

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

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ABSTRACT This study evaluated the effects of vitamin E (Vit E) and selenium (Se) supplementation on mRNA abundance of antioxidant, immune response, and tight junction genes, as well as taxonomic and functional profiles of ileal microbiota of broilers exposed to daily 4-h elevated temperature during d 28 to 35. A total of 640-day-old Cobb male broiler chicks were randomly allocated to 32 floor pens in a 2 × 2 factorial arrangement that included ambient temperature (thermoneutral [TN] or heat stress [HS]) and dietary treatments (basal diet or Vit E + Se). Vit E and organic Se were added to the basal diet at the rate of 250 mg/kg and 1 mg/kg, respectively. Liver and jejunum tissue samples were taken on d 27 (1 bird/pen), d 28 and d 35 (2 birds/pen) from birds for qPCR analysis. Data were subjected to a 2-way ANOVA using the GLM procedure of JMP. Ileal contents were taken on d 27 and d 35 for microbial profiling. Microbiota data were analyzed in QIIME 2 and significance between treatments identified linear

discriminant analysis effect size (LEfSe, $P < 0.05$). Dietary Vit E/Se significantly downregulated the mRNA levels of HSPs in liver and jejunal tissues of the HS-challenged birds both on d 28 and d 35. Moreover, mRNA abundance of TLR2, TNF α , IFN γ , IL-1 β , IL-10, and iNOS in the liver were significantly downregulated in birds fed the Vit E/Se diet on d 35. However, dietary treatment had no significant impact on oxidative stress, immunity, and gut integrity related genes analyzed in jejunal tissues on d 28 and d 35, except downregulation of IFN γ on d 35 ($P = 0.052$). LEfSe analysis revealed that *Lachnospiraceae* FE2018 and *Ruminococcaceae* NK4A214 groups was enriched in the Vit E/Se birds on d 35. Moreover, PICRUST analysis predicted significant functional differences among the treatment groups. In conclusion, dietary supplementation of Vit E/Se mitigated the negative effects of HS potentially via improving antioxidant status, regulating cytokine responses and modifying ileal microbiota and its function.

Key words: antioxidant, broiler, heat stress, immunity, microbiota

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INTRODUCTION

Current environmental challenges including prolonged durations of high heat in certain regions have presented a threat to plant and animal production worldwide (Chowdhury et al., 2020). Further, the need for agricultural products, especially protein sources, continues to rise with the growth rate of the world's

population (Bruinsma, 2009). Modern broilers are more efficient than other terrestrial food producing animals in converting plant-based nutrients into healthy protein (Macelline et al., 2021), making poultry a key source for supplying the increasing demand of animal-based protein in the future. However, these animals are susceptible to elevated temperatures due to their anatomic features (e.g., lack of sweat glands), physiological state, and greater metabolic activity (Sahin et al., 2009; Lara and Rostagno, 2013). Therefore, urgent management and nutritional strategies are needed to mitigate the negative effects of heat stress (HS) on poultry (Vandana et al., 2021).

High ambient temperature is one of the most common stressors in commercial broiler production resulting in reduced feed intake and weight gain, and increased

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mortality leading to significant economic losses (Lara and Rostagno, 2013). Besides its adverse effect on poultry production, HS also causes important pathophysiological changes at the cellular level including excessive production of reactive oxygen species (ROS), disturbed gut permeability, reduced energy metabolism, altered immune function and gut microbiota which further leads to increased susceptibility to pathogens and compromised overall health (Liu et al., 2016; Shi et al., 2019; Lian et al., 2020).

Birds respond to HS conditions by reprogramming several defense mechanisms including antioxidant enzymes, heat-shock proteins (HSPs), and cytokines to alleviate or reduce the negative effects of HS (Tan et al., 2010; Slimen et al., 2016; Emami et al., 2020). HSPs are important molecular chaperones that play a crucial role in folding/unfolding of polypeptides to achieve a functional structure and the repair of denatured proteins, or assist in their degradation after stress challenge (Whitley et al., 1999). Expression of HSPs, especially HSP70 and HSP90, significantly increased in response to elevated ambient temperature to protect and repair cells (Varasteh et al., 2015; Rajaei-Sharifabadi et al., 2017). HS-induced signaling events lead to upregulation of HSP70 through the HS-related increase in ROS level (Banerjee Mustafi et al., 2009). Along with the protective roles, HSPs also have regulatory functions during cellular stress as they inhibit the production of inflammatory cytokines (Ferat-Osorio et al., 2014) and modulate tight junction proteins (Dokladny et al., 2008).

Further, HS can induce alterations in cellular functions and disturbances in intestinal microbiome that need to be managed with several nutritional interventions to alleviate the negative impact of HS (Quinteiro-Filho et al., 2017; Rajaei-Sharifabadi et al., 2017; Siddiqui et al., 2020). Therefore, several nutritional strategies, especially non-drug-based feed additives, are being tested to maintain growth performance and overall health of the birds (Flees et al., 2021; Greene et al., 2021). Vit E and Se are important nutrients with antioxidant function. The combined dietary use of Vit E and Se is a more effective nutritional strategy than individual use as these feed additives act synergistically to modulate antioxidant activity (Harsini et al., 2012; Kumbhar et al., 2018). Even though these nutrients are present in the diets at certain levels, dietary addition of Vit E/Se above the recommended levels might have beneficial effects on performance, oxidative status, and immune response during a stress challenge (Harsini et al., 2012; Habibian et al., 2014; Surai, 2018).

As metabolically active organs, the liver and intestinal tissues, especially the jejunum, are highly responsive to HS conditions, thus such disturbances directly lead to reduced performance and increased susceptibility to disease. The impact of HS on oxidative balance, immune response and gut barrier function of broilers has been well documented; however, little is known about the combined use of additional Vit E/Se on the mechanisms of molecular regulation in the small intestine and liver along with the changes in the intestinal microbiota.

Therefore, the current study aimed to investigate the effects of dietary Vit E/Se supplementation on mRNA abundance of antioxidant, immune response, and tight junction genes, as well as on the ileum microbial profile of broiler chickens subjected to cyclic acute HS.

MATERIALS AND METHODS

Experimental Diets, Birds and Management

This project was approved and conducted under the guidelines of the Virginia Tech Institutional Animal Care and Use Committee. A total of 640 one-day old Cobb male chicks were randomly allocated to 32 floor pens in a 2 × 2 factorial arrangement that included ambient temperature (thermoneutral [TN] or heat stress [HS]) and dietary treatments (basal diet or Vit E/Se) as main factors. Four rooms were used (2 TN and 2 HS) each housing half of the 8 replicate pens per group. For the Vit E/Se diet, the basal diet was supplemented with 250 mg/kg Vit E (Rovimix E50, DSM Nutritional Products, Basel, Switzerland) and 1 mg/kg organic Se (Sel-Plex, Alltech Inc., Nicholasville, KY). Birds were raised in a controlled environment for 35 d. Starter and grower diets were based on corn-soybean meal formulations and provided to the birds from 0 to 21 d and 21 to 35 d of age, respectively. All diets were formulated to meet or exceed the NRC (1994) nutrient recommendations. Each pen was equipped with a bucket feeder and an automatic nipple drinker. Water and experimental diets (in mash form) were provided ad libitum throughout the study period.

