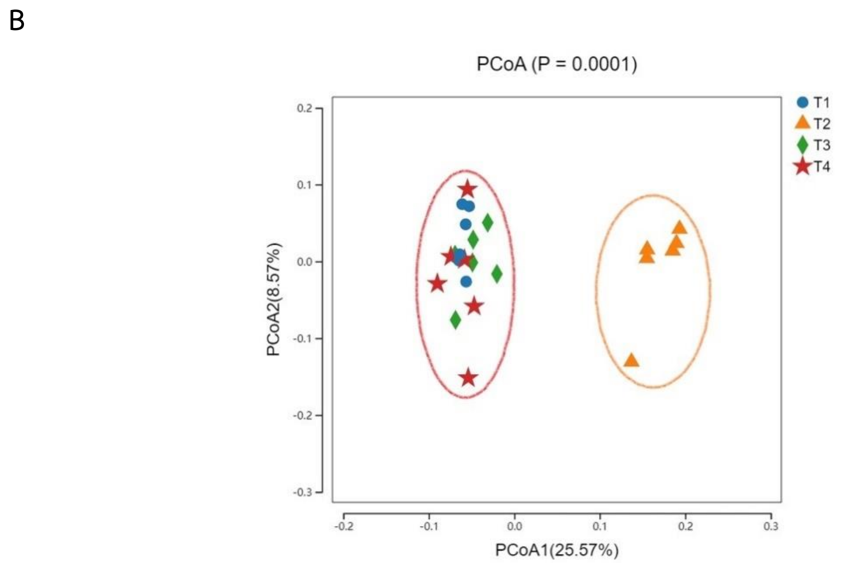


Figure 5.1 Effects of antioxidant supplementation in heat-stressed breeder hen diets under heat stress on microbial alpha diversity metrics (Coverage, Chao 1, Shannon entropy, and Simpson's index) (**A**), and principal coordinate analysis in heat-stressed hens (**B**).

$^{a-b}$ 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).

