MITIGATION OF HEAT STRESS IN POULTRY USING DRIED PLUM OR ALPHA-LIPOIC ACID SUPPLEMENT

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ABSTRACT

Introduction:

Heat stress is a significant problem in the poultry industry, causing a severe economic loss due to its detrimental effect on the health and performances of chickens. Dried plum (DP) is a good source of minerals, vitamins, antioxidants, and phenolic compounds, and plays a role in calcium homeostasis and cardiovascular dysfunctions. Alpha-lipoic acid (ALA), on the other hand, is water and fat-soluble antioxidant, which can be readily absorbed from the intestine resulting in maximum bioavailability. Moreover, ALA acts as a coenzyme in glucose metabolism and helps generate other antioxidants. Considering these health benefits and properties, we hypothesized that the dietary supplementation of DP or ALA would help to mitigate heat stress in poultry.

Objectives:

The purposes of this study were to: 1) to determine the effects of DP supplementation on growth performance, gut health and immune parameters of heat-stressed broiler chickens, and 2) to determine the effects of ALA supplementation on growth performance, gut microbiota, gut health and immune parameters of heat-stressed broiler chickens.

Methods:

Study 1: Day-old Cob-500 unsexed chicks (n=72) were randomly placed into three treatment groups (n=24/group): No heat stress (NHS), 2) Heat stress with basal diet (HS), and 3) Heat stress with dried plum (HS+DP). Birds were raised under the standard broiler rearing guidelines for the first 21 days. Afterward, birds in the HS and HS+DP groups were exposed to heat stress conditions (33°C for 8 hours during daylight) for 3 weeks, while those in those in the NHS group were reared under normal conditions. Inclusion of 2.5% DP was made on the diet of

the HS+DP group from 14 d onwards. Weekly body weight and feed intake were measured to calculate the average daily growth rate (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR). On day 42, birds were euthanized, a portion of ileum was excised for the gene expression and histomorphometry analysis. Cecum digesta was collected for the volatile fatty acids (VFAs) and microbial population analysis using 16S rRNA sequencing.

Study 2: Day-old Cob-500 unsexed chicks (n=72) were randomly placed into three treatment groups (n=24/group): No heat stress (NHS), 2) Heat stress with basal diet (HS), and 3) Heat stress with alpha-lipoic acid (HS+ALA) and were reared under the standard broiler rearing guidelines for the first 21 days. Afterward, birds in the HS and HS+ALA groups were exposed to heat stress conditions (33°C for 8 hours during daylight) for 3 weeks, while those in the NHS group were reared under normal conditions. Supplementation of ALA (500 mg/kg) was made on the diet of the HS+ALA group from 14 d onwards. All other experimental procedures and analyses were carried out as mentioned in the DP study.

Results:

Study 1: Supplementation of DP in the heat-stressed broilers significantly improved the final body weight, ADG, ADFI, efficiency; the expression of heat shock protein-related genes *(HSF1, HSF3, HSP70, HSP90)*, antioxidant-related genes (*SOD1, SOD2, GPX1, GPX3, PRDX1, TXN*), tight junction-related genes (*CLDN1*, *OCLN*), immune-related genes *(IL4, MUC2)* and major VFAs. The microbial analysis revealed significant enrichment of beneficial bacteria in DP supplemented broilers.

Study 2: Supplementation of ALA in the heat-stressed broilers significantly improve the final body weight, ADG, expression of *HSP90, PRDX1, GPX3, SOD2, OCLN, MUC2*, and major

VFAs. Finally, the microbial analysis revealed the significant abundance of beneficial bacteria *Lactobacillus* and *Peptostreptococcaceae* in the ALA supplemented broilers.

Conclusion:

This study identified dietary supplementation of DP to be the novel strategy to mitigate heat stress in poultry. Dietary supplementation of the DP improved both the growth performance and overall gastrointestinal physiology in heat-stressed broilers. Similarly, ALA improved body weight, gut microbiota, and other gut health parameters. Thus, the DP, and ALA supplementation can be considered as a potential remedy for heat stress in poultry.

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LIST OF ABBREVIATIONS

ADFI	Average daily feed intake
ADG	Average daily gain
ALA	Alpha-lipoic acid
ANOVA	Analysis of variance
CD	Crypt depth
cDNA	Complementary deoxyribonucleic acid
CLDN1	Claudin 1
DNA	Deoxyribonucleic acid
DP	Dried Plum
F	Forward
F	Forward
FCR	Feed conversion ratio
g	Grams
GC	Gas chromatography
GIT	Gastrointestinal tract
GPX1	Glutathione Peroxidase 1
GPX3	Glutathione Peroxidase 3
HS	Heat stress
HSF1	Heat shock factor 1
HSF3	Heat shock factor 3
HSP70	Heat Shock Protein 70
HSP90	Heat Shock Protein 90

IL4	Interleukin 4
kcal	kilo calorie
kg	Kilo gram
MUC2	Mucin 2
NFE2L2	Nuclear Factor, Erythroid 2 Like 2
OCLN	Occludin
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PRDX1	Peroxiredoxin 1
qPCR	Quantitative polymerase chain reaction
R	Reverse
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAS	Statistical analysis system
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
TMA	Trimethyl acetate
TXN	Thioredoxin
VFA	Volatile fatty acid
VH	Villus height

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

The poultry industry is growing across the world to fulfill the increasing demands of poultry meat and eggs. Poultry meat is a good source of white meat, contains a low amount of saturated fatty acids, and is rich in protein, vitamins, and minerals (Marangoni et al., 2015). Similarly, poultry eggs are the cheapest source of high-quality animal proteins. Besides vitamins, minerals, and proteins; eggs are also rich in antioxidants such as lutein and zeaxanthin which possess significant benefits for eye health (Zaheer, 2015). Considering these health benefits and affordable sources of nutrition, global consumption of the poultry meat and eggs have doubled in the past decade and is expected to be doubled by 2050. To meet such demands, there has been an improvement in chicken genetics in the past decade. Broiler chickens which were 905 gms at 52 days age in the 1950s are 4,202 gms now at the same age (Zuidhof et al., 2014). Similarly, recently improved laying hens lay around 300 eggs/year while laying hens in the early 90's used to lay 120 eggs per year. These improved breeds have higher metabolic rates and production performances. Compare to other livestock species, birds have inferior heat loss mechanisms. High stocking density of birds along with the high ambient temperature increases the propensity of heat stress (Goo et al., 2019a).

Heat stress (HS) is a major problem in the poultry industry affecting the health and performances of poultry, resulting in annual economic losses of $128 to $165 million in the poultry industry (St-Pierre et al., 2003). Heat stress in poultry is the condition where the chickens are unable to maintain a balance between body heat production and heat loss. Heat stress results from the interaction of different factors such as high environmental temperature, humidity, radiant heat, and airspeed; among them, high ambient temperature plays a major role. The normal body

temperature of the chicken is around 41-42°C, and the optimum ambient temperature to maximize growth for chicken is 18-20°C. The environmental temperature higher than 25°C elicits heat stress in poultry (Donkoh, 1989).

1.2 Biological changes in poultry due to heat stress

Heat stress in poultry results in severe behavioral, physiological, and neuroendocrine changes (Figure 1).

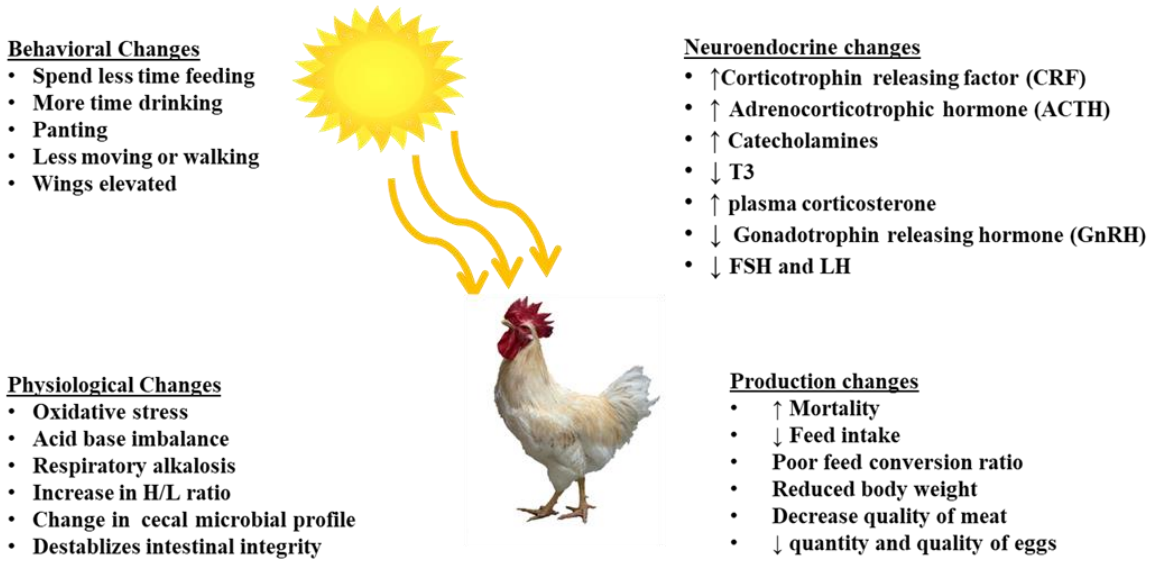


Figure 1 Effects of heat stress in poultry.

1.2.1 Physiological change

1.2.1.A Oxidative stress

Reactive oxygen species (ROS) are free radicals and peroxides which are normally produced within the cells and are removed by normal detoxifying mechanisms present within the cells. During the normal condition, activation of transcriptional factor Nrf2 causes the additional synthesis of a group of antioxidant molecules which deals with increased ROS produced inside the

cell (Surai et al., 2019). However, when there is an imbalance between these systems, either by higher production of ROS or by a decrease in the effectiveness of the antioxidant defense system, the cells are exposed to stress conditions commonly known as oxidative stress (Figure 2). Previous studies have shown that heat stress has been associated with oxidative stress in the cells (Estévez, 2015; Surai et al., 2019). Excess free radicals produced during the oxidative stress damage all the components of the cells including proteins, lipids, and DNA. Effects of oxidative stress depend upon its severity and range from small reversible changes to the apoptosis and cell death in case of severe oxidative stress (Mishra and Jha, 2019).

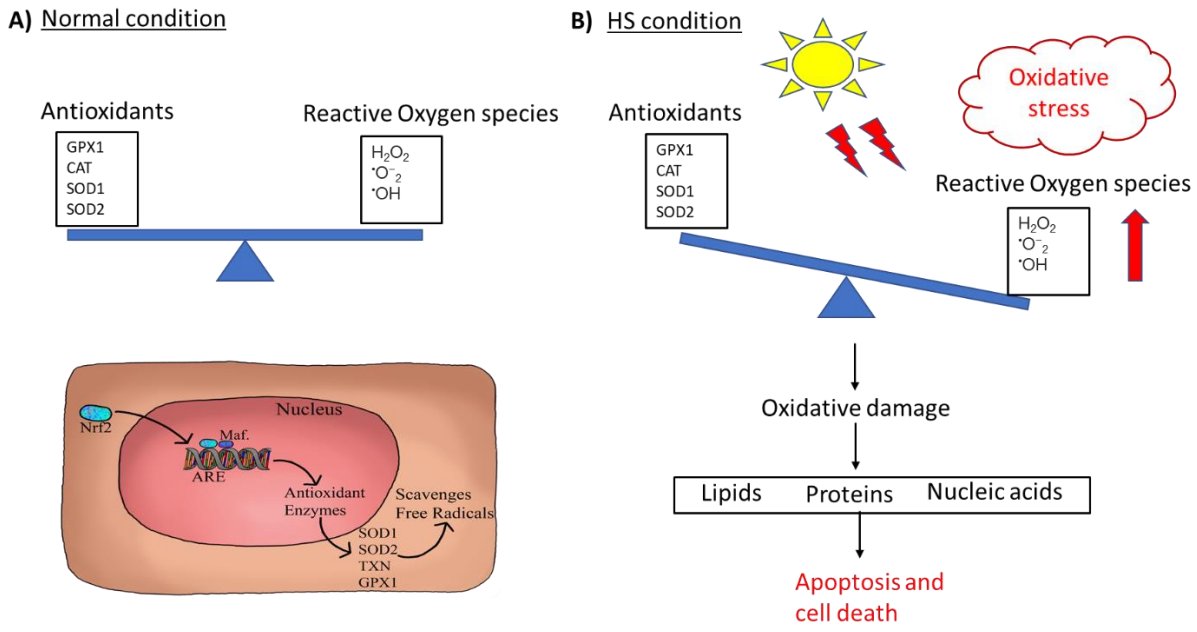


Figure 2 Schematic diagram showing the redox system: A) Normal condition, and B) Under heat stress condition.

1.2.1.B Acid-base imbalance

Birds possess unique features such as lack of sweat glands and have feathers throughout the body. These features make them pant- beak open breathing to cool the body during the time of

heat stress. During panting, excretion of the CO_2 occurs at a greater rate than the cellular production of the CO_2 which alters the normal bicarbonate buffer system in the blood. The reduction of CO_2 loss leads to a decrease in the concentration of carbonic acids (H_2CO_3) and hydrogen ions (H^+) with the increase in the concentration of the HCO_3^-, thus raising the blood pH i.e. blood becomes alkaline. To cope with this situation and maintain the normal blood pH, birds will start excreting more amount of HCO_3^- and retain H^+ from the kidney. This condition of alteration in the acid-base balance leads to respiratory alkalosis and metabolic alkalosis (Figure 3) and is associated with the decline in production performance in poultry (Borges et al., 2007).