Heat Stress Protocol

Heat stress was applied during the last week of the experiment (d 28–d 35). For the HS groups, room temperature was raised and maintained at 35 ± 1°C from 10 AM to 2 PM, then reduced again to 25°C ± 1 for the remainder of the day. Relative humidity was monitored and maintained at <50%. Birds in the TN groups were kept under recommended temperatures (25°C ± 1).

Total RNA Extraction and Reverse Transcription

On d 27 (1 bird/pen), d 28 and d 35 (2 birds/pen) birds were selected, euthanized, and jejunal sections (1 cm) and liver tissues were excised and snap-frozen in liquid nitrogen to later assess the mRNA abundance of oxidative stress, immunity, and gut integrity related genes. A 20–30 mg aliquot of jejunal and liver tissues was weighed into a 2 mL microcentrifuge tube and homogenized in 600 µL TRI Reagent (Zymo Research, Irvine, CA) by a TissueLyser II (Qiagen, Valencia, CA). Total RNA was extracted from the homogenate using the Direct-zol RNA Miniprep Plus Kit (Zymo Research, Irvine, CA) according to the manufacturer's recommendations. Total RNA concentration was determined at

optical density (**OD**) of 260 using a NanoDrop-1000 (Thermo Fisher Scientific, Waltham, MA), and RNA purity was verified by evaluating the 260/280 OD ratios. RNA integrity was evaluated by gel electrophoresis on 1.5% agarose gel in $0.5 \times$ TAE buffer. After extraction, 2 μ g total RNA were used to synthesize first-strand cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendation and the cDNA was stored at -20°C .

Quantitative Real-Time PCR

The mRNA abundance of oxidative stress related genes [superoxide dismutase (**SOD**), glutathione peroxidase (**GPx**), heat shock protein (**HSP**)70 and HSP90], cytokines [interleukin (**IL**)-1 β , IL-4, IL-10, IL-17A, IL-22, tumor necrosis factor alpha (**TNF α**), inducible nitric oxide synthase (**iNOS**), interferon gamma (**IFN γ**)], Toll-like receptor (**TLR**)2 and TLR4, tight junction proteins [occludin, zona occludens (**ZO**)-1], mechanistic target of rapamycin (**mTOR**) were determined by quantitative real-time PCR (ABI 7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA)

using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Details of primer sets are provided in [Table 1](#). Product specificity was confirmed by analysis of the melting curves produced by the ABI 7500 software (version 2.0.3). mRNA abundance was analyzed using glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) as an endogenous control. Average mRNA abundance relative to GAPDH for each sample was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method ([Livak and Schmittgen, 2001](#)). The calibrator for each gene was the average ΔCt value from the negative control group for each sampling day.

16S rRNA Gene Sequencing

On d 27 and d 35, one bird/pen was euthanized, ileal digesta were collected and snap-frozen in liquid nitrogen, and then stored at -80°C until further analysis. Bacterial DNA was extracted from ileal digesta using the Qiagen DNeasy PowerSoil Pro Kit (Qiagen GmbH, Hilden, Germany) based on the supplier's instructions. Quality and quantity of extracted DNA were measured using gel electrophoresis and spectrophotometer. All DNA samples were diluted to 300 ng/ μL and 50 μL of each for

Table 1. Sequences of primer pairs used for amplification of target and reference genes.

Gene ¹	Primer sequence	Size	Acc (reference)
SOD	GTGACCTCGCAATGTGACT GCAGTGTGGTCCGGTAAGAG	86	NM_205064.1
GPx	AAGAACTTCGGGCACCAGGA TGTACTGCGGGTTGGTCATC	214	NM_001277853.2
HSP70	CGTCAGTGCTGTGGACAAGAGTA CCTATCTCTGTTGGCTTCATCCT	145	NM_001006685
HSP90	GATCCCCAGACACATGCCAA CTCGGTAAGTGCAGGACTGG	105	NM_001109785
TLR2	CGGTCATCTCAGCTACACCA GCATCGCATGAAAGACAGGC	150	NM_204278
TLR4	CCACACACCTGCCTACATGAA GGATGGCAAGAGGACATATCAAA	63	NM_001030693
IL-1 β	CCCGCCTCCGCTACA CACGAAGCACTTCTGGTTGATG	66	NM_204524.1
IL-4	GCTCTCAGTGCCGCTGATG GAAACCTCTCCCTGGATGTCAT	60	NM_001007079.1
IL-10	CGCTGTCACCGCTTCTCA CGTCTCCTTGATCTGCTTGATG	63	NM_001004414.2
IL-17A	AGCTGGACCACAGCGTCAAC GGCGGAGGACGAGGATCT	57	NM_204460.1
IL-22	CTTCTACACAGCCCCCTACCTCCA GCTGACCGATGAGTCTGTTGTCAGTG	175	NM_001199614.1
TNF α	CCCATCCCTGGTCCGTAAC ATACGAAGTAAAGGCCGTCCC	77	MF000729.1
iNOS	CCTGTAAGGTTGGCTATTGG AGGCCTGTGAGAGTGTGCAA	66	NM_204961.1
IFN γ	AGTCAAAGCCGCACATCAAAC TTCACCTTCTTCACGCCATCA	82	NM_205149.1
Occludin	CCGTAACCCCGAGTTGGAT ATTGAGGCGGTCTGTTGATG	214	NM_205128.1
ZO-1	GGAGTACGAGCAGTCAACATAC GAGGCGCACGATCTTCATAA	101	XM_413773
mTOR	CATGTCAGGCACTGTGTCTATTCTC CTTTCGCCCTTGTTCTTCACT	77	XM_417614.6
GAPDH	CCTAGGATACACAGAGGACCAGGTT GGTGGAGGAATGGCTGTCA	64	NM_204305

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GPx: Glutathione peroxidase; HSP: Heat shock protein; IL: Interleukin; iNOS: Inducible nitric oxide synthase; IFN γ : Interferon gamma; SOD: Superoxide dismutase; TNF α : Tumor necrosis factor alpha; TLR: Toll Like Receptor; mTOR: Mechanistic target of rapamycin; ZO: Zonula occluden. For each gene, the primer sequence for forward (F) and reverse (R) (5'-3') primers, the amplicon size (bp) and the NCBI Accession number (Acc) used for the primer design are listed.

sequencing. The V4 region of the 16S rRNA gene was amplified using the Illumina MiSeq kit V3 (600-cycle format, 25 million reads, read length 2×300). Earth Microbiome Project primer sets 515F (Parada et al., 2016) and 806R (Apprill et al., 2015) generating 390 bp amplicons were used.

To assess the association between microbial community and dietary treatment within each sampling day, pairwise PERMANOVA analysis implemented in QIIME 2 was performed on an unweighted UniFrac distance matrix. The significance of PERMANOVA was obtained by 999 permutation tests. Relative abundance of taxa for each sampling day and treatment were calculated at the phylum and genus levels. The distinctive taxa between treatment groups were identified with linear discriminant analysis effect size (LEfSe), which was performed through Galaxy/Hutlab workflow platform (Segata et al., 2011). In order to predict the function of ileal microbiota, data analysis was performed through the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) pipeline (Weber et al., 2018). Then, PICRUSt outputs for the level 3 of the Kyoto Encyclopedia of Genes and Genomes (KEGG) were analyzed and illustrated with statistical analysis of the taxonomic and functional profiles (STAMP) software version 2.1.3 (Parks et al., 2014).