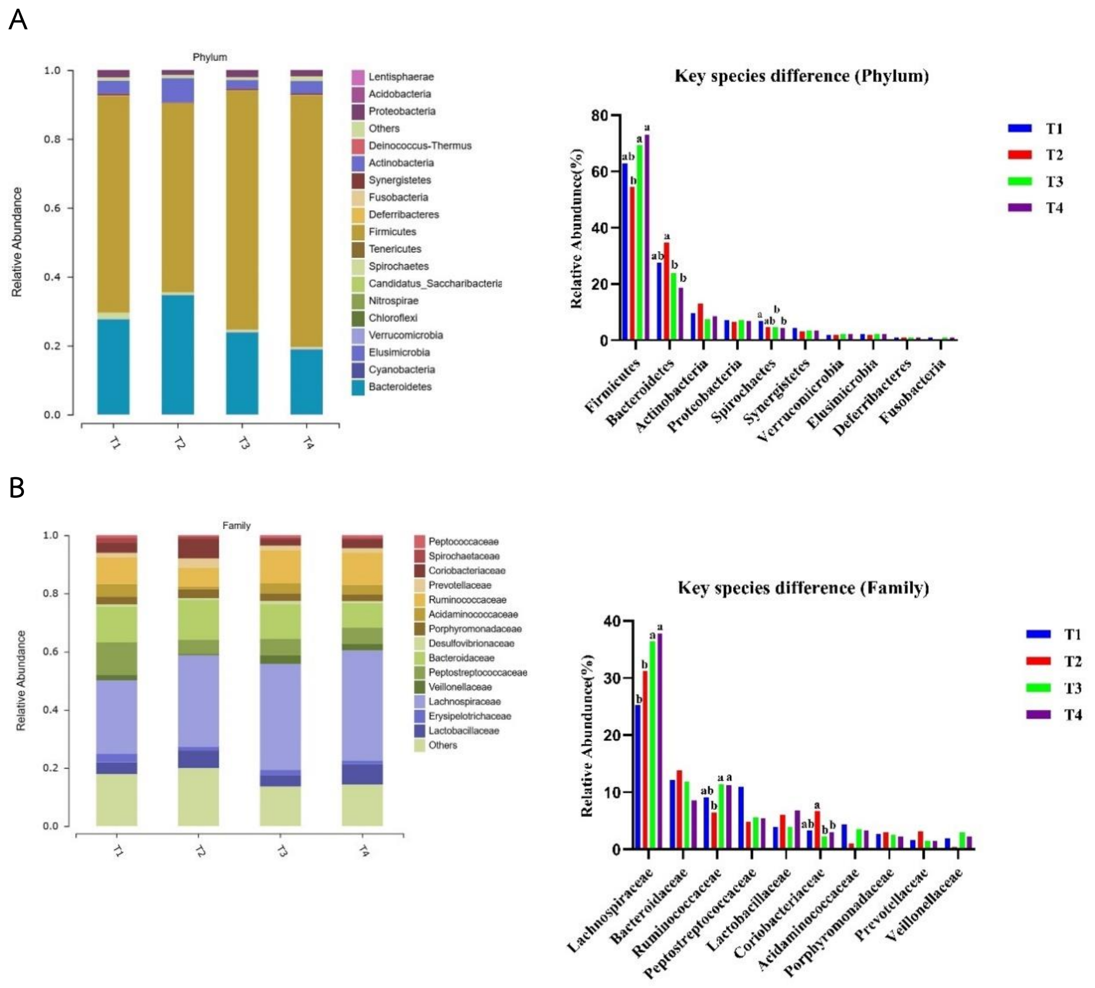


Figure 5.2 Effect of antioxidant supplementation in heat-stressed breeder hen diets on the relative abundance of microbiota and top 10 taxa in terms of phylum (**A**), family (**B**), and genus level (**C**).

[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.

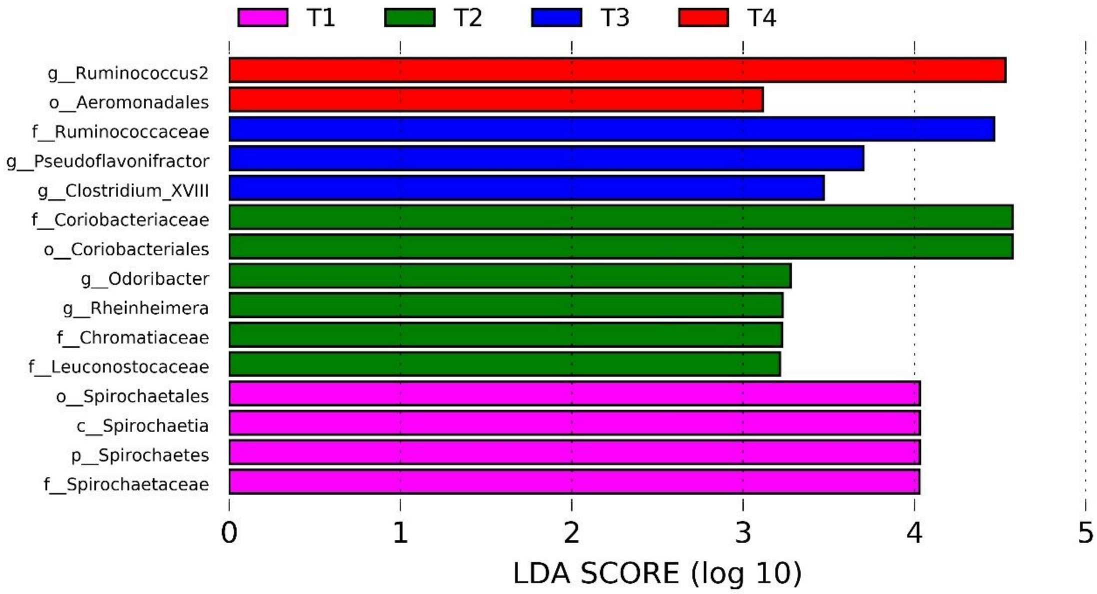


Figure 5.3 Effect of antioxidant supplementation in heat-stressed breeder hen diets on gut microbiota.