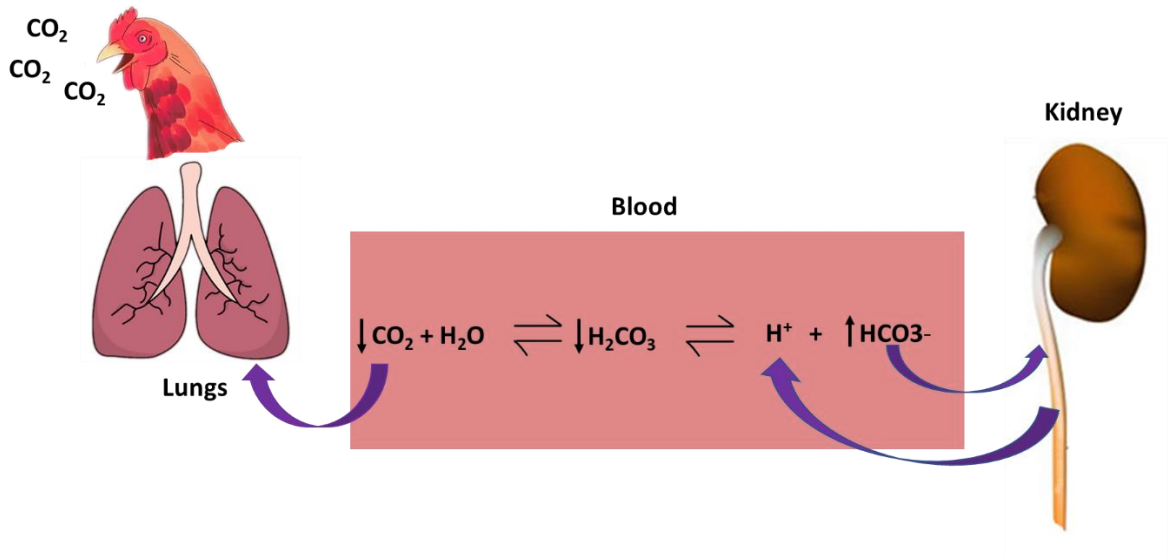


Figure 3 Schematic diagram showing an acid-base imbalance in poultry during heat stress.

In alkaline blood (higher pH), bone resorption is also impaired. The calcium ions remain retained in the bone. The resorbed calcium from bones is the major source of calcium for eggshell mineralization. Heat stress significantly limits the availability of calcium ions for eggshell formation (Roberts, 2004). Therefore, panting, a response to heat stress also deteriorates the eggshell quality in laying hens.

1.2.1.C Suppressed immunocompetence

Heat stress is known to suppress immunity in the chickens (Lara and Rostagno, 2013). As a result, the prevalence of contagious and infectious poultry disease is found relatively higher during the summer season in the tropical country. Besides this, the size of immune-related organs such as the spleen, thymus, and lymphoid organs are also found to regressed in the heat-stressed birds (Quinteiro-Filho et al., 2010; Ghazi et al., 2012). Antibodies are produced in the response of foreign antigens (bacteria, viruses, etc.). The level of antibodies is also found to be lowered in the heat-stressed birds (Bartlett and Smith, 2003). Likewise, total white blood cell counts (WBC) are found to be significantly lowered, whereas heterophils to lymphocytes (H/L) ratio is found to be higher in heat-stressed birds (Mashaly et al., 2004). Higher H/L ratio indicates depression of both T and B lymphocytes, which plays a role in antibody production and immune responses.

1.2.2 Neuroendocrine changes

The neuroendocrine system plays a crucial role in maintaining homeostasis and the normal functioning of the birds. Different hormones play a role in the growth and egg production in poultry (Mishra et al., 2019). In birds, the sympathoadrenal medullary (SAM) axis gets activated and regulates homeostasis during an early stage of heat stress. The increase in ambient temperature is perceived by the sympathetic nerves, which transmit the impulse to the adrenal medulla. Adrenal medulla in return increases the secretion of the catecholamines which causes a surge of glucose release in the blood, deplete liver glycogen, increase respiration rate, vasodilate the peripheral blood vessels, and increase neural sensitivity to cope with the stress (Siegel and Van Kampen, 1984; Kumari et al., 2018). However, when stress persists for a longer period, the hypothalamic-pituitary-adrenal (HPA) axis gets activated. In response to the stress, corticotrophin-releasing hormone (CRH) is released from the hypothalamus which causes releases of Adreno-cortical-

trophic hormone (ACTH) from the pituitary. ACTH increases the production and release of corticosteroid by the cortex of the adrenal gland (Smith and Vale, 2006). Corticosteroid functions to increase the blood glucose level through gluconeogenesis (Siegel and Van Kampen, 1984; Kumari et al., 2018). Thyroid hormones, triiodothyronine (T3) and thyroxine (T4), released from the thyroid gland, also play a crucial role in maintaining metabolic rate in the birds. Previous studies in heat-stressed birds have shown that T3 concentration was lowered in the heat-stressed birds (Etches et al., 2008; Star et al., 2008; Quinteiro-Filho et al., 2012) whereas T4 concentration was found inconsistent in another study (Etches et al., 2008). Besides this, secretion of the gonadotrophin-releasing hormones, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) are also found to be impaired in heat-stressed birds (Nawab et al., 2018). Moreover, sex hormones – plasma progesterone, plasma testosterone, and plasma estradiol, were also found to be lowered in heat-stressed white leghorns (Rozenboim et al., 2007).

1.2.3 Behavioral changes

When birds are exposed to a higher environmental temperature, birds try to dissipate excess heat produced inside the body, which is manifested by specific behavioral changes in the birds. Chickens in the thermal stress condition are found to spend less time walking and standing, consume less amount of feed and more water, spread wings, and cover their body surface in the litter. In addition to this, the characteristics of the panting sign are also observed in heat-stressed birds.

These major physiological, neuroendocrine, and behavioral changes lead to a decrease in feed intake, reduced final body weight, decreased quality of meat and eggs, an increase in the feed conversion ratio, and an increase in mortality in poultry. Thus, heat stress has been of paramount

importance in the poultry industry considering global warming and economic loss resulting from it. To cope with this problem, different strategies have been employed by researchers and farmers.

1.3 Major strategies to mitigate heat stress in poultry

Major strategies that have been used to mitigate the detrimental effects of heat stress in poultry are discussed in this review paper.

1.3.1 Feeding strategies

1.3.1.A Feed restriction

Restricting the feed during the hot period of the day has been a common practice in the tropics. Feed restriction in the broilers is found to reduce the rectal temperature and minimize the mortality (Abhu-Dieyeh, 2006; Uzum and Toplu, 2013). Uzum and Toplu (2013) found that restricting the feed for 8 h a day to broilers during the hot period improved the feed efficiency and shorter tonic immobility. Similarly, in the case of broiler hens, controlled feeding was found to reduce heat production by 23 % (MacLeod and Hocking, 1993). Yet, this approach is not widely used in the poultry industry, as it results in reduced growth rate and delayed marketing age of the bird (Francis et al., 1991; Wiernusz and Teeter, 1996; Abhu-Dieyeh, 2006; Uzum and Toplu, 2013).

1.3.1.B Dual feeding regime

Practical observations have shown that feed restriction results in overcrowding and rush at a re-feeding time, resulting in some additional mortality. Thus, the dual feeding regime has been devised so to make sure birds get access to feed throughout the day. In this feeding regime, the protein-rich diet is provided during the cooler and the energy-rich diet during the warmer period of the day. Studies have shown that providing protein-rich diet 16 to 9 h and energy-rich diet during

the 9 to 16 h during the heat stress period, were found to significantly reduce the body temperature (De Basilio et al., 2001; Lozano et al., 2006) and mortality in the heat-stressed broilers (De Basilio et al., 2001). However, this approach couldn't enhance the growth and feed conversion in heat-stressed birds (Lozano et al., 2006).

1.3.1.C Wet feeding

Wet feeding stimulates predigestion, improves absorption of the nutrients from the gut, and accelerates the action of the digestive enzyme on the feed (Syafwan et al., 2011). In broilers, wet feeding is found to improve the feed intake, body weight, and weight of the GI tract and GI organs (Moritz et al., 2001; Shariatmadari and Forbes, 2005). In laying hens, wet feed feeding at high temperature was found to increase dry matter intake, egg weight, and egg production (Lin et al., 2006). Although this approach was found to have beneficial effects in HS birds, it is less common among poultry farmers due to the risk of fungal growth in the feed causing mycotoxicosis in the birds and other logistic limitations of wet feeding.

1.3.1.D Adding fat in the diet

Higher energy diets are found to be effective in partially mitigating the heat stress effects in poultry. During metabolism, fat produces lower heat increment as compared to protein and carbohydrates. Considering this, the supplementation of fat in the diet has been a general practice in hot climatic places to increase the energy level and ameliorate the detrimental effects of heat stress. Supplementation of fat in the poultry diet not only helps to increase the nutrient utilization in the GI tract by lowering the rate of food passage (Mateos et al., 1982) but also helps to increase the energy value of the other feed constituents (Mateos and Sell, 1981). Adding fat at the level of 5% to the diet in heat-stressed laying hens are found to increase feed intake by 17% (Daghir, 2008). Similarly, significant improvement in the broiler performance was observed when 5% fat diet was

provided (Ghazalah et al., 2008). Albeit of its benefit, adding fat was found to significantly increase the abdominal fat in HS broilers (Ghazalah et al., 2008), which is not desirable.

1.3.1.E Supplementation of vitamins, minerals, and electrolytes

i Vitamin E

Vitamin E (alpha-tocopherol) is a fat-soluble antioxidant that helps to scavenge the free radicals and helps in the proliferation of the cells involved in the immune system such as lymphocytes, macrophages and plasma cells against oxidative damages (Meydani and Blumberg, 1993; Puthpongsiriporn et al., 2001). Dietary supplementation with vitamin E in heat-stressed laying birds was found to improve the egg production, egg weight, eggshell thickness, egg specific gravity, and haugh unit (Khan et al., 2011). The liver is an essential organ for egg formation as it helps in the synthesis and release of egg yolk protein-vitellogenin. Yardibi and Hosturk (2008) reported that vitamin E helps to improve the egg production by preventing liver damage in heat-stressed birds, thus, facilitate the synthesis and release of vitellogenin. Similarly, broilers supplemented with 250 mg/kg vitamin E of feed was found to reduce the liver and serum malondialdehyde (MDA) concentration, and increased serum and liver vitamin E and A concentration in HS condition (Sahin and Kucuk, 2001).

ii Vitamin A

Vitamin A is related to antibody production and T cell proliferation (Sklan et al., 1994). Supplementation of the higher level of vitamin A (6000 and 9000 IU/ kg of feed) was able to increase the egg weight in heat-stressed laying hens (Lin et al., 2002). They also reported that hens exposed to the heat stress immediately after Newcastle disease virus (NDV) vaccination require a higher amount of vitamin A for an adequate level of antibody production. In broilers, supplementation of vitamin A (15000 IU/kg of feed) was found to increase live weight gain,

improve feed efficiency, and decrease the serum MDA concentration in heat-stressed birds (Kucuk et al., 2003).

iii Vitamin C

Although poultry can synthesize vitamin C, the amount is limited during heat stress conditions. Thus, the supplementation of dietary vitamin C is found to be an effective strategy to attenuate the negative effect of heat stress in poultry. In poultry, supplementation of vitamin C (250 mg/kg of feed) has been found to improve the growth rate, nutrient utilization, egg production, and quality, immune response, and antioxidant status in heat-stressed birds as reviewed by (Khan et al., 2012). Supplementation of vitamin C in the diet of heat-stressed Japanese quail lowered the serum concentration of MDA, homocysteine, and adrenal corticotropin hormone (Sahin et al., 2003). In broilers, dietary supplementation of 200 mg ascorbic acid per kg of feed was found to improve body weight gain and effective feed gain ratio (Njoku, 1986).

iv Zinc

Zinc is an essential nutrient required for activating more than 300 different enzymes. Zinc is associated with the antioxidant defense system, normal immune function, and skeletal development (Prasad and Kucuk, 2002). Zinc is also essential for the synthesis of metallothionein, which acts as the free radical scavengers (Oteiza et al., 1996). Moreover, zinc is also associated with the carbonic anhydrase enzyme which plays an essential role in the formation of the carbonate-key compound for the eggshell formation (Balnave and Muheereza, 1997). Supplementation of zinc help to suppress the free radicals by being part of some of the antioxidant's enzymes such as superoxide dismutase and glutathione. In broilers, supplementation of the organic form of zinc (40 mg /kg of feed) was effective in improving body mass growth, reducing the level of lipid peroxide, and increasing the activity of superoxide dismutase enzyme

during summer (Rao et al., 2016). Supplementation of 30 mg of Zn and 600 mg of Mg/kg of feed was found to improve live weight gain, feed intake, and hot and chilled dressing percentage in heat-stressed quails (Kucuk, 2008). In another experiment supplementing 60 mg/kg zinc in the feed of egg-laying Japanese quail was found to reduce the MDA concentration, increase serum vitamin C and vitamin E level and also increase egg production (Sahin and Kucuk, 2003). In laying hens, dietary supplementation of 80 mg/kg of zinc (Moreng et al., 1992) or 100 mg/kg of zinc (Balnave and Zhang, 1993) in the form of Zn-methionine is found to improve the eggshell thickness and mitigate the eggshell defects seen in heat-stressed laying hens.

v Chromium

Chromium is an essential mineral, which is an integral component of chromodulin and is essential for insulin functioning (Vincent, 2000). Moreover, chromium is also involved in carbohydrate, protein, lipid, and nucleic acid metabolism (Hayirli, 2005). Sahin et al. (2002a) experimented to determine the effects of chromium supplementation (chromium picolinate CrPic) of various levels (200, 400, 800 or 1200 µg/ kg of the diet) in heat-stressed broilers, where they found that increased supplementation of the chromium was associated with the increase in the body weight, feed intake, and carcass quality. They also observed a decreased level of serum corticosterone concentration, decreased serum glucose and cholesterol concentration, and increased serum insulin level. Moreover, the organic form of chromium supplemented as chromium methionine was also found to improve the cellular and humoral immune responses (Jahanian and Rasouli, 2015). In laying hens, dietary supplementation of 0.4 – 2 mg Cr/kg as CrPic was found to improve immune response, egg quality, haugh unit (Li et al., 2001; Sahin et al., 2002b) and reduced serum glucose, cholesterol and triglyceride concentration (Torki et al., 2014).