Statistical Analysis

Data were subjected to a 2-way ANOVA using the GLM procedure of JMP (Pro13). The models included ambient temperature (TN and HS) and dietary treatments (basal diet or VitE/Se) as the main factors, and the 2-way interactions Tukey's multiple range test was only carried out for significant interactions and was performed using simple effect analysis. The probability $P < 0.05$ was considered significant unless otherwise noted.

RESULTS

mRNA Abundance of Oxidative Stress, Immunity, and Gut Integrity Related Genes in the Jejunum and Liver on d 27

Jejunum: The effects of dietary Vit E/Se administration on the mRNA abundance of oxidative stress, immunity, and gut integrity related genes in the jejunum of broilers are shown in Table 2. mRNA abundance of genes involved in oxidative stress, immunity, and gut integrity were not influenced by Vit E/Se supplementation under standard commercial conditions.

Liver: Dietary Vit E/Se supplementation significantly downregulated mRNA levels of TLR2 and iNOS as compared to the basal diet group (Table 2). However, no differences were observed between control and treatment groups for the other targets examined.

Table 2. Effect of dietary supplementation of Vit E/Se on relative mRNA abundance of several genes in the jejunum and liver tissues of broiler chickens on d 27.¹

Item	Treatment groups ²		Statistics	
	Control	Vit E/Se	SEM	P-value
Jejunum				
HSP70	1.04	0.88	0.05	0.099
HSP90	1.03	0.96	0.06	0.539
TLR2	1.05	1.23	0.09	0.300
TLR4	1.16	1.31	0.12	0.511
IL-1 β	1.10	1.10	0.08	0.988
IL-4	1.14	1.08	0.10	0.781
IL-10	1.24	1.13	0.17	0.761
TNF α	1.11	1.19	0.09	0.637
iNOS	1.10	1.11	0.06	0.900
IFN γ	1.07	1.20	0.09	0.454
Occludin	1.04	1.17	0.06	0.261
ZO-1	1.11	1.31	0.11	0.397
Liver				
HSP70	1.17	0.88	0.11	0.204
HSP90	1.04	0.85	0.06	0.118
SOD	1.05	1.04	0.05	0.934
GPx	1.09	0.84	0.07	0.079
mTOR	1.17	1.01	0.14	0.594
TLR2	1.07	0.79	0.06	0.023
TLR4	1.71	1.10	0.28	0.290
IL-1 β	1.09	0.80	0.08	0.106
IL-4	1.37	0.86	0.16	0.110
IL-10	1.19	1.08	0.17	0.773
TNF α	1.17	0.89	0.11	0.218
iNOS	1.07	0.77	0.07	0.031
IFN γ	1.14	0.97	0.11	0.477

¹Data represent mean values of 16 replicates per treatment.

²Control: birds fed a basal diet; Vit E/Se: birds fed a basal diet supplemented with 250 mg/kg vitamin E and 1 mg/kg selenium.

mRNA Abundance of Oxidative Stress, Immunity, and Gut Integrity Related Genes in the Jejunum and Liver on d 28

Jejunum: A significant interaction was observed in mRNA abundance of HSP70 and HSP90 between dietary treatment and ambient temperature in jejunal tissues (Table 3). Dietary treatment significantly downregulated ($P < 0.05$) mRNA levels of HSP70, HSP90, and IL-4, and upregulated ($P = 0.017$) that of IL-17A on d 28. Heat stress significantly upregulated HSP70, HSP90, IL-4, IL-22, IFN γ , and ZO-1 levels and downregulated those of TLR2, TNF α , and occludin.

Liver: A significant interaction was observed in abundance of HSP90 between dietary treatment and ambient temperature in liver tissues (Table 4). Supplementation of Vit E/Se reduced ($P < 0.05$) mRNA abundance of HSP90 and GPx. Heat stress significantly upregulated SOD, GPx, HSP70, HSP90, TLR4, and downregulated that of mTOR, TNF α , and IFN γ on d 28.

mRNA Abundance of Oxidative Stress, Immunity, and Gut Integrity Related Genes in the Jejunum and Liver on d 35

Jejunum: No significant interaction effect on oxidative stress, immunity and gut integrity related markers was observed between diet and ambient temperature (Table 5). Dietary treatment had no significant impact

Table 3. Effect of dietary supplementation of Vit E/Se on relative mRNA abundance of oxidative stress, immunity, and gut integrity related genes in the jejunum of heat-stressed broiler chickens on d 28.¹

Item	Treatments ²								RMSE	Statistics ³		
	Control		Vit E/Se		Diet		Ambient temperature			P-value		
	TN	HS	TN	HS	Control	Vit E/Se	TN	HS		Diet	AT	D × AT
HSP70	1.13 ^c	13.31 ^a	1.34 ^c	4.31 ^b	7.22	2.82	1.23	8.81	3.82	<0.001	<0.001	<0.001
HSP90	1.11 ^c	5.42 ^a	1.36 ^c	2.66 ^b	3.26	2.01	1.23	4.04	1.43	0.002	<0.001	<0.001
TLR2	1.09	0.80	1.20	0.87	0.94	1.04	1.14	0.84	0.49	0.453	0.016	0.848
TLR4	1.14	1.15	1.39	1.17	1.14	1.28	1.26	1.16	0.66	0.414	0.529	0.472
IL-1β	1.14	1.03	1.20	1.00	1.09	1.10	1.17	1.02	0.60	0.930	0.306	0.749
IL-4	1.39	3.57	1.10	2.56	2.48	1.83	1.25	3.06	1.18	0.032	<0.001	0.228
IL-10	1.34	1.92	1.78	2.14	1.63	1.96	1.56	2.03	1.34	0.337	0.174	0.741
IL-17A	1.11	0.83	1.60	1.33	0.97	1.47	1.36	1.09	0.79	0.017	0.186	0.974
IL-22	1.28	3.92	1.62	4.45	2.60	3.03	1.45	4.18	2.07	0.447	<0.001	0.860
TNFα	1.27	0.82	1.48	0.73	1.05	1.10	1.37	0.77	0.83	0.759	0.002	0.406
iNOS	1.23	1.91	1.48	1.59	1.57	1.54	1.36	1.75	0.92	0.872	0.090	0.217
IFNγ	1.23	1.88	1.14	1.64	1.55	1.39	1.18	1.76	0.85	0.435	0.009	0.719
Occludin	1.05	0.85	1.11	0.88	0.95	1.00	1.08	0.87	0.33	0.579	0.015	0.843
ZO-1	1.32	2.40	1.61	2.42	1.86	2.01	1.46	2.41	0.92	0.500	<0.001	0.557

^{a-c}Means with different superscripts in the same row are significantly different ($P < 0.05$).

¹Data represent mean values of 16 replicates per treatment.

²Control: birds fed a basal diet; Vit E/Se: birds fed a basal diet supplemented with 250 mg/kg vitamin E and 1 mg/kg selenium; TN: thermoneutral; HS: heat stress

³AT: Ambient Temperature; D: Diet.