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

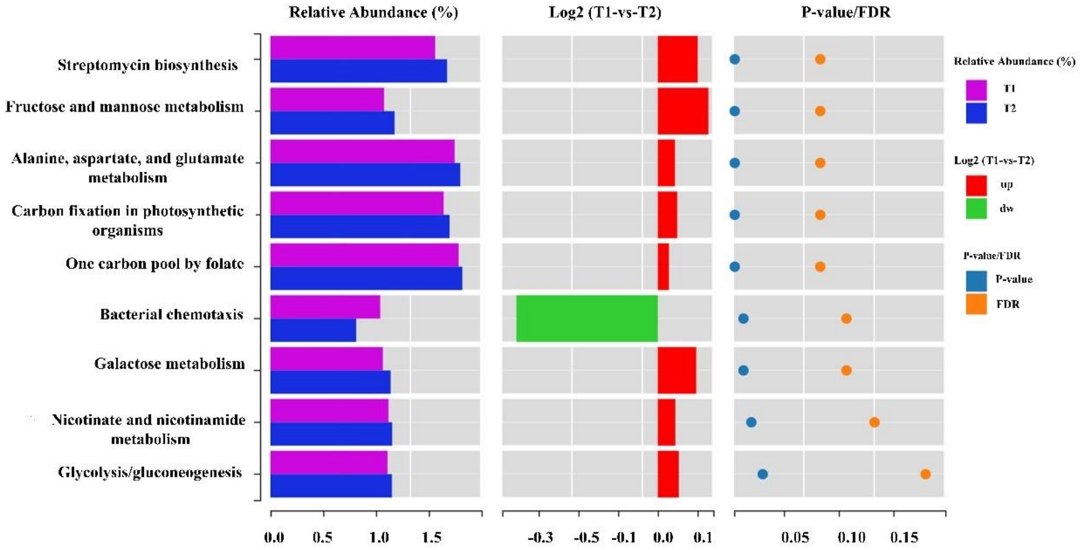
$^{a\text{-}b}$ Means the effect of treatment was statistically different at $P < 0.05$.

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.

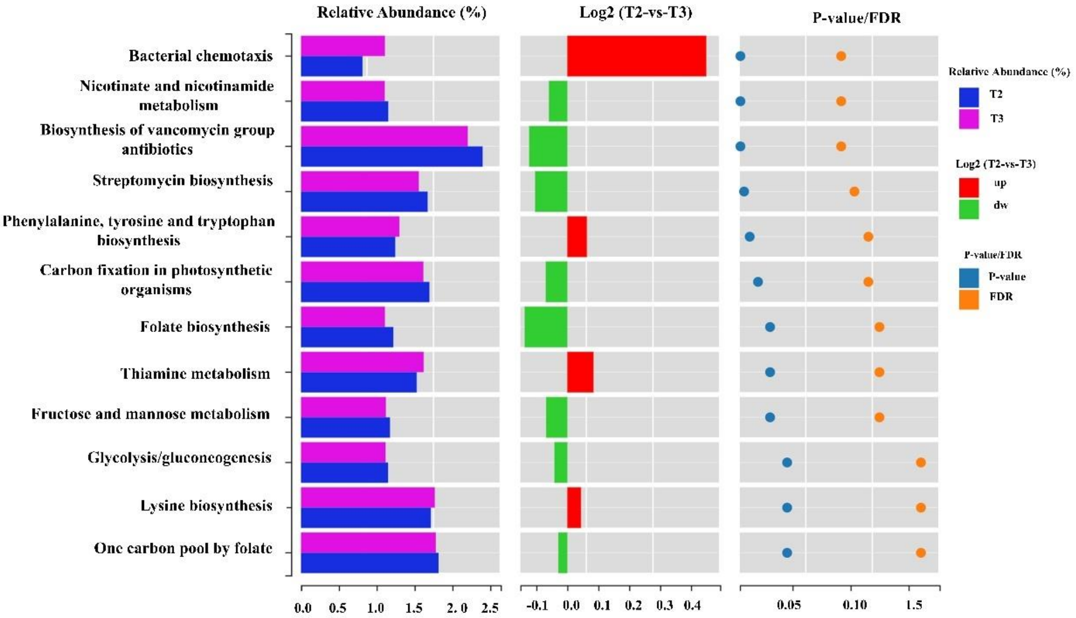
5.4.5 Functional prediction of cecal microbiota