vi Selenium

Selenium is a vital component of at least 25 different selenoproteins. Most of these selenoproteins perform as an enzyme inside the cell (Nazıroğlu et al., 2012; Zhou et al., 2013). Two different forms of selenium i.e. inorganic forms (sodium selenite and selenite) and organic forms (selenomethionine and selenium-yeast) are used as supplements for poultry, where organic forms are found to be easily absorbed than the inorganic forms (Yang et al., 2012). Rahimi et al. (2011) found dietary supplementation if 0.3 mg/kg selenium improved the live weight and feed conversion ratio. Similarly, supplementation of 0.1 or 0.2 mg/kg sodium selenite was found to improve the carcass quality and performance of quails reared under high temperature (Sahin and Kucuk, 2001). Supplementation of the selenized yeast in the diet of laying hen has improved the egg weight, egg production, haugh units, and eggshell strength (Siske et al., 2000). In laying quails, there were linear increases in feed intake, body weight, and egg production and improved feed efficiency upon selenium supplementation (0.15 and 0.30 mg/kg of sodium selenite or selenomethionine) to quails reared under heat stress (Sahin et al., 2008). They reported that haugh units and eggshell weights were also increased when both organic and inorganic selenium were supplemented.

vii Electrolytes

Panting in heat-stressed birds alter the acid-base balance in the blood plasma and ultimately leads to respiratory alkalosis. This acid-base imbalance can be recovered by supplementation of the electrolytes such as NH_4Cl, $NaHCO_3$, KCL. During respiratory alkalosis, birds excrete more amount of bicarbonate ions from the kidney to restore the normal blood pH. These bicarbonates ions are coupled with Na^+ and K^+ ions before being excited through the kidney. Ultimately, the loss of ions results in acid-base imbalance (Ahmad et al., 2008). Thus, sodium and potassium

supplementation is preferred in heat-stressed birds to increase the blood pH and blood HCO_3^-, while chloride is supplemented to reduce these parameters (Hurwitz et al., 1973). A higher range of dietary electrolyte balance (DEB) (i.e. 200-300 mEq/kg) has been suggested to ameliorate the detrimental effect of heat stress in poultry (Mushtaq et al., 2013). Several researchers had found $NaHCO_3$ as the salt of choice during heat stress as they contain Na and HCO_3 (reviewed by Mushtaq et. al., 2013). Moreover, supplementation of sodium bicarbonate in heat stress laying hens is also found to improve the eggshell quality (Balnave and Muheereza, 1997). Benton et al. (1998) reported that $NaHCO_3$ can be incorporated into broiler diets up to 0.5% to improve the performance of heat-stressed broiler chicken. Similarly, Smith and Teeter (1987) found dietary levels of 1.5-2.0% K from KCl was effective in improving FCR during chronic heat stress conditions. Besides supplementing these salts in the diet; supplementation of electrolytes such as 0.2% NH4Cl or 0.15% KCl, 0.6% KCl, and 0.2% NaHCO3 in drink water is also found to improve the performance in heat-stressed birds. (Lin et al., 2006).

1.3.1.F Supplementation of phytochemicals

i Lycopene

Lycopene is a predominant carotenoid mainly found in the tomatoes and tomatoes product. Sahin et al. (2016) used 200 or 400 mg/kg lycopene in heat-stressed broilers where they found an increased level of dietary lycopene was able to improve the cumulative feed intake, body weight, and decreased feed conversion ratio. Previous studies have also shown lycopene improves the antioxidant enzymes (SOD, GSH-Px) and significantly reduces the MDA concentration in broilers (reviewed by Arain et al., 2018). In the case of laying hens, dietary supplementation of the lycopene was found to improve the oxidative status of laying hens, enhance vitamin levels in the egg, and also improve egg oxidative stability and yolk color (Arain et al., 2018).

ii Resveratrol

Resveratrol is natural bioactive polyphenols mainly found in grapes, peanuts, berries, and turmeric. Previous studies have shown that supplementation of resveratrol 400 mg/kg feed has enhanced the antioxidant capacity in birds (Hu et al., 2019). He et al. (2019a) found that supplementation of the resveratrol improved the average daily gain, decrease the rectal temperature, lowered the level of corticosterone, adrenocorticotropin hormone, cholesterol, and MDA while increasing the level of triiodothyronine, glutathione, total superoxide dismutase, catalase, and glutathione peroxidase. In another study, Zhang et al. (2017a) found that resveratrol not only improved the final body weight but also improved gut health parameters such as microbial profile, villus-crypt structure, and expression of the tight junction and adherence junction related genes. Interestingly, resveratrol is also found to improve the meat quality in heat-stressed broilers by increasing the muscle total antioxidant capacity (T-AOC) and activity of antioxidant enzymes (CAT, GSH-PX) (Zhang et al., 2017b). In laying hens, supplementation of 200 mg/kg feed resveratrol was found to improve the egg production, while 400 mg/kg feed reduced the total serum cholesterol and triglycerides, reduced egg cholesterol content, improved antioxidant activity, and improved egg sensory scores (Zhang et al., 2019).

iii Epigallocatechin gallate (EGCG)

Epigallocatechin gallate (EGCG) is the polyphenols present in the green tea extract that possess higher antioxidant and anti-inflammatory properties. Luo et al. (2017) used the different dosages (0, 300 and 600 mg/kg feed) of the EGCG in the heat-stressed broiler, where they found a linear increase in body weight, feed intake, and level of serum total protein, glucose and alkaline phosphatase activity in heat-stressed birds. In a similar experiment, Xue et al. (2017) found that the inclusion of the EGCG also improved the antioxidant enzymes (GSH-Px, SOD, and catalase)

in the liver and serum, along with the improvement in the body weight. Sahin et al. (2010) used 200 or 400 mg of EGCG/kg diet in heat-stressed female quails, where they found that increased supplementation of the EGCG linearly increased feed intake, egg production, hepatic SOD, CAT, and GSH-Px activity and linear decrease in the hepatic MDA level.

iv Curcumin

Curcumin is the major polyphenols extracted from turmeric and has found to possess antioxidant properties. As it is absorbed readily by animals, its's use as a potential compound to mitigate heat stress in poultry has got considerable attention in recent days. Previous studies have shown that curcumin was able to improve the growth performance of heat-stressed birds (Zhang et al., 2015, 2018a; b). Zhang et al. (2018a) found that the inclusion of curcumin at 50 and 100 mg/ kg feed improved the heat stress condition. Curcumins are found to reduce the mitochondrial MDA level and the ROS production by increasing the activity of Mn-SOD, GSH-PX, Glutathione S-transferase (GSST) (Zhang et al., 2015) and increase gene expression of thioredoxin 2 and peroxiredoxin-3 (Zhang et al., 2018a).

Table 1 Summary of the beneficial effects of vitamins, minerals, and phytochemicals in heat-stressed poultry.

Supplements	Beneficial effects on HS birds	References
Vitamin E	➢ Prevent liver damage, facilitate the synthesis and release of vitellogenin; ↑ egg production.	Yardibi and Hosturk, 2008
	➢ ↓liver and serum MDA concentration; ↑ increased serum and liver vitamin E and A concentration	Sahin et al., 2001
Vitamin A	➢ ↑ egg weight in HS layers	Lin et al., 2002
	➢ ↑live weight gain, improve feed efficiency and ↓serum MDA concentration	Kucuk et al., 2003
Vitamin C	➢ improve the growth rate, nutrient utilization, egg production, and quality, immune response, and antioxidant status	Khan et al., 2012
	➢ ↓ the serum concentration of MDA, homocysteine, and adrenal corticotrophin hormone	Sahin et.al., 2003
	➢ improve body weight gain and effective feed gain ratio	Njoku, 1986
Zinc	➢ improve body mass growth, ↓ level of the lipid peroxide, ↑ activity of superoxide dismutase	Rao et al., 2016
	➢ improve live weight gain, feed intake, and hot and chilled dressing percentage	Kucuk, 2008
	➢ ↓ malondialdehyde concentration, ↑serum vitamin C and vitamin E level, ↑ egg production	Sahin and Kucuk, 2003
	➢ improve the eggshell thickness and mitigate the eggshell defects	Balnave and Zhang, 1993; Moreng et al., 1992
Chromium	➢ ↑ body weight, feed intake, and carcass quality; ↓ the level of serum corticosterone concentration; ↓ serum glucose and cholesterol concentration; ↑ serum insulin level.	Sahin et al., 2002a
	➢ improve the cellular and humoral immune responses	Jahanian and Rasouli, 2015
	➢ ↑ immune response, egg quality, haugh unit	Li et al., 2001; Sahin et al., 2002b

	➢ ↓ serum glucose, cholesterol and triglyceride concentration	Torki et al., 2014
Sodium Bicarbonate	➢ improve the eggshell quality	Balnave and Muheereza, 1997
KCL	➢ 1.5-2.0% K from KCl effective in improving FCR	Smith and Teeter, 1987
Lycopene	➢ ↑ cumulative feed intake, body weight, and ↓ feed conversion ratio.	Sahin et al., 2016
	➢ ↑ antioxidant level enzymes (SOD, GSH-Px) and ↓ the MDA concentration	Arain et al., 2018
	➢ ↑ oxidative status of laying hens, enhance vitamin levels in the egg and improve egg oxidative stability and yolk color	Arain et al., 2018
Resveratrol	➢ ↑ average daily gain, ↓ rectal temperature, ↓ corticosterone, adrenocorticotropin hormone, cholesterol, and malonaldehyde; ↑ triiodothyronine, glutathione, total superoxide dismutase, catalase, and glutathione peroxidase.	He et al., 2019a
	➢ Improve microbial profile, villus-crypt structure, and expression of the tight junction and adherence junction related genes	Zhang et al., 2017a
	➢ ↑ muscle total antioxidant capacity (T-AOC) and activity of antioxidant enzymes (CAT, GSH-PX)	Zhang et al., 2017b
	➢ ↓ total serum cholesterol and triglycerides, ↓ egg cholesterol content, ↑ antioxidant activity, and ↑ egg sensory scores	Zhang et al., 2019
Epigallocatechin gallate (EGCG)	➢ increase in body weight, feed intake, and level of serum total protein, glucose, and alkaline phosphatase activity	Luo et al., 2017
	➢ improve the antioxidant enzymes (GSH-Px, SOD, and catalase) in the liver and serum	Xue et al., 2017
	➢ linearly increase feed intake, egg production, hepatic SOD, CAT, and GSH-Px activity and linear decrease in the hepatic MDA level.	Sahin et al., 2010
Curcumin	➢ ↓ mitochondrial MDA level and ↑activity of Mn-SOD, GSH-PX, Glutathione S-transferase (GSST)	Zhang et al., 2015
	➢ ↑ gene expression of thioredoxin 2 and peroxiredoxin-3	Zhang et al., 2018a

1.3.2 Genetic approach

Improved broilers lines have a higher metabolic rate, as a result, are more susceptible to the heat stress condition. Thus, poultry lines incorporating some of the genes that help to reduce heat stress can be developed to have a high magnitude of production in the hot and arid areas.

1.3.2.A Naked neck genes

The Na gene is the single dominant autosomal gene that helps to reduce feathers in the neck region thus help to dissipate heat through the neck region in the birds. Naked neck genes reduce the feather by 20% and 40% in Na/na (heterozygous naked neck) and Na/Na (homozygous naked neck), respectively as compared to normal siblings (na/na) (Merat, 1986). Na gene broiler is associated with the increase in breast muscle, increase in the body weight (Cahaner et al., 1993; Azoulay et al., 2011), reduce abdominal fat (Rajkumar et al., 2010), body temperature (Yalcin et al., 1997). In another study, Rajkumar et al. (2011) reported that the total cholesterol level in plasma and H/L ratio was significantly lowered in the naked necked birds as compared to normal. Laying birds with the naked neck gene are found to have improved egg mass, number, and quality under hot temperatures (Fathi et al., 2013).

1.3.2.B Fizzle gene

The fizzle (F) gene causes the curving of the outline of the feather, resulting in a reduced featherweight, reduced insulating property of the feather cover, and an increase in heat radiation from the body. Zerjal et al. (2013) reported that F mutation in its homozygous state in laying hens was found to improve the egg production and quality traits by increasing the magnitude of heat dissipation as compared to heterozygous carriers and normal feathered hens. Sharifi et al. (2010) found a significant interaction between feathering genotype (*FF*) and environmental temperature for all reproductive traits (egg production, hatchability, and chick production) except sexual

maturity under heat stress, they observe a sharp reduction in all reproductive traits except sexual maturity for normally feathered hens compared with frizzle-feathered hens. In contrast, under temperate conditions (19°C), egg production and the number of chicks of the *FF* genotype were reduced and sexual maturity was delayed.

The beneficial effect of the F gene as compared to the Na gene is lower in the broilers at high temperatures. However, there are an additive effect in the double heterozygous (Na/Na F/f) broilers (Yunis and Cahaner, 1999)

1.3.2.C Dwarf (dw)

The dwarf gene is a sex-linked recessive gene that is found to reduce the body weight by about 40% and 30% in homozygous males and females, respectively, and lower metabolic heat production in breeders (Daghir, 2008). There has been a discrepancy regarding the advantage of the dw gene in the heat-stressed laying hens. Decuypere et al. (1991) concluded that the inherent heat tolerance of dw genotype in laying hen was uncertain. It has been found that the dw gene in fast-growing broiler chickens under chronic heat stress conditions was unable to improve heat tolerance (Deeb and Cahaner, 2001).