Table 4. Effect of dietary supplementation of Vit E/Se on relative mRNA abundance of oxidative stress and immunity related genes in the liver of heat-stressed broiler chickens on d 28.¹

Item	Treatments ²								RMSE	Statistics ³		
	Control		Vit E/Se		Diet		Ambient Temperature			P-value		
	TN	HS	TN	HS	Control	Vit E/Se	TN	HS		Diet	AT	D × AT
HSP70	1.09	8.28	1.15	6.14	4.68	3.64	1.12	7.21	5.33	0.463	<0.001	0.436
HSP90	1.16 ^a	4.86 ^a	1.25 ^b	2.00 ^b	3.01	1.62	1.20	3.43	2.42	0.026	0.001	0.018
SOD	1.05	1.31	1.01	1.30	1.18	1.15	1.03	1.30	0.42	0.814	0.013	0.864
GPx	1.10	2.53	0.83	1.64	1.82	1.22	0.97	2.10	1.04	0.032	<0.001	0.246
mTOR	1.05	0.73	1.07	0.79	0.89	0.93	1.06	0.76	0.33	0.665	0.001	0.818
TLR2	1.08	1.09	0.87	1.07	1.09	0.97	0.98	1.08	0.45	0.293	0.363	0.418
TLR4	1.17	2.31	1.30	2.08	1.74	1.69	1.24	2.19	0.80	0.792	<0.001	0.361
IL-1β	1.12	1.01	1.16	1.10	1.06	1.13	1.14	1.06	0.53	0.611	0.553	0.850
IL-4	1.13	1.02	1.38	1.01	1.07	1.19	1.25	1.02	0.54	0.386	0.090	0.341
IL-10	1.14	0.89	0.96	0.80	1.01	0.88	1.04	0.84	0.57	0.355	0.164	0.757
TNFα	1.11	0.66	1.01	0.63	0.87	0.82	1.05	0.64	0.44	0.579	<0.001	0.748
iNOS	1.15	1.31	1.40	1.19	1.23	1.30	1.28	1.25	0.78	0.728	0.904	0.336
IFNγ	1.14	0.82	1.29	0.76	0.97	1.03	1.21	0.79	0.69	0.747	0.007	0.495

^{a,b}Means with different superscripts in the same row are significantly different ($P < 0.05$).

¹Data represent mean values of 16 replicates per treatment.

²Control: birds fed a basal diet; Vit E/Se: birds fed a basal diet supplemented with 250 mg/kg vitamin E and 1 mg/kg selenium; TN: thermoneutral; HS: heat stress

³AT: Ambient Temperature; D: Diet.

Table 5. Effect of dietary supplementation of Vit E/Se on relative mRNA abundance of oxidative stress, immunity, and gut integrity related genes in the jejunum of heat-stressed broiler chickens on d 35.¹

Item	Treatments ²								RMSE	Statistics ³		
	Control		Vit E/Se		Diet		Ambient Temperature			P-value		
	TN	HS	TN	HS	Control	Vit E/Se	TN	HS		Diet	AT	D × AT
HSP70	1.05	8.08	1.03	6.54	4.20	3.28	1.04	7.38	4.06	0.482	<0.001	0.494
HSP90	1.09	4.52	1.10	3.86	2.75	2.39	1.10	4.20	1.69	0.454	<0.001	0.451
TLR2	1.05	1.88	0.89	1.72	1.46	1.31	0.97	1.80	0.98	0.518	0.001	0.983
TLR4	1.07	0.93	0.84	1.20	1.00	1.02	0.96	1.07	0.53	0.871	0.416	0.064
IL-1β	1.09	1.23	0.88	1.08	1.16	0.98	0.99	1.15	0.53	0.187	0.214	0.824
IL-4	1.08	2.02	0.96	1.75	1.55	1.35	1.02	1.88	1.13	0.488	0.003	0.780
IL-10	1.14	1.68	0.74	1.42	1.41	1.08	0.94	1.55	0.76	0.087	0.002	0.737
IL-17A	1.12	0.47	1.33	0.42	0.79	0.87	1.22	0.44	0.55	0.564	<0.001	0.346
IL-22	1.07	2.17	0.88	1.47	1.62	1.18	0.98	1.82	1.09	0.121	0.004	0.355
TNFα	1.04	1.09	0.95	1.09	1.06	1.02	1.00	1.09	0.46	0.709	0.420	0.701
iNOS	1.05	1.15	0.90	1.15	1.10	1.02	0.97	1.15	0.48	0.500	0.141	0.537
IFNγ	1.13	0.72	0.84	0.53	0.93	0.68	0.98	0.63	0.49	0.052	0.005	0.696
Occludin	1.06	2.16	0.96	2.41	1.61	1.68	1.01	2.28	0.91	0.753	<0.001	0.450
ZO-1	1.06	2.10	0.94	2.23	1.58	1.58	1.00	2.17	0.86	0.976	<0.001	0.566

¹Data represent mean values of 16 replicates per treatment.

²Control: birds fed a basal diet; Vit E/Se: birds fed a basal diet supplemented with 250 mg/kg vitamin E and 1 mg/kg selenium; TN: thermoneutral; HS: heat stress

³AT: Ambient Temperature; D: Diet.

Table 6. Effect of dietary supplementation of Vit E/Se on relative mRNA abundance of oxidative stress and immunity related genes in the liver of heat-stressed broiler chickens on d 35.¹

Item	Treatments ²				Diet		Ambient Temperature		RMSE	Statistics ³		
	Control		Vit E/Se		Control	Vit E/Se	TN	HS		P-value		
	TN	HS	TN	HS						Diet	AT	D × AT
HSP70	1.14 ^c	13.96 ^a	1.02 ^c	4.97 ^b	7.55	3.00	1.08	9.47	4.49	0.001	<0.001	0.001
HSP90	1.24 ^c	5.97 ^a	1.25 ^c	3.65 ^b	3.60	2.45	1.25	4.81	1.74	0.015	<0.001	0.014
SOD	1.06	1.19	1.13	1.06	1.13	1.10	1.10	1.13	0.37	0.755	0.750	0.299
GPx	1.13	2.31	1.16	2.39	1.72	1.77	1.14	2.35	1.26	0.866	<0.001	0.941
mTOR	1.09	0.85	0.82	0.70	0.97	0.76	0.96	0.78	0.35	0.018	0.046	0.515
TLR2	1.20	1.00	0.85	0.60	1.10	0.73	1.02	0.80	0.53	0.006	0.096	0.851
TLR4	1.22	1.00	0.84	0.77	1.11	0.81	1.03	0.88	0.65	0.070	0.370	0.641
IL-1β	1.09	0.37	0.82	0.34	0.73	0.58	0.96	0.36	0.29	0.047	<0.001	0.101
IL-4	1.05	0.88	0.95	0.99	0.96	0.97	1.00	0.93	0.46	0.944	0.553	0.352
IL-10	1.21	1.46	0.70	0.68	1.33	0.69	0.95	1.07	0.78	0.002	0.552	0.497
TNFα	1.11	0.93	0.82	0.61	1.02	0.72	0.97	0.77	0.52	0.025	0.141	0.935
iNOS	1.06	1.02	0.65	0.98	1.04	0.82	0.85	1.00	0.43	0.050	0.185	0.092
IFNγ	1.26	0.85	0.74	0.40	1.05	0.57	1.00	0.62	0.56	0.001	0.009	0.778

^{a-c}Means with different superscripts in the same row are significantly different ($P < 0.05$).

¹Data represent mean values of 16 replicates per treatment.

²Control: birds fed a basal diet; Vit E/Se: birds fed a basal diet supplemented with 250 mg/kg vitamin E and 1 mg/kg selenium; TN: thermoneutral; HS: heat stress

³AT: Ambient Temperature; D: Diet.

on oxidative stress, immunity, and gut integrity related genes analyzed in jejunum on d 35. However, mRNA abundance of IFNγ decreased ($P = 0.052$) in Vit E/Se treatment. Heat stress caused an increase in mRNA levels of HSP70, HP90, TLR2 IL-4, IL-10, IL-22, occludin, ZO-1, and a decrease in IL-17A and IFNγ.