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


Relative Abundance (%)
Log2 (T1-vs-T2)
P-value/FDR
Streptomycin biosynthesis
Fructose and mannose metabolism
Alanine, aspartate, and glutamate metabolism
Carbon fixation in photosynthetic organisms
One carbon pool by folate
Bacterial chemotaxis
Galactose metabolism
Nicotinate and nicotinamide metabolism
Glycolysis/gluconeogenesis
0.0
0.5
1.0
1.5
-0.3
-0.5
-0.1
0.0
0.1
0.05
0.10
0.15
Relative Abundance (%)
T1
T2
Log2 (T1-vs-T2)
up
dw
P-value/FDR
P-value
FDR

B


Relative Abundance (%)
Log2 (T2-vs-T3)
P-value/FDR
Bacterial chemotaxis
Nicotinate and nicotinamide metabolism
Biosynthesis of vancomycin group antibiotics
Streptomycin biosynthesis
Phenylalanine, tyrosine and tryptophan biosynthesis
Carbon fixation in photosynthetic organisms
Folate biosynthesis
Thiamine metabolism
Fructose and mannose metabolism
Glycolysis/gluconeogenesis
Lysine biosynthesis
One carbon pool by folate
0.0
0.5
1.0
1.5
2.0
2.5
-0.1
0.0
0.1
0.2
0.3
0.4
0.05
0.10
1.5
Relarive Abundance (%)
T2
T3
Log2 (T2-vs-T3)
up
dw
P-value/FDR
P-value
FDR

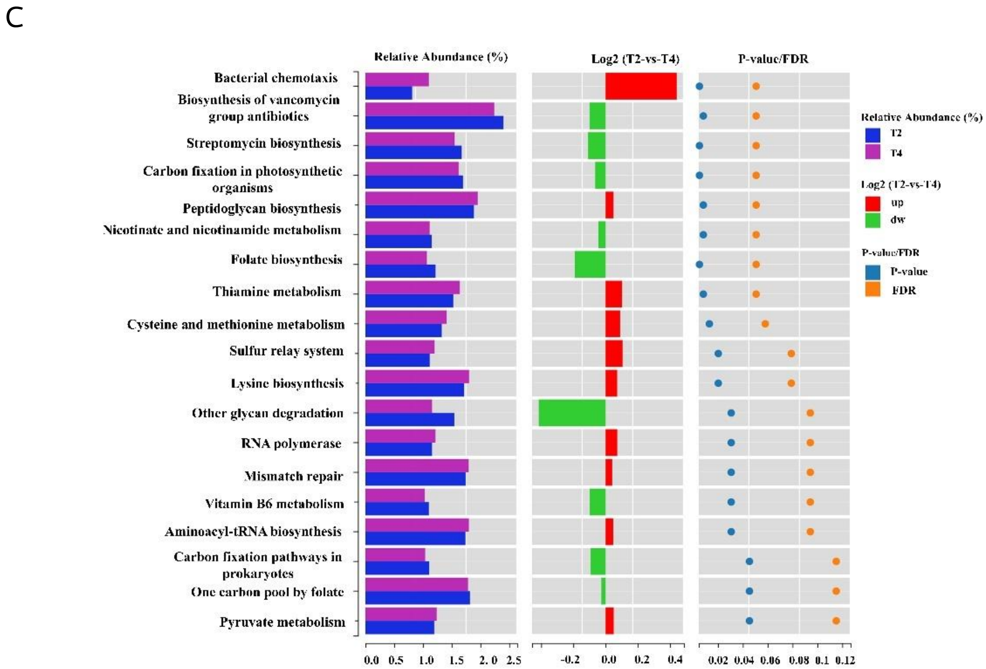


Figure 5.4 Predicted functions of cecal microbiota in heat-stressed breeder hens receiving dietary antioxidants at KEGG level 3 among the different groups: differentially regulated metabolic pathways in T1 vs. T2 (A), T2 vs. T3 (B), T2 vs. T4 (C).