1.3.3 Housing

Naturally ventilated open-type housing is most common in the tropics, which should be oriented in the east-west direction. The width of such a house should not exceed 12 m, while the length of the building can depend upon the convenience. In the case of long buildings, doors should be placed at an interval of 15-30 m. It is recommended to have a side-wall height of at least 2.1 m along with curtails that can be raised or lowered easily. Regarding the roof, a roof slope of 45°C is recommended as it reduces the heat gain of the roof from direct solar radiation. It has been observed that farmers used different local materials such as thatched and bamboo to insulate the

roof. In the case of an uninsulated metal roof, a sprinkling roof with cold water has also been a common practice to reduce heat load in the poultry house. Moreover, in this kind of housing, fans (either suspended from the interior building structures or vertical ceiling fans), interior fogging, and sprinkling systems have been used effectively.

With the advancement of technologies, there has been a surge in the use of a closed house system. Closed housed systems equipped with air conditioning, cooling pads, cool perches, and exhaust fan are found effective in attenuating the negative effect of heat stress in the poultry. However, using this equipped machinery has not been proven to cost-effective for the farmers in the tropics.

1.3.4 Others

In addition to these strategies, some additional strategies have been applied to mitigate heat stress in poultry. In early heat conditioning (EHC), broiler chicks are exposed to high temperature (36°C) for 24 h at 3 to 5 d of age (Lin et al., 2006), while in early feed restriction (EFR) chicks are restricted for about 60% of feed on day 4, 5 and 6 (Lin et al., 2006). Similarly, reducing the stocking density of birds (Park et al., 2018; Goo et al., 2019b), and thinning the litter during the summer seasons are also found to be an effective strategy to ameliorate heat stress in poultry.

1.4 Dried plum and Alpha-lipoic acid

1.4.1 Dried plum

Dried plum (DP), *Prunus domestica L.*, has the highest oxygen radical absorbance capacity (ORAC) score among the 22 most commonly consumed vegetables and fruits (Castaldi and Degen, 2003). They contain a good amount of antioxidant (fat-soluble carotenoids, alpha-tocopherol, etc.), polyphenolic compounds (such as chlorogenic acids, proanthocyanidins, etc.), sorbitol, and fibers. DP is also a good source of several vitamins (vitamin A, C, K1, B1, B2, and niacin), and minerals (Ca, K, Mg, Se, and Zn) (Stacewicz-Sapuntzakis, 2013). The nutritional composition of DP is shown in Table 2. Several studies with the mouse model and postmenopausal women have highlighted the role of DP in the calcium absorption and bone health (Arjmandi et al., 2017). Additionally, DP is found to have many gastrointestinal and cardiovascular benefits, improves immune function, and mitigates neural/cognitive defects (Stacewicz-Sapuntzakis, 2013).

Table 2 Nutritional composition of DP.

	Dried Plums
Serving (g)	100
Energy (kcal)	240
Total carbohydrate (g)	63.88
Total sugars (g)	38.13
Glucose (g)	25.46
Fructose (g)	12.45
Sucrose (g)	0.15
Starch	5.11
Total dietary fiber (g)	7.1
Sorbitol (g)	12
Protein (g)	2.18
Fat (g)	0.38
Moisture (g)	30.92
Ca (mg)	43
K (mg)	732
Fe (mg)	0.93
Mg (mg)	41
P (mg)	69
Cu (mg)	0.281
Mn (mg)	0.299
Se (µg)	0.3
Zn (mg)	0.44
Vitamin A (µg RAE)	39
Beta-carotene (µg)	394
Alpha-carotene (µg)	57
Beta-cryptoxanthin (µg)	93
Lutein + zeaxanthin (µg)	148
Vitamin C (mg)	0.6
Vitamin E (mg a-tocopherol)	0.43
Vitamin K1(µg	59.5
Thiamin (B1) (mg)	0.051
Riboflavin (B2) (mg)	0.186
Niacin (mg)	1.882
Panthotenic acid (mg)	0.422
Vitamin B6 (mg)	0.205
Folate (µg	4.00
Choline (mg)	10.1

(Stacewicz-Sapuntzakis, 2013)

1.4.2 Alpha-lipoic acid

Alpha-lipoic acid (1, 2-dithiolane-3-pentanoic acid; ALA) is a naturally occurring dithiol synthesized from octanoic acid in the mitochondria. They are produced in a small amount inside the cell. It is also known as "a Universal antioxidant" as it possesses all the criteria required from an ideal antioxidant (Packer et al., 1995). It is both water and fat-soluble antioxidants; hence they are readily absorbed from the intestine and can easily cross the blood-brain barrier result in optimum bioavailability. Besides this, both ALA and dihydrolipoic acid (DHLA)- the reduced form of ALA, can quench free radicals both in the liquid and aqueous domains (Sohaib et al., 2017). Moreover, ALA and DHLA also possess metal-chelating activity, acts as a coenzyme in glucose metabolism, and also generate other antioxidants such as ascorbate, vitamin E and glutathione (GST) (Packer et al., 1995; Bilska and Wodek, 2005). The structure of ALA and DHLA is shown in Figure 4.

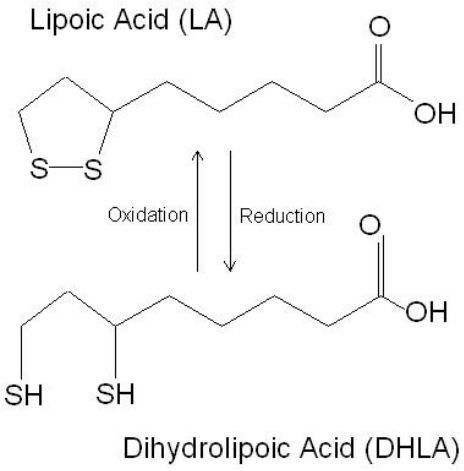


Figure 4 Structure of lipoic acid and dihydrolipoic acid.

Considering these benefits of ALA, there have been several studies of ALA as a potential feed supplement in the feed of poultry, both in normal and oxidative stress conditions. Studies have shown that dietary fortification of ALA was able to improve the growth performance indices, immunological, biochemical characteristics, lipid metabolism, and oxidative stress in poultry. Likewise, the storability of poultry meat and meat product was also found to increase with the fortification of ALA in the feed (Sohaib et al., 2017).

Guo et al. (2014) reported that the inclusion of dietary ALA (500 mg/kg feed) improved feed intake and body weight gain and reduced abdominal fat. Furthermore, they reported that supplementation was able to enhance the activities of superoxide dismutase, catalase, and glutathione peroxidase enzymes and decreased activity of xanthine oxidase activity of liver in the arbor acre chickens.

1.5 Rationale of the study

The human population is growing exponentially across the world. In 2017, the human population was 7.7 billion, and it is predicted to increase to 9.6 billion by 2050. To feed such an enormous population, it will require a 70% increase in the current food production system (FAO, 2009). Similar to an increase in overall food demand, an increased population demands an increase in the production of animal proteins. Poultry meat is the most consumed animal meat throughout the world, and its demand is expected to be doubled in the next decade (Alexandratos and Bruinsma, 2012).

At the same time, our earth is warming at an alarming rate, and the global average temperature will likely increase by 4°C by 2100 A.D. (New et al., 2011). With the growing population and rising global temperature, heat stress in poultry threatens protein food security.

Moreover, Heat stress has been a significant problem in the poultry industry and is estimated to cause an annual economic loss of $128 million to the U.S. poultry industry (St-Pierre et al., 2003). Therefore, to solve this problem, various approaches have been tried in the poultry industry. However, only a few of them were partly effective in mitigating the heat stress in poultry at a nominal cost. Thus, considering the beneficial effects of DP and ALA, this study was designed to examine the potential of these compounds to attenuate heat stress in poultry by supplementing them in the feed and secure relatively cheap source of meat ensuring protein food security.

1.6 Hypothesis

Considering the health benefits and the nutritive value of DP and ALA, we hypothesized that supplementing the DP, and the ALA will help to mitigate heat stress in the poultry.

1.7 Objectives

The objectives of this study were:

- To determine the effects DP supplementation on the growth performance, gut microbiota, gut health, and immune parameters of heat-stressed broiler chickens.
- To determine the effects ALA supplementation on the growth performance, gut microbiota, gut health, and immune parameters of heat-stressed broiler chickens.

CHAPTER 2: MATERIALS AND METHODS

2.1 Effects of dietary dried plum supplementation on heat-stressed broilers

2.1.1 Animals and husbandry practices

All the animal procedures in the experiment were carried out following the approved protocol from the University of Hawaii Institutional Animal Care and Use Committee (Approval no. 17-2605). Day-old Cobb-500 unsexed chicks (n=72) were sourced from a local hatchery, weighed individually, winged tagged, and placed equally and randomly into 24 pens (4 birds/pen), making 6 replicated of each treatment (n=24/treatment). The treatment groups were: 1) No heat stress (NHS), 2) Heat stress with basal diet (HS), and 3) heat stress with a basal diet supplemented with dried plum (HS+DP). Birds were raised on the floor pen system under the standard broiler rearing conditions for the first 21 days. After 21 days, birds in the HS, and HS+DP were exposed to 33-35°C (during the day- 8 am to 6 pm) and at 21-22°C (during the night) with 50% relative humidity for 3 weeks (Figure 5). Birds in the NHS group were reared at the normal room temperature (22°C-24°C) with 50% relative humidity. All birds were provided with *ad libitum* feed and water throughout the experimental period and were monitored twice a day (in the morning and the evening) for health conditions.

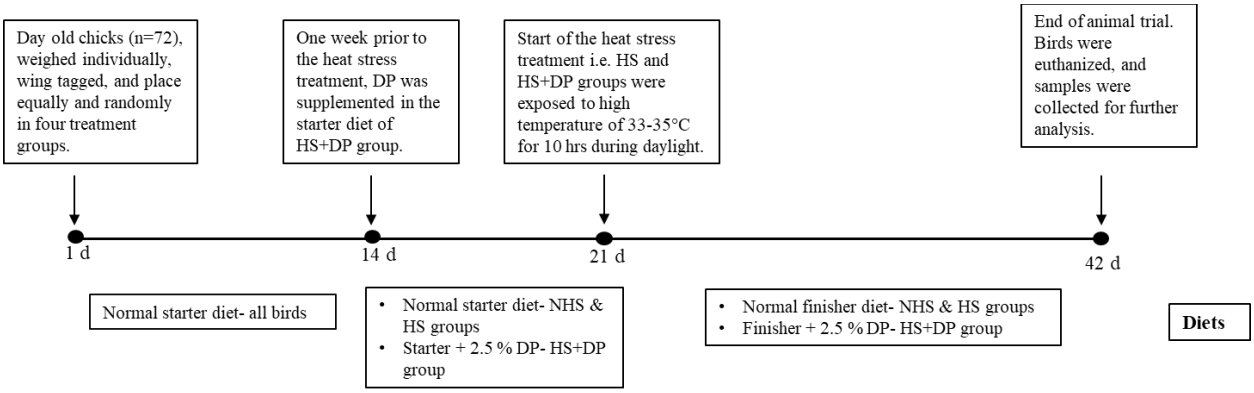


Figure 5 Timeline showing different events carried out for DP study.

2.1.2 Diet

The diets were prepared in two phases: starter (1-21 d) and finisher (22-42 d), to meet the nutrients requirements of broilers (NRC, 1994). All birds were provided with the standard starter feed for the first 14 days, afterward, the inclusion of 2.5% of DP was made in the starter feed of the HS+DP group from 14 to 21 d, while other two groups (HS and NHS) were provided with the normal starter diet. From 22 to 42 d, NHS and HS birds were provided with the normal finisher diet, and the inclusion of 2.5 % DP was made in the finisher diet of the HS+DP group. As DP hasn't been fortified in poultry diet, the dosage of DP (2.5% of total feed) was determined based on a previous study done in post-menopause women (100 gm/day) (Arjmandi et al., 2017). Thus, prepared diets were stored in airtight plastic containers until fed to birds. The diet formulation and their nutrient profiles are presented in Table 3.

Table 3 Ingredients and nutrient composition of the experimental diets for DP study.

	Starter		Finisher	
Ingredients %	normal	with DP	normal	with DP
Corn	54.86	53.36	63.14	61
SBM	39.5	38.5	29.6	29
Dry plum	0	2.5	0	2.5
Soybean oil	2	2	4.5	4.74
Limestone	1.27	1.27	0.85	0.85
Monocalcium phosphate	0.75	0.75	0.5	0.5
Lysine	0.23	0.23	0.18	0.18
Methionine	0.14	0.14	0.12	0.12
Threonine	0.2	0.2	0.16	0.16
NaCl	0.43	0.43	0.35	0.35
Sodium bicarbonate	0.12	0.12	0.1	0.1
Vitamin + Mineral mix*	0.5	0.5	0.5	0.5
Calculated nutrient contents, %				
MEn, kcal/kg	2909	2903	3203	3207
CP	22.09	21.96	18.07	18.08
Ca	0.75	0.75	0.52	0.52
Total P	0.57	0.56	0.47	0.46
digP	0.30	0.30	0.23	0.23
Lysine	1.39	1.36	1.10	1.08
Methionine	0.48	0.47	0.41	0.41
Cystine	0.43	0.41	0.38	0.37
Threonine	1.03	1.01	0.85	0.83
Tryptophan	0.33	0.32	0.26	0.25
Methionine + Cysteine	0.91	0.89	0.8	0.78
Arginine	1.61	1.57	1.31	1.28
Valine	1.22	1.19	1.03	1.00
Isoleucine	0.93	0.91	0.76	0.75
Leucine	1.89	1.84	1.63	1.59
Choline (mg/kg)	1419	1382	1200	1170
dig Lys	1.25	1.22	0.99	0.97
dig Met	0.45	0.45	0.39	0.38
dig Thr	0.85	0.83	0.69	0.68
NDF	9.13	8.89	8.78	8.53
CF	3.97	4.29	3.46	3.8
Na	0.22	0.22	0.18	0.18
Cl	0.30	0.30	0.25	0.25

*Providing the following (per kg of diet): vitamin A (trans-retinyl acetate), 10,000 IU; vitamin D_3 (cholecalciferol), 3,000 IU; vitamin E (all-rac-tocopherol-acetate), 30 mg; vitamin B_1, 2 mg; vitamin B_2, 8 mg; vitamin B_6, 4 mg; vitamin B_{12} (cyanocobalamin), 0.025 mg; vitamin K_3 (bisulphatemenadione complex), 3mg; choline (choline chloride), 250 mg; nicotinic acid, 60 mg; pantothenic acid (D-calcium pantothenate), 15 mg; folic acid, 1.5 mg; betaíne anhydrous, 80 mg; D-biotin, 0.15 mg; zinc (ZnO), 80 mg; manganese (MnO), 70 mg iron ($FeCO_3$), 60 mg; copper ($CuSO_4 \cdot 5H_2O$), 8 mg; iodine (KI), 2 mg; selenium (Na_2SeO_3), 0.2 mg.