Liver: There was significant interaction between dietary and ambient temperature treatments in terms of liver HSP70 and HSP90 mRNA levels (Table 6). Dietary Vit E/Se treatment significantly downregulated mRNA levels of HSP70, HSP90, mTOR, TLR2, IL-1β, IL-10, TNFα, iNOS, and IFNγ on d 35. Heat stress significantly upregulated GPx, HSP70, and HSP90, and downregulated mTOR, IL-1β and IFNγ.

Composition of Ileal Microbiota

The relative abundance of amplicon sequence variants of ileal microbiota was analyzed at different ranking levels from phylum to genus. The dominant phyla across the groups were *Firmicutes*, *Bacteroidetes*, *Tenericutes*, and *Proteobacteria* together contributing more than 99% of the whole phyla on d 27 (Supplementary Figure 1) and d 35 (Supplementary Figure 2). The LeFSe analysis revealed shifts in ileal microbiota communities in response to dietary Vit E/Se supplementation or heat challenge. On d 27, relative abundance of *Anaeroplasmataceae*, *Christensenellaceae*, *Sellimonas*, *Eubacterium*

rectale group, *Ruminococcaceae* UCG_005, *Ruminococcus gawreavii* group, and *Anaeroplasma* was lower in Vit E/Se group compared to control. However, *Lachnospiraceae* FE2018 and *Ruminococcaceae* NK4A214 groups were higher in Vit E/Se group on d 35 (Supplementary Table 1). Moreover, TN birds had greater abundance of *Porphyromonadaceae*, *Alcaligenaceae*, *Enterococcaceae*, *Parabacteroides*, *Parasutterella*, and *Enterococcus* compared to HS birds while relative abundance of *Clostridiales vadinBB60* group and *Erysipelatoclostridium* was higher in the HS group on d 35 (Supplementary Table 2).

Predicted Functions of Ileal Microbiota

There were 8 pathways at KEGG level 3 with distinctive enrichment between control and Vit E/Se groups on d 27 (Figure 1). Among these, the Vit E/Se group microbiota had lower numbers of functional genes associated with genetic information including mismatch repair, nucleotide excision repair, DNA replication, and DNA replication proteins compared to the control group. There were 19 pathways at KEGG level 3 with distinctive enrichment between control and Vit E/Se groups under HS conditions on d 35 (Figure 2A). In the HS group, ileal microbiota of the control group was enriched in functions involved in tyrosine metabolism, PPAR signaling

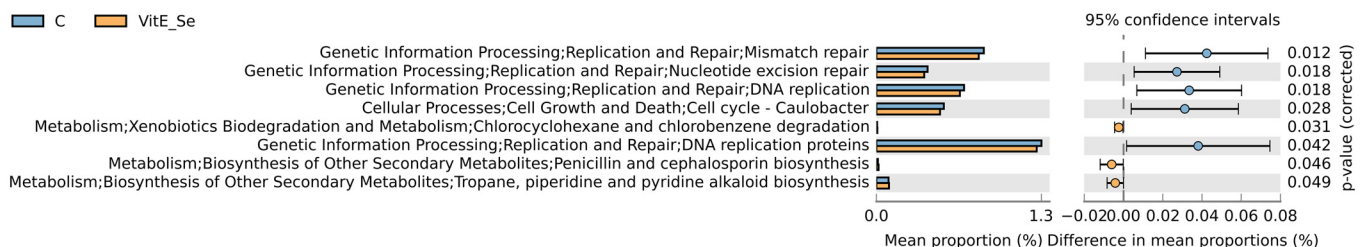


Figure 1. Predicted functions of ileal microbiota in broiler chickens at KEGG level 3 ($n = 8$ /treatment). Differentially regulated metabolic pathways in C vs. Vit E/Se group on d 27. Control (C): birds fed a basal diet; Vit E/Se: birds fed a basal diet supplemented with 250 mg/kg Vit E and 1 mg/kg selenium.

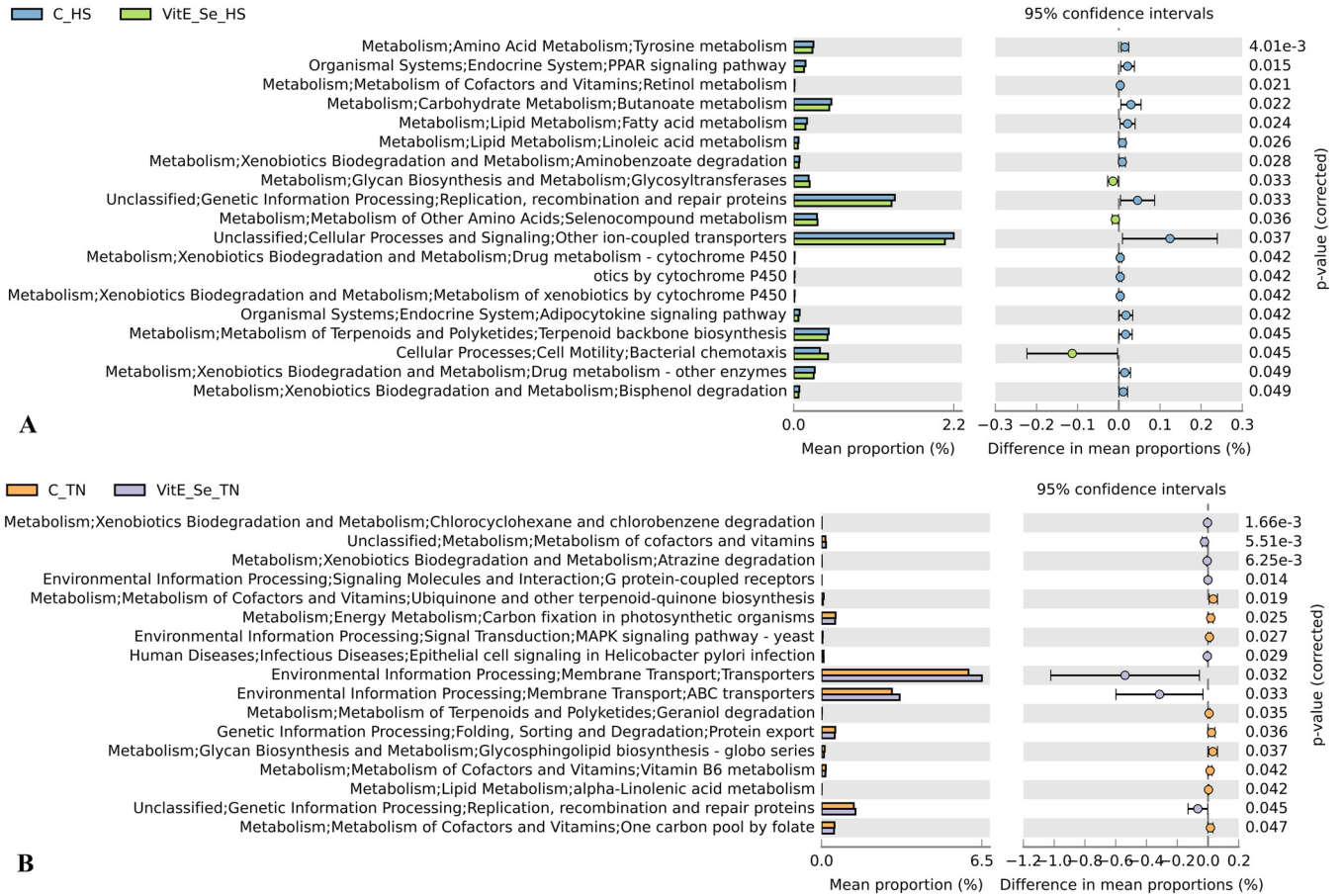


Figure 2. Predicted functions of ileal microbiota in broiler chickens at KEGG level 3 (n = 8/treatment). Differentially regulated metabolic pathways in C vs. Vit E/Se group during TN (**2A**) and HS (**2B**) conditions on d 35. Control (C): birds fed a basal diet; Vit E/Se: birds fed a basal diet supplemented with 250 mg/kg Vit E and 1 mg/kg selenium. TN: thermoneutral; HS: heat stress.