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 ($P < 0.05$).

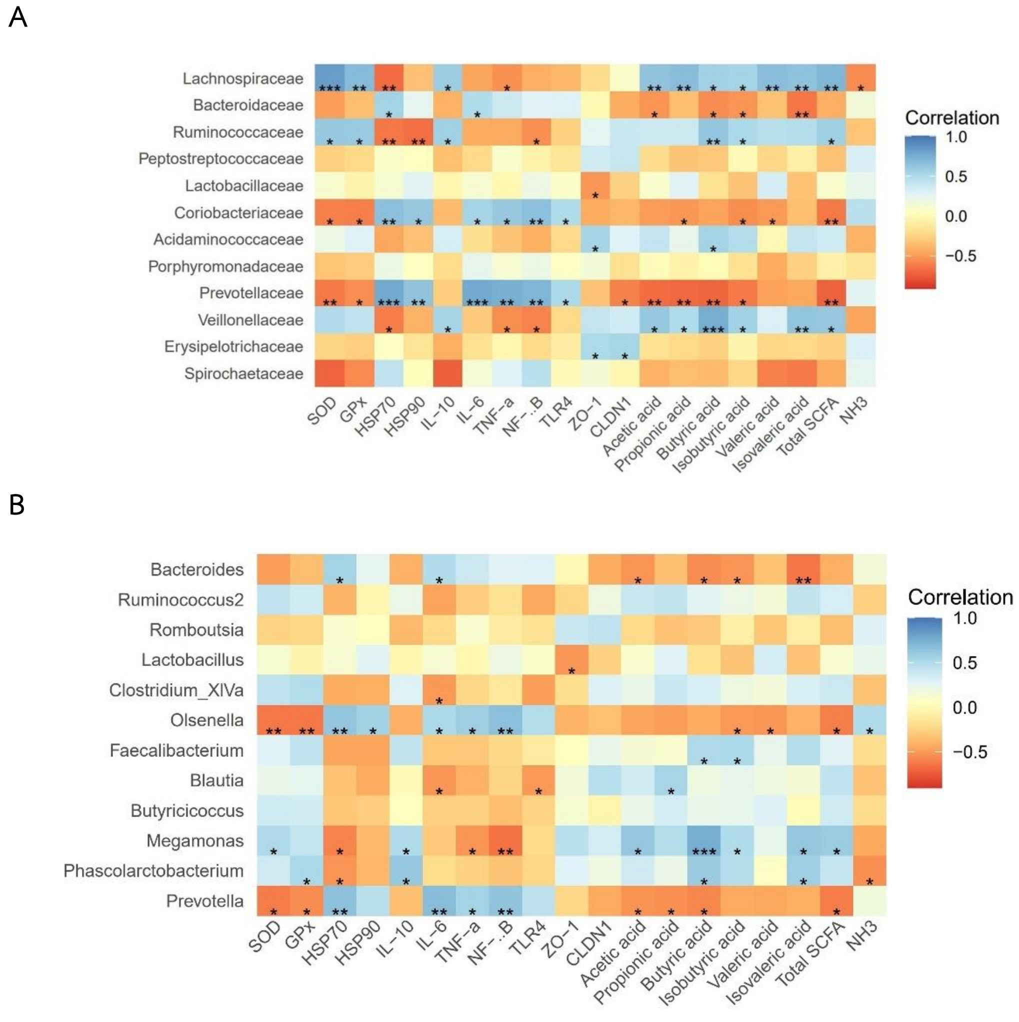


Figure 5.5 Spearman correlation analysis between different parameters and cecal microbial composition at the genus levels in heat-stressed breeder hens among the treatment groups. The row names represent the genera, and the column names represent the different parameters. The red and blue

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

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