2.1.3 Growth performance

Birds were weighed at the start of the trial. Afterward, weekly (7, 14, 21, 28, 35, and 42 d) body weight and feed intake per replicate pen were measured. Based on these data, average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were calculated.

2.1.4 Sample collection

At the end of the trial (42 d), two birds/pen (n=12) from each treatment group were euthanized by carbon dioxide asphyxiation. For gene expression study, small pieces of the ileum (5 cm posterior to the ileocecal transition) were collected (n= 6 per treatment; one from each pen), snap-frozen, and stored at -80°C until RNA extraction. For the microbiota and VFA study, caecum was excised and wrapped separately in aluminum foil, which was snap-frozen at -80°C. For ileum histomorphology, approximately 1 cm of the ileum sample (6 cm posterior to the ileocecal junction) was excised, washed with 0.9% normal saline to remove the digesta and fixed in 10% neutral buffer formalin for 24 hours.

2.1.5 Total RNA extraction

Total RNAs were isolated from the frozen ileum tissues (50-100 mg) using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The process of RNA extraction involved four major procedures: tissue homogenization, phase separation, total RNA precipitation, and RNA wash, and resuspension. Firstly, 100 mg of 1 mm size beads were added

in a sterile Eppendorf tube which was followed by adding 300 µL of TRIzol in that sterile tube. Approximately 100 mg of the ileum tissue was then added in the tube containing TRIzol and beads. Tissue samples were homogenized by using Bullet Blender Cr (Next Advance, Inc., Troy, USA) setting the speed at 8 for 3 minutes. The homogenized tissue was centrifuged at 10,000 rpm for 1 min at 4°C. The supernatant was transferred into a new sterile tube where 700 µL of TRIzol was added and allowed to stand at room temperature for 5 minutes. Then, 0.2 mL of chloroform was added in the sample, shacked vigorously for 15 seconds, and allowed to incubate at room temperature for 5 minutes. The resultant mixture was centrifuged at 10,000 rpm for 15 minutes at 4°C, which result in separation of the homogenate into three distinct layers: an upper aqueous layer containing the RNA, the bottom organic phase containing protein and the middle interface with DNA. Avoiding the interphase, the upper clear liquid was pipetted and transferred into a new Eppendorf tube. Then, 0.5 mL of isopropanol was added, mixed, incubated for 5 minutes, and finally centrifuged at 10,000 rpm for 10 minutes at 4°C for precipitating the total RNA. The RNA pellet was washed by adding 1 mL of 75% ethanol after removing the supernatant which was then centrifuged at 14,000 rpm at 4°C for 5 minutes. The supernatant ethanol was then removed and allowed to air dry by inverting on the clean Kim wipe for 10 minutes. The pellet was resuspended in nuclease-free water (Thermo Scientific, Waltham, MA) and incubated on a heating plate at 60°C for 10 minutes.

The concentration of total RNA was determined by using NanoDrop one (Thermo Fisher Scientific, Madison, WI). The quality of RNA was determined by running samples on 2 % agarose gel. RNA samples were stored at -80°C until further analysis.

2.1.6 Complementary DNA synthesis (cDNA)

The synthesis of cDNA was carried out by using 1 µg of total RNA (20 µl reaction of reverse transcriptase mixture) using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Firstly, RNA samples were diluted by using nuclease-free water to prepare a concentration of 1 µg per 10 µL. Then, 10 µL reverse transcriptase (RT) master mix was prepared on ice by using 2 µL of 10X RT Buffer, 0.8 µL of 25X dNTP (100nM), 2 µL of 10X RT Random Primer, and 1 µL of Multiscribe RTase enzyme (Applied Biosystems, Foster City, CA). Afterward, 10 µL of RNA sample and 10 µL of RT master mix were added and mixed properly in a 200 µL PCR tube. Thus, prepared samples were kept on the ice until it was loaded on the thermocycler. Finally, samples were loaded in the thermocycler by setting the following run condition: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and 4°C for infinity. The newly synthesized cDNA (20 µL) was diluted (25X) with 480 µL of nuclease-free water. Finally, cDNA was stored at -20°C until quantitative real-time PCR (qPCR) assay.

2.1.7 Quantitative real-time PCR (qPCR) assay

StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA) was used to carry out the qPCR assay where 10 µL reaction mixture containing 3 µL of cDNA and 7 µL of PCR mix was used. Firstly, the PCR mix was prepared by adding 5 µL of PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and 1 µL each of forward and reverse primers (Table 4) specific to the gene target. Specific primer pairs for the detection of each gene were designed using the NCBI Primer-Blast tool. The PCR mix (7 µL) was then loaded in the 96-well PCR plate; afterward, 3 µL of cDNA was loaded, mixed, and the plate was sealed with clear optical adhesive films (Applied Biosystems, Foster City, CA). The PCR plate was then inserted onto the StepOne Plus machine. The amplification conditions were 50°C for 2 minutes (hold), 95°C for 2

minutes (hold), followed by 40 repeat cycles of 95°C for 15 seconds (denaturation), 60°C for 15 seconds (annealing), and 72°C for 1 minute (extension). A melting curve was also generated to confirm the sequence-specific products or any primer dimmers. Further, the specificity of the qPCR product was confirmed using 2% gel electrophoresis.

To determine the most stable housing keeping genes in the ileal tissues, expression of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Beta-actin (β-actin), and TATA-Box Binding Protein (TBP) were analyzed on triplicate. Further, their expression in the ileum tissue was compared across the treatment. β-actin was found to be the most stable in the ileum and was used to normalize the target gens. Afterward, target genes were analyzed in duplicates, and an average value was taken for each experimental replicate. The expression level was determined using the cycle threshold (Ct) values following the standard curve method after normalization with B-actin. The fold change for each gene was calculated by the $2^{-\Delta\Delta Ct}$ method. To determine the fold change, firstly the average of Ct values of a housekeeping gene (B-actin) and the target genes in the experimental and control conditions were used i.e., Tested Experimental (TE), Tested Control (TC), Housekeeping Gene Experimental (HE), and Housekeeping Gene Control (HC). Afterward, the differences in the experimental (Δ CtE =TE-HE) and the control condition (Δ CtC = TC-HC) were determined.

Finally, the difference between ΔCTE and ΔCTC (i.e., ΔCTE- ΔCTC) was calculated to get the Double Delta Ct value (Δ ΔCt). Thus, the obtained fold change value was presented as a mean ± standard error on the bar diagram.

Table 4 Primers used to quantify the expression of the genes by qPCR.

Gene	Accession no.	Primer Sequence	Amplicon (bp)
SOD1	NM_205064.1	F: CAACACAAATGGGTGTACCA	119
		R: CTCCCTTTGCAGTCACATTG	
SOD2	NM_204211.1	F: CCTTCGCAAACTTCAAGGAG	160
		R: AGCAATGGAATGAGACCTGT	
GPX1	NM_001277853.2	F: AATTCGGGCACCAGGAGAA	101
		R: CTCGAACATGGTGAAGTTGG	
GPX3	NM_001163232.2	F: GAGGGAGAAGGTGAAATGCT	192
		R: CCCAGCTCATTTTGTAGTGC	
TXN	NM_205453.1	F: GGCAATCTGGCTGATTTTGA	79
		R: ACCATGTGGCAGAGAAATCA	
PRDX1	NM_001271932.1	F: GGTATTGCATACAGGGGTCT	101
		R: AGGGTCTCATCAACAGAACG	
NFE2L2	NM_205117.1	F: CCCTGCCCTTAGAGATTAGAC	248
		R:CAAGTTCATGTCCTTTTCTCTGC	
HSF1	NM_001305256.1	F: AAGGAGGTGCTCCCAAAGTA	221
		R: TTCTTTATGCTGGACACGCTG	
HSF3	NM_001305041.1	F: TTCAGCGATGTGTTTAACCCT	244
		R: GGAGGTCTTTTGGATCCTCT	
HSP90AA1	NM_001109785.1	F: GATAACGGTGAACCTTTGGG	120
		R: GGGTAGCCAATGAACTGAGA	
HSP70	NM_001006685.1	F: TCTCATCAAGCGTAACACCAC	104
		R: TCTCACCTTCATACACCTGGAC	
OCLN	NM_205128	F: CCGAGGACAGCCCTCAATAC	82
		R: CTTTGGTAGTCTGGGCTCCG	
CLDN1	NM_001013611	F: TACCCCAAAAATGCCCCCTC	109
		R: GCGGCATTGTAGTGTCCTCT	
MUN2	NM_001318434	F: GTGGTCTGTGTGGCAACTT	71
		R: GTCTCTTGCAGCCCATTCCT	
IL4	NM_001030693	F: TGTGCCCACGCTGTGCTTACA	155
		R: CTTGTGGCAGTGCTGGCTCTCC	

*F=Forward; R= Reverse

2.1.8 Ileum histomorphology

At the end of the trial, one chicken was randomly selected from each pen (n=6/ treatment) and ileum (approximately 1 cm) sample, mid-way between the Meckel's diverticulum and ileocecal junction, was excised which was then flushed with 0.9% normal saline to clear the intestinal digesta and fixed overnight in 10 % neutral buffer formalin. Samples were then transferred in 70% ethanol, dehydrated by different concentrations of alcohol (70%, 80%, 95%, and 100%), and was finally embedded in paraffin blocks for histological sectioning (Histology core facility, John A. Burns School of Medicine, UH Manoa). The embedded ileum tissue was then sectioned at 6 µm thickness and stained with hematoxylin and eosin staining. A total of 6 intact, well oriented villus-crypt units were selected in triplicate (18 measurements for each sample). Sections were observed under an 8X objective lens, and images were taken using an Olympus microscope (U-TV0.63XC, Tokyo, Japan). Intestinal morphological parameters such as villus height (VH)- distance from the tip of villus to the crypt, crypt depth (CD)- distance from villus base to the submucosa, and the ratio of villus height to crypt depth (VH/CD) were measured along with the apparent villus surface area by using Infinite Analyze software (Lumenera Corporation, Ottawa, ON, CA).

2.1.9 Volatile fatty acids (VFAs)

For VFAs analysis, cecal content samples (two chickens per pen, n=12 per treatment) were collected, wrapped in aluminum foil, and immediately preserved at -80°C until further analysis. During analysis, samples were thawed, 200 mg of the cecal content was weighted in the Eppendorf tube and diluted by adding 200 µl of distilled water. Then, 100 µL of TMA (Trimethyl acetic acid, as internal standard), and 200 µl of 25% metaphosphoric acid was added. Finally, 800 µL of distilled water was added in the tube to make 1500 µl of total volume. The tube was then vortexed

to homogenize the sample and centrifuged at 12,000× g for 15 minutes at 4°C. Afterward, 500 µL of the supernatant was transferred in the GC vial and VFA was analyzed using a gas chromatograph (TRACE 1300 gas chromatograph; Thermo Scientific, Waltham, MA) coupled with a 30 m × 0.53 mm internal diameter column (Teknokroma TRB-FFAP, Teknokroma, Barcelona, Spain) and flame ionization detector. The injector-port and flame ionization detector temperatures were fixed at 230°C and 250°C, respectively. In the temperature program, the initial temperature was held at 120°C for 4 min after injection and then increased at 4°C/min to 160°C, where it was held for 4 min. Helium was used as a carrier gas. The injection volume was set at 0.5 µL and analyses were performed. The run time for each analysis was set for 15 min. An aqueous stock standard solution was prepared with different concentrations of 0, 0.5, 1, 2, 4, 6, and 8 mM with a final volume of 1500 µL. All the stock standard solutions were stored at −20°C until used. The molar concentration of each VFA was calculated using methyl valeric acid, and the molar percentage of an individual VFA was calculated by dividing the micromolar (µM) concentration of the individual VFA by the µM sum of all the VFA multiplied by 100. Individual VFA concentrations were calculated as a percentage of the total VFA content to determine the treatment effect if any.

2.1.10 DNA extraction

DNA was extracted from the cecal content by using the QIAamp® DNA Stool Mini Kit (Cat. no. 51604, QIAGEN, Hilden, Germany) following the manufacturer's instruction. Firstly, 200 mg of the cecal content was weight in the 2 mL microcentrifuge tube and was placed on the ice. Then, 1 mL of InhibinEX buffer was added and homogenized. Bacteria in the homogenized cecal contents were lysed by placing a microcentrifuge tube in the water bath having a temperature of 70°C for 7 minutes. The sample was then centrifuged at 15,000 g for 6 minutes to pellet stool particles. In the new 1.5 mL microcentrifuge tube, 15 µL of proteinase K, 200 µl of supernatant,

and 200 µL of Buffer AL were added, mixed through, and incubated at 70°C for 10 minutes. Afterward, 200 µL of ethanol (100 %) was added to the lysate and mixed thoroughly. Then 600 µL of this lysate was applied to the QIAamp spin column which was centrifuged (15,000 g for 3 minutes), and the tube containing filtrate was discarded. The QIAamp spin column was then placed in the new 2 mL collection tube, 500 µL of Buffer AW1 was added, the spin column was centrifuged (15,000 g for 3 minutes), and the filtrate was discarded. Again, the QIAamp spin column was placed in the new 2 mL collection tube, 500 µl of Buffer AW2 was added, the spin column was centrifuged (15,000 g for 3 minutes), and the filtrate was discarded. Finally, the QIAamp spin column was transferred into a new, labeled 1.5 mL microcentrifuge tube, and the DNA was eluted by adding 100 µL Buffer ATE directly onto the QIAamp membrane, followed by incubation at room temperature for 1 minute, and centrifuging at 15,000 g for 1 minute. The concentration of the eluted DNA was determined by using NanoPhotometer®P330 (IMPLEN, Los Angeles, CA). Thus, obtained DNA was preserved at -20°C until used.