pathway, retinol metabolism, butanoate metabolism, fatty acid and linoleic acid metabolism, replication, recombination and repair proteins, adipocytokine signaling pathway and bisphenol degradation compared to Vit E/Se group. Conversely, ileal microbiota of the Vit E/Se group subjected to HS were enriched

in functions associated with the glycosyltransferases, seleno-compound metabolism, and bacterial chemotaxis compared to control group. There were 17 pathways at KEGG level 3 with distinctive enrichment between control and Vit E/Se groups under TN conditions on d 35 (**Figure 2B**). Among these,

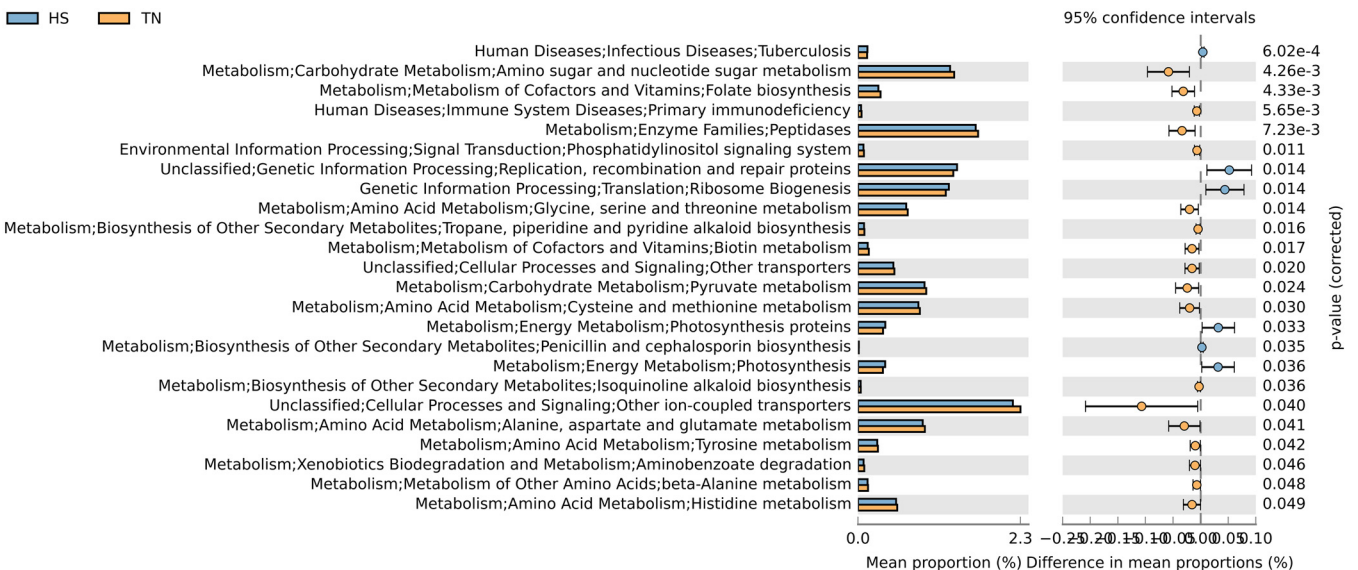


Figure 3. Predicted functions of ileal microbiota in broiler chickens at KEGG level 3 (n = 16/treatment). Differentially regulated metabolic pathways in TN vs. HS condition on d 35. TN: thermoneutral; HS: heat stress.

the Vit E/Se group had higher number of functional genes involved in cofactor and vitamin metabolism, membrane transports, and replication, recombination and repair proteins compared to control group. At third level of KEGG hierarchy, there were 24 differentially enriched pathways (Figure 3) between TN and HS groups (regardless of dietary treatments). Among them, metabolic pathways associated with carbohydrates (sugar and pyruvate), amino acids metabolism (glycine, serine and threonine, cysteine and methionine, alanine, aspartate and glutamate, tyrosine, and histidine), peptidases, folate biosynthesis, and biotin were enriched in the TN group compared to HS. Pathways involved in ribosome biogenesis and replication, recombination, and repair proteins were enriched in the HS group as compared to TN birds.

DISCUSSION

Environmental challenges cause several changes in the birds' physiological and immunological responses. Under HS conditions, the immune system, intestinal barrier function, and oxidative stability are compromised in broilers, resulting in reduced performance and welfare as well as increased mortality and susceptibility to pathogens (Lara and Rostagno, 2013). Therefore, several management and nutritional strategies are applied in modern poultry production systems to reduce or eliminate the effects of such challenges in order to maintain bird's growth and health (Emami et al., 2020). Dietary Vit E and/or Se supplementation is a well-known nutritional strategy that improves the antioxidant status of the host through eliminating free radicals to alleviate oxidative stress (Singh et al., 2006). In this context, the central hypothesis of the present study was that the adverse effects of heat stress on the immune response, oxidative stability, gut integrity, and intestinal microbiota can be mitigated by incorporating Vit E and organic Se into poultry diets.

Dietary Vit E/Se supplementation did not affect mRNA abundance of oxidative stress, immunity and gut integrity related genes in the jejunum on d 27, one day before the HS challenge. Similarly, mRNA abundance of oxidative stress and immunity related genes in the liver were not influenced by dietary treatments, except for a downregulation of TLR2 and iNOS on d 27. This outcome was not unexpected as under standard production practices, dietary supplementation of Vit E/Se above the recommended levels is not needed as these additives exert more pronounced effects during a challenge (Surai, 2018; Surai et al., 2019a).

Generally, protein synthesis is reduced under HS conditions except for a group of highly conserved proteins known as HSPs (Gu et al., 2012). These proteins, also called molecular chaperones, are responsible for the correct folding/unfolding of polypeptides to achieve a functional shape and to repair denatured proteins or assist in their degradation after stress (Whitley et al., 1999).