2.1.11 16S rRNA gene sequencing

The V3 and V4 hypervariable regions of the 16S rRNA gene were considered in this study. The 16S rRNA gene sequencing involved different steps: 1) 1st stage PCR: Amplicon PCR, 2) PCR Clean-Up, 3) 2nd Stage PCR: Index PCR, 4) 2nd PCR Clean-Up 5) Library quantification, normalization, and pooling and 6) Library denaturing and MiSeq sample loading. Amplicon PCR was carried out using Platinum® Taq DNA Polymerase High-Fidelity (Invitrogen, Life Technologies Corporation, Grand Island, NY). The specific sequence of 16S rRNA gene was amplified from genomic DNA using forward primer (5′-CCTACGGGNGGCWGCAG-3′) and reverse primer (5′-GACTACHVGGGTATCTAATCC- 3′) according to Illumina MiSeq protocol (Klindworth et al., 2013). The adapter overhang nucleotide was added to the gene-specific

sequences. The overhang forward and reverse adapter sequences that were added to 5′ end of the locus-specific primers (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′) and (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′), respectively. The amplicon PCR cycle consisted of initial denaturation step of heating PCR plate at 95°C on a thermal cycler for 3 minutes followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and an extension step at 72°C for 5 minutes, which was then followed by a hold at 4°C. The PCR amplicon was run on 1% agarose gel to ascertain the size of the amplified product to be around 550 bp. The 16S V3 and V4 amplicon were then purified to remove the primers and primer-dimer species; for that Illumina 16S rRNA PCR clean-up, the protocol was followed. All procedures were followed following the protocol except Mag-Bind Total Pure NGS beads (Omega Bio-Tek) were used instead of AMPure XP beads and 70% ethanol instead of 80% ethanol. After the clean-up of the amplicon PCR product, 2nd stage PCR (index PCR) was performed for multiplexing by attaching Nextera XT dual indices and Illumina sequencing adapters to the target amplicon. The PCR conditions used for index PCR were 95°C for 3 minutes; 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds; followed by an extension at 72°C for 5 minutes and a final hold at 4°C. The cleaning of the index PCR product was carried similarly as done in the previous clean-up step. After PCR clean-up, the libraries were quantified using the Quant-iT PicoGreen dsDNA Assay Kit, normalized, and pooled. The library pools (0.7 ng) were run on a Bioanalyzer High Sensitivity DNA chip to verify the size of the final library to be around 630 bp. The normalized and pooled amplicons were sequenced on the Illumina MiSeq desktop sequencer (2×300 bp paired-end run) at the University of Hawaii at Manoa Advanced Studies in Genomics, Proteomics, and Bioinformatics core facility.

2.1.12 Microbial bioinformatics analysis

Microbial bioinformatics analysis was done by CLC Genomics Workbench 12.0.1 and the CLC Microbial Genomics module. The procedures for sequencing analysis were followed as described in the OTU clustering step by step tutorial. Firstly, the sequencing files (ending with 'fastq') were imported in the CLC workbench, files were then paired setting the minimum distance to 200 and maximum distance to 500. The reads were then trimmed, and the read with the low coverage was removed from the analysis. For filtering the reads with lower coverages, 100 and 50 were set as the minimum number of reads and 5 minimum percent from the median, respectively. The samples that do not fulfill these requirements were discarded from further analysis. Thus, obtained reads were then clustered as operational taxonomical units (OTUs), based on 97% sequence similarity against the Greengenes v13_8 97% database using the CLC Microbial Genomics module. For alpha and beta diversity analysis, the phylogenetic tree was constructed using a maximum likelihood approach based on multiple sequence alignment (MSA) of the OTU sequences generated by MUSCLE in the workbench. The alpha diversity was estimated by calculating Chao 1, Simpson's index, and Shanon entropy then visualized with a boxplot. Beta-diversity was estimated by calculating Bray-Curtis, unweighted UniFrac, and weighted UniFrac distances and was visualized with principal coordinate analysis. Permutational multivariate analysis of variance (PERMANOVA) analysis was carried out to measure the significance of beta diversity. Differentially abundant taxa (family, class, genus, etc.) were identified by using one-way ANOVA, and mean between the treatment groups was done by using Tuckey's test in R-studio.

2.1.13 Statistical analysis

The data were analyzed using the SAS V9.3 (SAS Inc., Cary, NC) and multiple mean comparisons between different treatment groups were carried out by Fisher's Least significant difference (LSD) test function after performing a one-way analysis of variance (ANOVA). Kruskal- Wallis pairwise test and pairwise PERMANOVA test was carried out to determine significant differences between treatment groups by using the CLC Microbial Genomics module for alpha and beta diversity, respectively. Statistical significance was set at $p<0.05$.

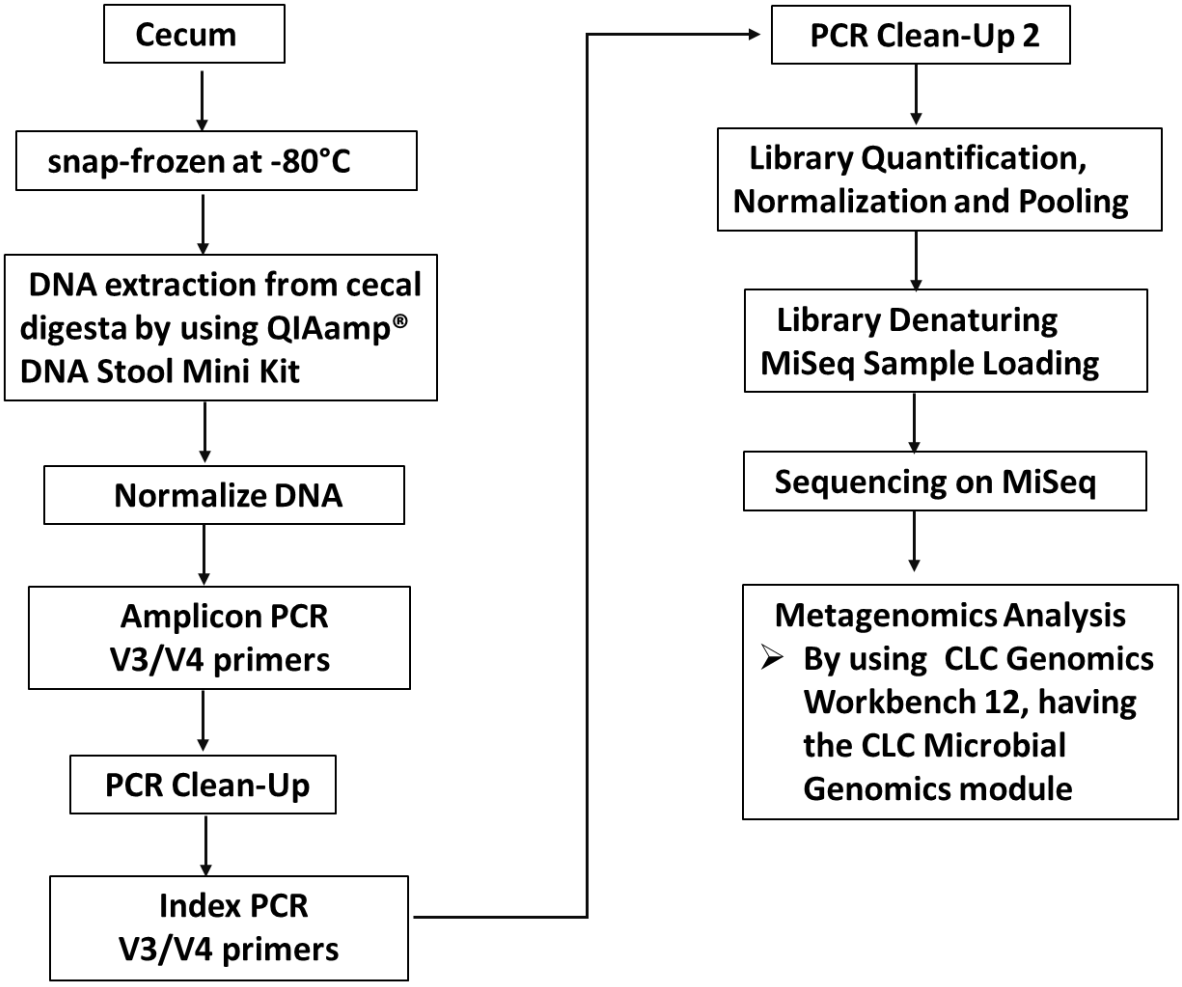


Figure 6 General overview of the workflow for 16S rRNA gene-based metagenomic analysis of cecal microbiota.

2.2 Effects of dietary alpha-lipoic acid supplementation on heat-stressed broilers

2.2.1 Animals and husbandry practices

All the animal procedures in the experiment were carried out following the approved protocol from the University of Hawaii Institutional Animal Care and Use Committee (Approval no. 17-2605). For this study, control and heat-stressed birds were utilized from the DP study (2.1). Briefly, Day-old Cobb-500 unsexed chicks (n=72) were sourced from a local hatchery, weighed individually, winged tagged, and placed equally and randomly into 24 pens (4 birds/pen), making 6 replicated of each treatment (n=24/treatment). The treatment groups were: 1) No heat stress (NHS), 2) Heat stress with basal diet (HS), and 3) heat stress with alpha-lipoic acid (HS+ALA). Birds were raised on the floor pen system under the standard broiler rearing conditions for the first 21 days. After 21 days, birds in the HS, and HS+ALA were exposed to 33-35°C (during the day-8 am to 6 pm) and at 21-22°C (during the night) with 50% relative humidity for 3 weeks. Birds in the NHS group were reared at the normal room temperature (22°C-24°C) with 50% relative humidity. All birds were provided with *ad libitum* feed and water throughout the experimental period and were monitored twice a day (in the morning and the evening) for health conditions.

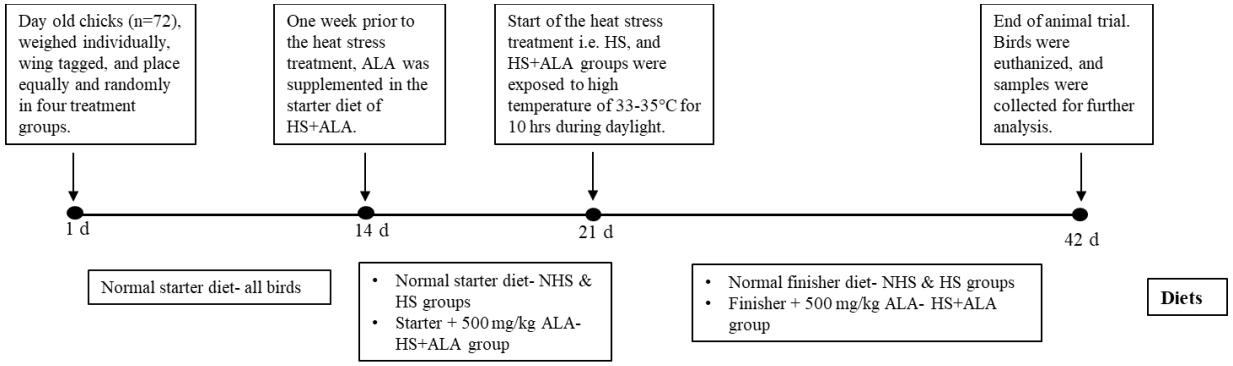


Figure 7 Timeline showing different events carried out for ALA study.

2.2.2 Diet

The diets were prepared in two phases: starter (1-21 d) and finisher (22-42 d), to meet the nutrients requirements of broilers (NRC, 1994). All birds were provided with the standard starter feed for the first 14 days. Afterward, 500 mg/kg ALA was supplemented on the starter feed of the HS+ALA group from 14 to 21 d, while the other two groups (HS and NHS) were provided with the standard starter diet. From 22 to 42 d, NHS and HS birds were provided with the normal finisher diets, and supplementation of 500 mg/kg ALA was made on the finisher diet of the HS+ALA group. The dosage rate of ALA was considered as 500 mg/kg feed based on the study carried out by Guo et al. (2014) in broiler birds. The diet formulation and their nutrient profiles are presented in Table 5.

Table 5 Ingredients and nutrient composition of the experimental diets for ALA study.

	Starter		Finisher	
Ingredients %	normal	with ALA	normal	with ALA
Corn	54.86	54.86	63.14	63.14
SBM	39.5	39.5	29.6	29.6
Soybean oil	2	2	4.5	4.5
Limestone	1.27	1.27	0.85	0.85
Monocalcium phosphate	0.75	0.75	0.5	0.5
Lysine	0.23	0.23	0.18	0.18
Methionine	0.14	0.14	0.12	0.12
Threonine	0.2	0.2	0.16	0.16
NaCl	0.43	0.43	0.35	0.35
Sodium bicarbonate	0.12	0.12	0.1	0.1
Vitamin + Mineral mix*	0.5	0.5	0.5	0.5
ALA, mg/kg (top dressing)	0	500	0	500
Calculated nutrient contents, %				
MEn, kcal/kg	2909	2909	3203	3203
CP	22.09	22.09	18.07	18.07
Ca	0.75	0.75	0.52	0.52
Total P	0.57	0.57	0.47	0.47
digP	0.3	0.3	0.23	0.23
Lysine	1.39	1.39	1.10	1.10
Methionine	0.48	0.48	0.41	0.41
Cystine	0.43	0.43	0.38	0.38
Threonine	1.03	1.03	0.85	0.85
Tryptophan	0.33	0.33	0.26	0.26
Methionine + Cysteine	0.91	0.91	0.8	0.8
Arginine	1.61	1.61	1.31	1.31
Valine	1.22	1.22	1.03	1.03
Isoleucine	0.93	0.93	0.76	0.76
Leucine	1.89	1.89	1.63	1.63
dig Lys	1.25	1.25	0.99	0.99
dig Met	0.45	0.45	0.39	0.39
dig Thr	0.85	0.85	0.69	0.69
NDF	9.13	9.13	8.78	8.78
CF	3.97	3.97	3.46	3.46
Na	0.22	0.22	0.18	0.18
Cl	0.30	0.30	0.25	0.25
Choline (mg/kg)	1419	1419	1200	1200

*Providing the following (per kg of diet): vitamin A (trans-retinyl acetate), 10,000 IU; vitamin D_3 (cholecalciferol), 3,000 IU; vitamin E (all-rac-tocopherol-acetate), 30 mg; vitamin B_1, 2 mg; vitamin B_2, 8 mg; vitamin B_6, 4 mg; vitamin B_{12} (cyanocobalamin), 0.025 mg; vitamin K_3 (bisulphatemenadione complex), 3mg; choline (choline chloride), 250 mg; nicotinic acid, 60 mg; pantothenic acid (D-calcium pantothenate), 15 mg; folic acid, 1.5 mg; betaíne anhydrous, 80 mg; D-biotin, 0.15 mg; zinc (ZnO), 80 mg; manganese (MnO), 70 mg iron ($FeCO_3$), 60 mg; copper ($CuSO_4 \cdot 5H_2O$), 8 mg; iodine (KI), 2 mg; selenium (Na_2SeO_3), 0.2 mg.

2.2.3 Analysis

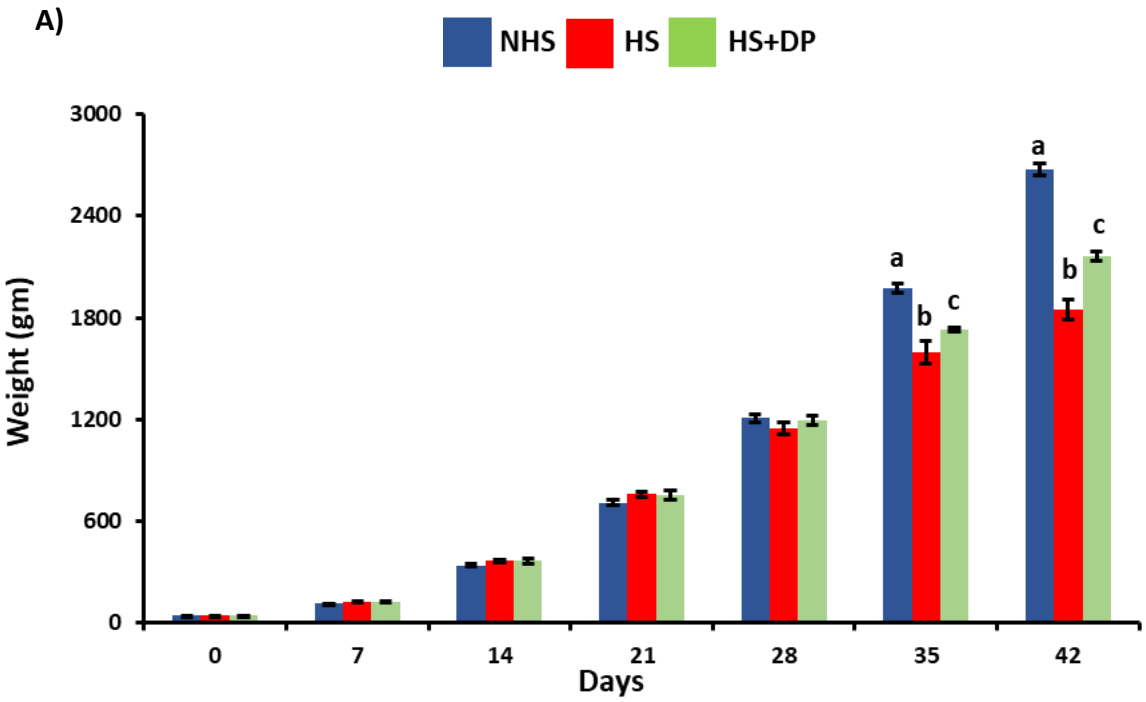
All other experimental procedures: sampling, RNA and DNA extraction, cDNA synthesis, qPCR assay, ileum histomorphology, VFAs analysis, 16S rRNA sequencing, microbial analysis, and statistical analysis were carried out similarly to DP study.

CHAPTER 3: RESULTS

3.1 Effects of dietary supplementation of dried plum on heat-stressed broilers

3.1.1 Growth performance

The effect of DP supplementation on the growth performance of heat-stressed broilers is shown in Figure 8. Supplementation of the dried plum significantly improved the body weight in heat-stressed broilers. There was no significant difference between the treatment groups on body weight until 28 days. However, from 35 d onwards, body weight was significantly decreased in the broilers under the heat stress as compared to the broilers without heat stress. At the same time, supplementation of the 2.5% DP significantly improved the body weight in the heat-stressed broiler birds. The ADG was significantly decreased in the heat-stressed broiler birds from 21-28 days onwards, and DP supplementation significantly improved the ADG from 28 days onwards. The ADFI was also significantly lowered in the heat-stressed birds from 21days onwards, whereas DP significantly improved the ADFI from 28 days onward in heat-stressed birds. During the heat stress period (21-42 d), FCR was significantly higher in the HS group, while supplementation of the DP significantly lowered FCR in the heat stress broiler birds.


A)
NHS
HS
HS+DP
Weight (gm)
3000
2400
1800
1200
600
0
0
7
14
21
28
35
42
Days
a
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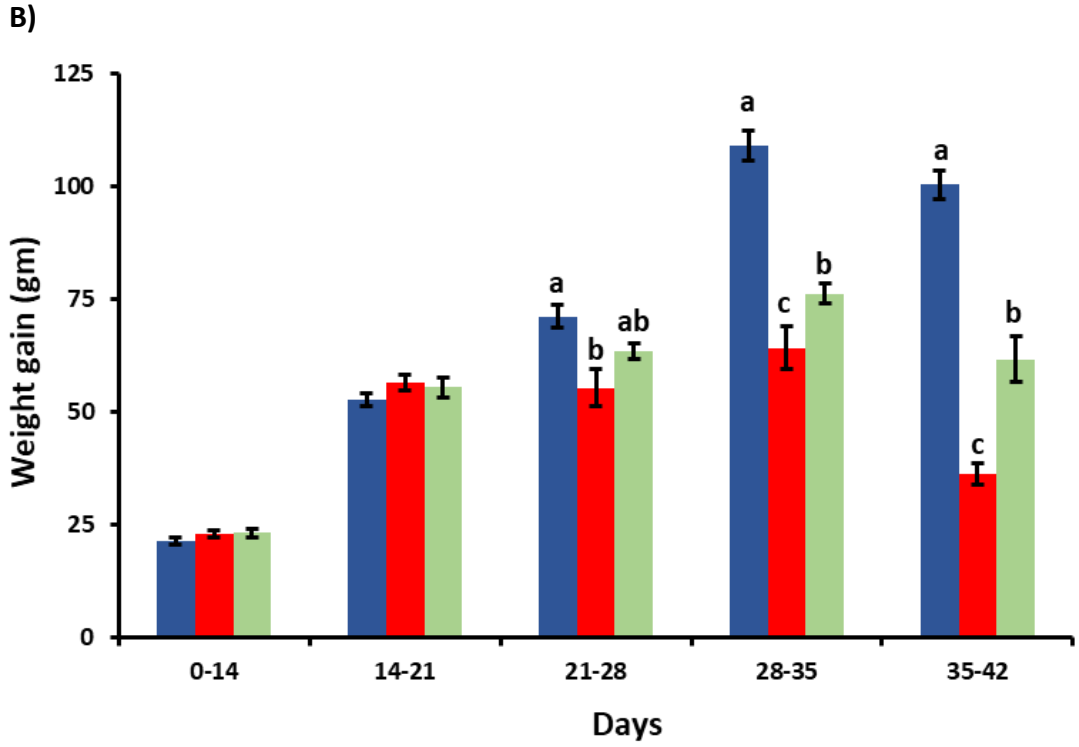
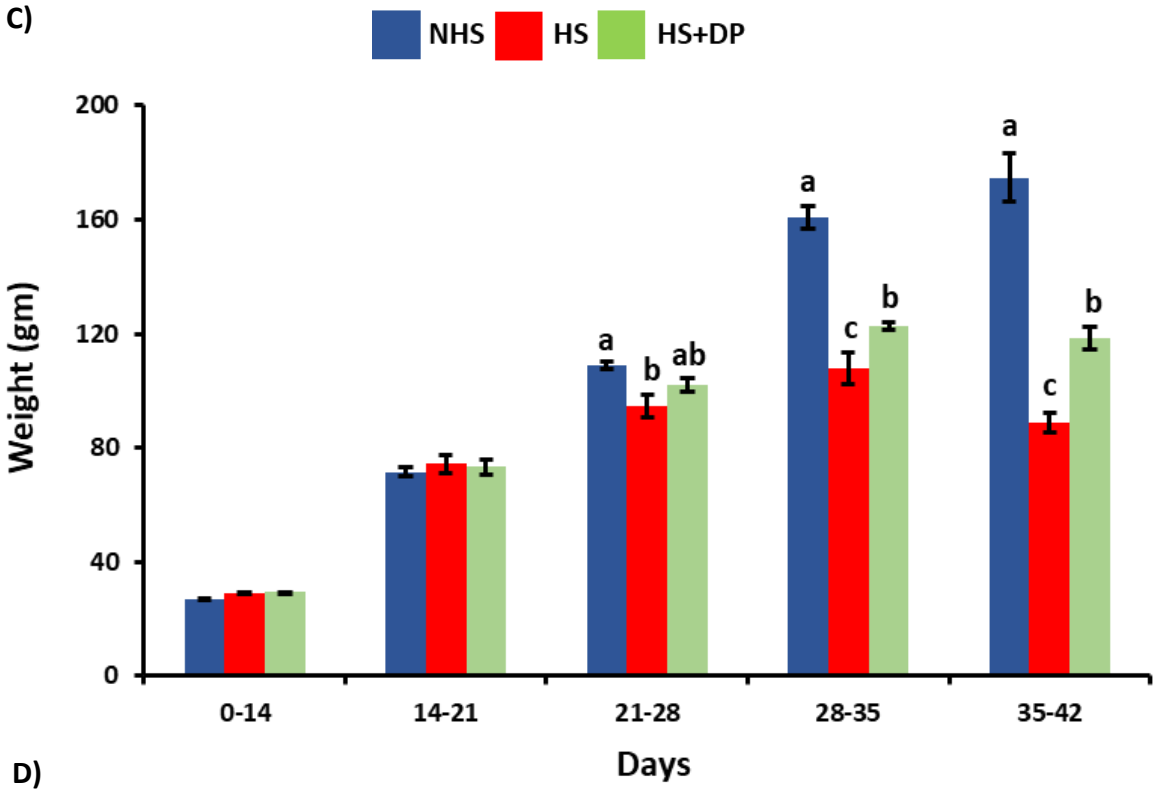

B)
Weight gain (gm)
125
100
75
50
25
0
0-14
14-21
21-28
28-35
35-42
Days
a
b
ab
a
c
b
a
c
b



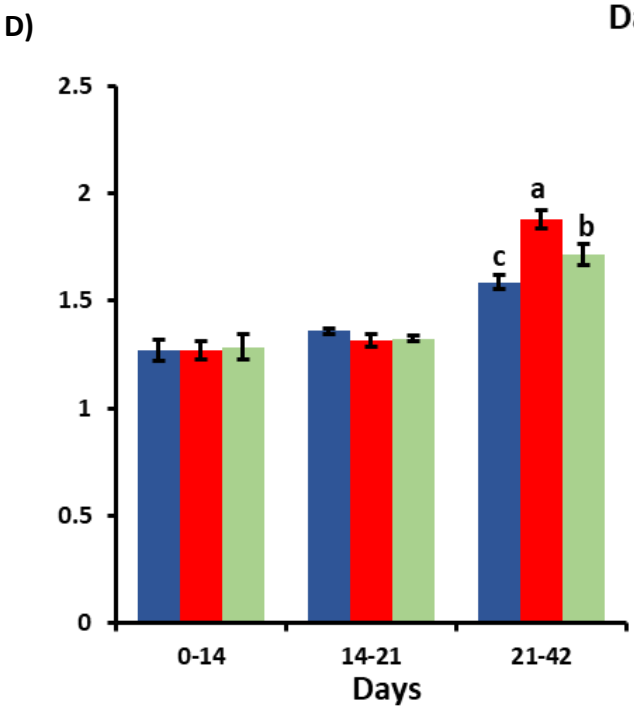


Figure 8 Effects of DP on the growth performance of heat-stressed broilers. A) Bodyweight, B) ADG, C) ADFI, and D) FCR. Data presented as the mean± SEM. The effect of treatment was statistically different at * $p<0.05$ for body weight, ADG and ADFI; and at * $p<0$.

3.1.2 Effects of dried plum on the intestinal gene expression

3.1.2.A Expression of heat shock protein-related genes

The effect of DP supplementation on the mRNA expression of the heat shock-related genes (*HSF1*, *HSF3*, *HSP70*, and *HSP90*) in heat-stressed broiler birds is summarized in Figure 9. There were no significant differences in the expression of the *HSF1* and *HSF3* between the NHS and HS groups. However, the expressions of the *HSF1* and *HSF3* mRNA were significantly increased in the heat-stressed birds supplemented with the DP compared to the HS group.