The mRNA abundance of HSPs, along with the oxidative stress markers such as SOD, CAT, and GPx, help to evaluate the effectiveness of dietary antioxidants under thermal stress conditions. Heat stress-associated increase in the mRNA and protein abundance of HSP70 and HSP90 were recently reported (Rimoldi et al., 2015; Liu et al., 2016; Santos et al., 2019). As expected, we observed a significant upregulation in HSP70 and HSP90 mRNA abundance in jejunal and liver tissues both at the start (d 28) and end (d 35) of the HS challenge. Consistent with our hypothesis, the mRNA levels of HSP70 and HSP90 were regulated by the dietary Vit E/Se treatment, where a significant downregulation was observed in HS birds. Similarly, other studies reported that dietary Vit E (Jang et al., 2014) and Vit E/Se (Kumbhar et al., 2018) significantly decreased the mRNA abundance of HSPs in several tissues of heat stressed broilers. Elevated environmental temperatures induce oxidative stress, which further leads to an increase in ROS level and an imbalance in redox state of cells (Gorman et al., 1999; Banerjee Mustafi et al., 2009). Hyperthermia-related overexpression of HSP70 is dependent on the activation of p38 Mitogen-activated protein kinase (p38MAPK), which is stimulated by the presence of ROS (Banerjee Mustafi et al., 2009). It is known that unfavorable effects of ROS are eliminated by enzymatic and nonenzymatic antioxidant defense mechanisms and free radical scavenging antioxidants, such as Vit E, Vit C, and Coenzyme Q10 (CoQ10) (Surai et al., 2019b). Such nutrients support the first level of antioxidant defense mechanisms and thereby reduce the need for the HSP response (Surai et al., 2019b). Therefore, downregulation of HSP70 and HSP90 in heat-stressed broilers fed Vit E/Se supplemented diet are likely due to the well-known antioxidant properties of these additives.

SOD and GPx are the first line of antioxidant defense system and play an important role in maintaining the oxidative stability of the host and cooperate to eliminate superoxide anions and hydrogen peroxide in cells (Gu et al., 2012). The expression of these antioxidant enzymes is regulated by mutable factors including cellular oxidative state and several antioxidant substances (Rodriguez et al., 2004) and also dietary nonenzymatic antioxidants (Surai et al., 2019a). Heat stress, being a promoter of oxidative stress (Emami et al., 2020), increased liver mRNA abundance of GPx (d 28 and d 35) and SOD (d 28). Such upregulations strongly correlate with the increase of HSPs mRNA abundance in the liver (Gu et al., 2012), as a strategy to eliminate excessive ROS caused by HS and to protect cells from oxidation. However, regardless of dietary Vit E/Se treatment, high temperature did not influence mRNA abundance of SOD on d 35. Even though increased SOD synthesis against oxidative stress has been evidenced under HS, extreme stress conditions cause a decrease in SOD activity (Surai et al., 2019b). Similarly, no noticeable change in SOD activity was observed in birds (Lin et al., 2006; Willemssen et al., 2011; Rimoldi et al., 2015) and rats (Morrison et al., 2005) exposed to HS. This observation

is most likely an adaptation strategy against prolonged heat challenge as birds show differential expression patterns during acute and chronic HS conditions (Xie et al., 2015). Unexpectedly, there were no interaction or diet main effect on SOD and GPx mRNA levels except a downregulation of GPx in HS birds fed Vit E/Se supplemented diets on d 28. Unlike previous reports (Harsini et al., 2012; Kumbhar et al., 2018), additional Se, a component of the GPx, did not upregulate mRNA abundance of this enzyme in the current study. This outcome could be attributed to the ROS scavenging activity of Vit E (Harsij et al., 2020). Another potential explanation for the observed decrease in GPx mRNA abundance is that other antioxidant enzymes such as CAT might compensate the redox status (Ajuwon et al., 2015). Both GPx and CAT neutralize H₂O₂ to water and molecular oxygen, consequently completing the dismutation process initiated by SOD (Ajuwon et al., 2015; Ighodaro and Akinloye, 2018).

Cytokines are regulatory small proteins involved in immune cell development, inflammation, and immune responses (Hedger and Meinhardt, 2003). Hyperthermia influences the immune response and alters cytokine expression and consequently leads to changes in cell energy metabolism and reduced performance (Quinteiro-Filho et al., 2010; Siddiqui et al., 2020). Heat stress-related suppression of proinflammatory immune response and induction of anti-inflammatory cytokine production were evidenced in previous studies (Siddiqui et al., 2020; Yan et al., 2020; Emami et al., 2021; Greene et al., 2021). Similarly, HS significantly suppressed the pro-inflammatory cytokines TNF α , IFN γ and IL-17A in the jejunum, and IL-1 β , TNF α and IFN γ in the liver. However, upregulation of the anti-inflammatory cytokines IL-4 and IL-10 was only noted in the jejunum of HS birds. Although IL-4 and IL-10 exert an anti-inflammatory role, the absence of IL-4 and IL-10 upregulation in the liver could have been dampened by elevated levels of other anti-inflammatory elements, such as transforming growth factor (TGF)- β and HSPs (Lee and Repasky, 2012). Chronic HS at 31 \pm 1°C for 6 d upregulated TGF- β in cecal tonsils, while IL-10 expression remained unaltered in both spleen and cecal tonsils (Quinteiro-Filho et al., 2017). Further, elevated HSP70 during HS was accompanied with downregulated IL-6 and TNF α levels and upregulated IL-10 mRNA abundance in the small intestine of broilers (Siddiqui et al., 2020). Similarly, our data corroborate with the fact that HSP70 diminishes the TNF α , a pro-inflammatory cytokine, through the recruitment of heat shock transcription factor 1 (HSF-1) to the cytokine promoter (Ferat-Osorio et al., 2014). Suppressed inflammatory response during HS, possibly caused by increased HSF-1, exerts a competitive inhibition on nuclear factor kappa B (NF- κ B) a regulator of pro-inflammatory cytokine production (Song et al., 2008). Therefore, modulated cytokine response in the jejunum and the liver of the HS broilers could be related to HSP upregulation as a regulatory function to protect cells (Ferat-Osorio et al., 2014) and prevent tissue damage

(Lee and Repasky, 2012). Along with the HS induced rapid change in cytokine expression, dietary Vit E/Se showed additional modulation of the immune response by decreasing the mRNA levels of TLR2, IL-1 β , IL-10, TNF α , iNOS, and IFN γ especially in the liver on d 35. Dietary Vit E and Se can act synergistically and influence antioxidant and immune responses during oxidative stress (Habibian et al., 2014; Dalia et al., 2018). In agreement with these findings, Khan et al. (2019) reported that dietary Se-enriched probiotics (containing viable *Lactobacillus acidophilus* and *Saccharomyces cerevisiae*) reduced liver IL-1 β , TNF α , and IFN γ mRNA abundance through the inhibition of NF- κ B in birds exposed to natural high temperature (ranges 30 to 39°C during daytime). Both pro- and anti-inflammatory cytokine responses in intestinal tissues were downregulated with increasing level of dietary Vit E under normal conditions (Pitargue et al., 2019). Vit E and Se, alone or combined, have anti-inflammatory characteristics, which lower pro-inflammatory cytokine expression through NF- κ B (Sen and Packer, 1996; Duntas, 2009) and mitogen activated protein kinase (MAPK) pathways (Zhang et al., 2014). Similar results are also evidenced using several dietary antioxidants such as resveratrol (Sahin et al., 2012) and Vit C (Jang et al., 2014). Therefore, the observed cytokine modulation in the present study appears to be associated with antioxidant characteristics of Vit E/Se. Moreover, it can be speculated that the anti-inflammatory modulatory effect of Vit E/Se is more noticeable under prolonged stress conditions as we observed more pronounced cytokine mRNA modulation on d 35 compared to d 28.

mTOR is a serine/threonine kinase that plays a regulatory role in a variety of cellular functions including cell growth, differentiation, protein synthesis, and autophagy (Sarbasov et al., 2005; Fazolini et al., 2015; Du et al., 2019). We found that mTOR expression in liver tissues was significantly decreased in birds exposed to HS (d 28 and d 35) and birds fed Vit E/Se supplemented diet (only d 35). A recent study also reported that heat exposure suppressed mTOR activity in chickens (He et al., 2019). Potentially, reduction of mTOR is a protective mechanism by slowing down protein synthesis burden during HS (Sun et al., 2015). However, there are limited reports regarding the effects of Vit E/Se combination on mTOR activity in broilers. More recently, CoQ10 treatment significantly upregulated autophagy and suppressed the PI3K/Akt/mTOR pathway to protect primary chicken myocardial cells exposed to HS (Xu et al., 2019). Similarly, decrease in mTOR mRNA abundance was also reported in HS broilers fed Noni- (a plant source rich in a variety of phytochemicals and antioxidants) supplemented diets (Rajaei-Sharifabadi et al., 2017). Therefore, our findings suggest that additional Vit E/Se supplementation might decrease mRNA abundance of mTOR in the liver to mitigate HS-induced oxidative stress.

Heat stress-induced hypoxia disturbs the antioxidant balance and immune response that leads to epithelial

damage and loss of barrier integrity (Lian et al., 2020). Tight junction proteins, which seal the paracellular space between adjacent epithelial cells, are highly responsive to HS. We herein, observed a main effect of HS on TJ proteins with a significant modulation during thermal challenge. In conjunction with our results, Varasteh et al. (2015) reported significant increase in E-cadherin, claudin-1, claudin-5, and ZO-1 protein expressions in the small intestine of broilers exposed to HS. Heat stress induced upregulation of HSF-1 increases the occludin protein expression by binding the occludin promoter and improves the participation of occludin in junctional complex (Dokladny et al., 2008). Therefore, it is possible that enhanced abundance of HSPs in this study might be related to the increased mRNA levels of TJ protein genes under HS conditions (Lian et al., 2020). Moreover, observed upregulation in TJs coincide with the IL-22 mRNA upregulation, which is a cytokine critical for maintenance of intestinal barrier function and tissue regeneration (Wang et al., 2017). Intestinal integrity-related effects of Vit E/Se are not broadly studied during a HS challenge. Contrary to our expectation, dietary Vit E/Se supplementation did not influence jejunal TJs mRNA abundance, likely related to the duration and severity of HS applied in the current study.

The intestinal microbiota play an important role in health and disease conditions of animals. Colonization, composition, growth, and diversity of this dynamic environment is influenced by many factors including diet and stress (Shi et al., 2019; Yang et al., 2020). Dietary antioxidants may regulate the microbiota composition and help to alleviate the adverse effects of several stressors on gut microbiota (Yang et al., 2020). LEfSe analysis revealed that, at the genus level, dietary Vit E/Se supplementation significantly enriched *Lachnospiraceae* FE2018 and *Ruminococcaceae* NK4A214 groups compared to control birds on d 35. *Lachnospiraceae* and *Ruminococcaceae* families are known to produce butyrate by fermenting polysaccharides and degrade resistant starch and non-starch polysaccharides (Yacoubi et al., 2018; Chen and Yu, 2020). Selenium nanoparticles (0.9 mg/kg) improved intestinal health by increasing abundance of *Lactobacillus* and *Faecalibacterium*, and butyric acid in broilers (Gangadoo et al., 2018). Under healthy conditions, intestinal lumen is strictly anoxic; however oxidative stress can change the oxygen gradient and shift the equilibrium of microbiota from anaerobic to facultative anaerobic groups (Dam et al., 2019). Such shift may decrease the butyrate producers and anaerobic fermenters of complex carbohydrates possibly leading to improper nutrient absorption and pathogen colonization (Million and Raoult, 2018; Dam et al., 2019). Butyrate is a preferred energy source for enterocytes and could enhance the epithelial regeneration; therefore, it can improve the host energy metabolism and performance (Calik and Ergun, 2015). Accordingly, along with their antioxidant effects on host metabolism, higher relative abundance of these two microbial groups might have contributed to the

improved performance in HS birds fed Vit E/Se diets. Heat challenge could alter the composition of ileal and cecal microbiota (Wang et al., 2018; Shi et al., 2019). Our results showed that heat challenge had no significant effect on relative abundance of major phyla of ileal microbiota. However, at the family and genus levels, we identified distinctive features, which coincide with previous findings (Wang et al., 2018). We observed an increase in *Clostridiales vadinBB60* group and *Erysipelatoclostridium* and a decrease in *Porphyromonadaceae* and *Enterococcaceae* in HS birds. This result aligns with the general opinion that HS change the gastrointestinal microbiota. In addition to changes in the ileal microbiota composition, the PICRUST analysis revealed significant functional differences among the treatment groups. In birds subjected to HS, Vit E/Se supplementation downregulated the microbial genes associated with amino acid, retinol, carbohydrate and lipid metabolism along with ion-coupled transporters and replication recombination and repair proteins compared to control. Elevated levels of ROS have DNA-damaging effect which trigger several DNA repair pathways in a cell (Brierley and Martin, 2013). Therefore, the reduction in the replication recombination and repair proteins is likely due to the antioxidant features and ROS scavenger effects of Vit E and Se. This finding also coincides with the enriched selenocompound metabolism in Vit E/Se group. Although we are not certain about the enriched nutrient metabolism pathways in HS-control group compared to HS-Vit E/Se group, this might be related to the stress adaptation strategy to overcome growth limiting conditions (Tran and Bonilla, 2021). Unlike HS conditions, ileal microbiota of the birds fed Vit E/Se supplemented diets and raised under TN conditions showed significantly enriched microbial functions related to the membrane transporters and replication, recombination and repair proteins. Membrane transport is related to the survival of the bacteria in gastrointestinal environment (Lyons et al., 2017). Thus, adding 250 mg/kg Vit E and 1 mg/kg Se can improve the renewal of bacteria and maintain vigorous vitality by influencing functions related to replication and repair and membrane transport (Tian et al., 2020). However, the modes of action of Vit E/Se on predicted functions of ileal microbiota could be different between optimal and suboptimal thermal conditions. When examining the direct physiological effects of HS on predicted microbial functions regardless of dietary treatments (Figure 3), nutrient metabolism pathways such as carbohydrate, amino acid, peptidases, folate biosynthesis, and biotin were adversely affected by heat challenge, suggesting differences in metabolism between broilers in different temperature conditions. Further investigation regarding the use of natural antioxidants on intestinal microbiome and metabolome needed for better understanding of its effect on the host under different stress conditions.

Based on the presented findings, HS challenge significantly upregulated the mRNA abundance of HSPs, antioxidant enzymes, and TJ proteins and downregulated pro-inflammatory cytokines as well as suppressed the

nutrient metabolism pathways of the ileal microbiota. Dietary supplementation of Vit E/Se helped to maintain the antioxidant balance especially by modulating mRNA abundance of antioxidant and immunity related genes in jejunal and liver tissues of the HS-challenged broilers. Ileal microbial profiling and functional analysis revealed taxonomic and functional signatures associated with dietary supplementation of Vit E/Se and HS challenge. In conclusion, the present study revealed that dietary supplementation of Vit E/Se mitigated the negative effects of HS by improving antioxidant status, regulating cytokine responses, and modulating ileal microbiota and its function.

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DISCLOSURES

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.psj.2022.101858](https://doi.org/10.1016/j.psj.2022.101858).

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