The mRNA expression of the *HSP90* was significantly decreased in the HS group as compared to the NHS group, and DP supplementation was able to significantly improve the expression of the *HSP90* in heat-stressed birds. Unlike the *HSP90*, the expression of *HSP70* was not affected by heat stress, but the mRNA expression of the *HSP70* was significantly increased in the heat-stressed broiler birds supplemented with the DP.

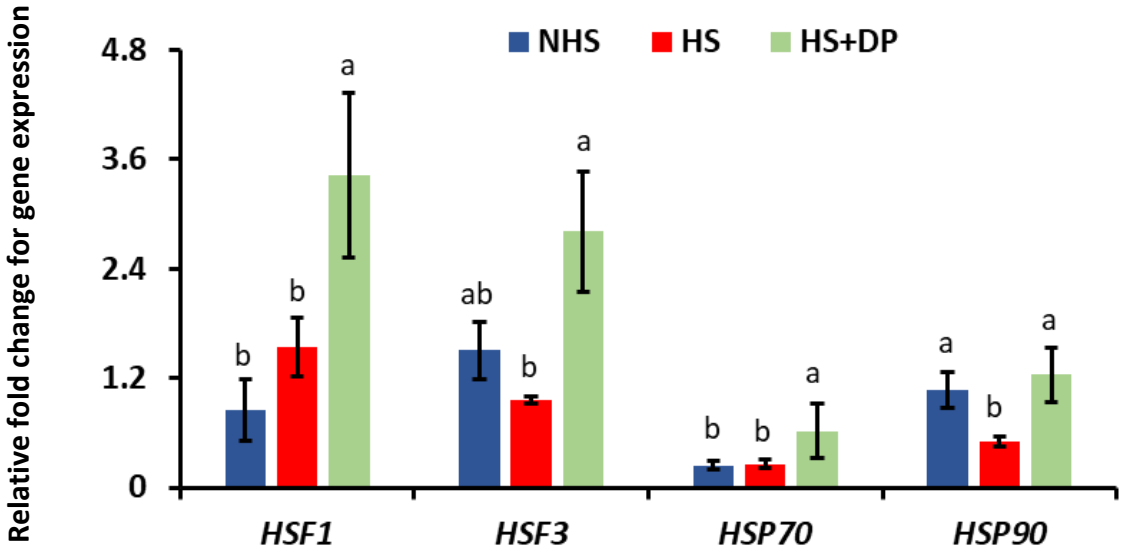


Figure 9 Effects of DP supplementation on the expression of heat shock protein-related genes.

3.1.2.B Expression of antioxidant related genes

The expression profile of the antioxidant genes analyzed during the study is shown in Figure 10. The dietary supplementation of DP significantly increased the mRNA expression of the *SOD1*, *SOD2*, *GPX1*, *GPX3*, *PRDX1*, *TXN*, and NRF2 in the heat-stressed broilers birds as compared to the HS group. The expression of *SOD2*, *GPX3*, and *NRF2* mRNA remained unchanged between the NHS group and the HS group.

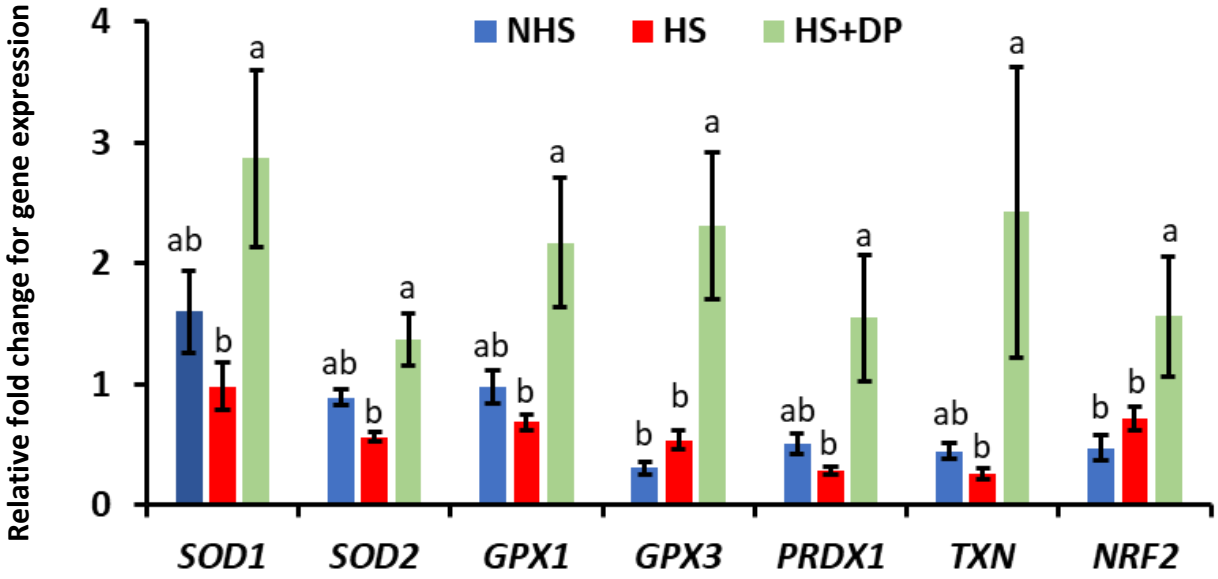


Figure 10 Effects of DP supplementation on the expression of antioxidants related genes.

3.1.2.C Expression of tight-junction related genes

The effect of DP supplementation on the mRNA expression of the tight-junction related genes (*OCLN* and *CLDN1*) is shown in Figure 11. The expression of *OCLN* and *CLDN1* was significantly higher in the HS birds supplemented with DP. There was no significant difference in the expression of these genes between the NHS and HS group; however, the expression of these genes was numerically lower in the HS group as compared to the NHS group.

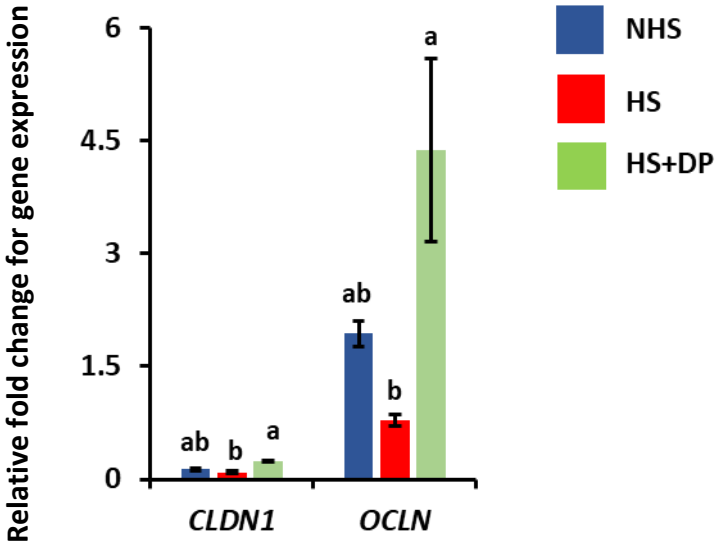


Figure 11 Effects of DP on the expression of tight-junction related genes.

3.1.2.D Expression of immune-related genes

The effect of DP supplementation on the expression of immune-related genes (*IL4* and *MUC2*) in heat-stressed broiler birds is shown in Figure 12. The mRNA expression of the *IL4* was significantly decreased in the HS group as compared to the NHS group, while DP supplementation significantly increased the expression of *IL4* in heat-stressed birds. Supplementing the DP in the heat-stressed broilers birds significantly improves the mRNA expression of the *MUC2* as compared to the HS groups. Although the mRNA expression of the *MUC2* was lowered in the HS group as compared to the NHS group, there was no significant difference in the expression of this gene in between these two groups.

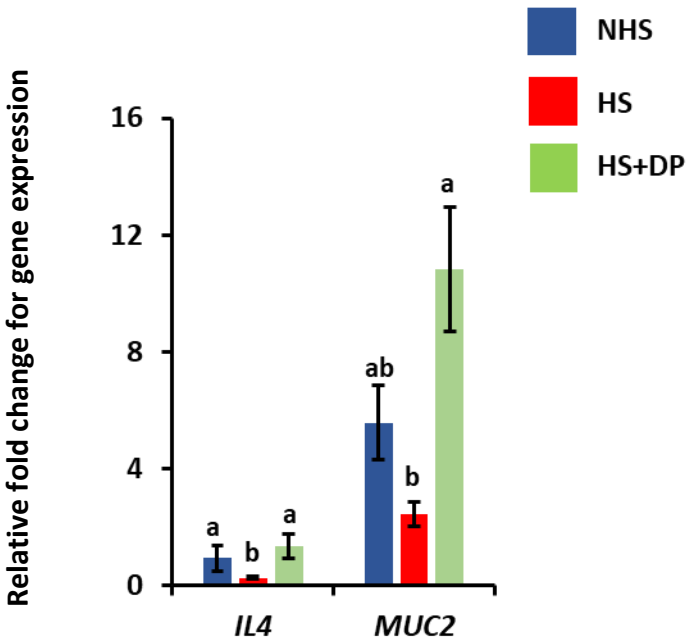


Figure 12 Effects of DP supplementation on the expression of immune-related genes.

3.1.3 Ileum histomorphology

The effect of heat stress and DP supplementation on gut histomorphology is shown in Figure 13. The villus height and villus height to crypt depth ratio were significantly lower in the HS group as compared NHS group while supplementing DP in heat-stressed broiler birds were found to improve these parameters. Villus surface area was also found to be significantly lower in the heat-stressed birds. However, dietary supplementation of DP could not improve the surface area in heat-stressed broiler birds. No significant difference was observed for the crypt depth.

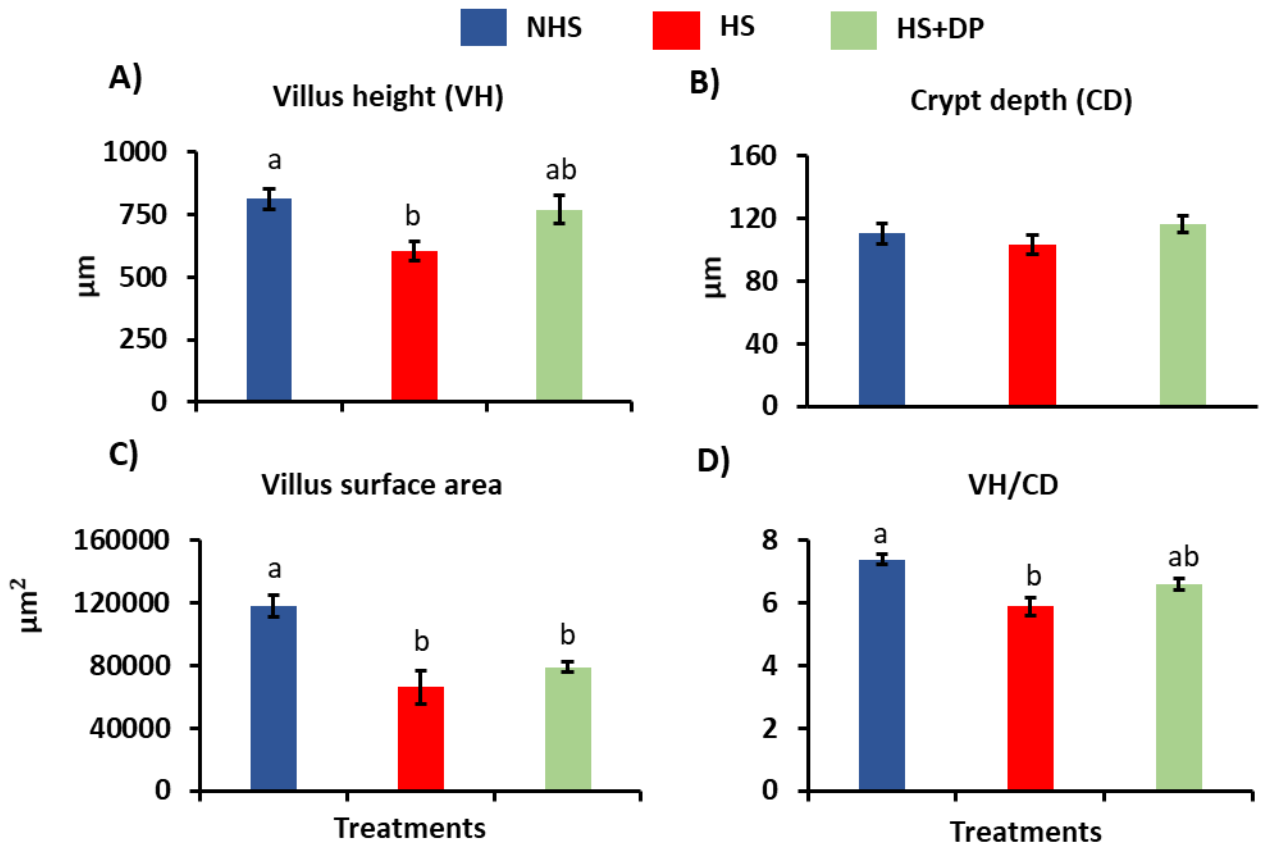


Figure 13 Effects of DP supplementation on the ileum histomorphology of the heat-stressed broilers. A) Villus height (VH), B) Crypt depth (CD), C) Villus surface area, D) Villus height (VH): Crypt depth (CD). The effect of treatment was statistically different at * $p<0.05$.

3.1.4 Volatile fatty acids

The effect of DP supplementation on the major volatile fatty acids in the cecal digesta of the heat-stressed broiler birds is shown in Figure 14. Propionate was significantly decreased in the cecal digesta of HS groups as compared to the NHS groups. Dietary supplementation of the DP significantly increased propionate and acetate in the heat-stressed birds as compared to the HS group. There was no significant difference in the concentration of the butyrate between treatment groups. Overall, total volatile fatty acids were significantly higher in the DP supplementation groups (HS+DP), while it was significantly lower in the HS group.

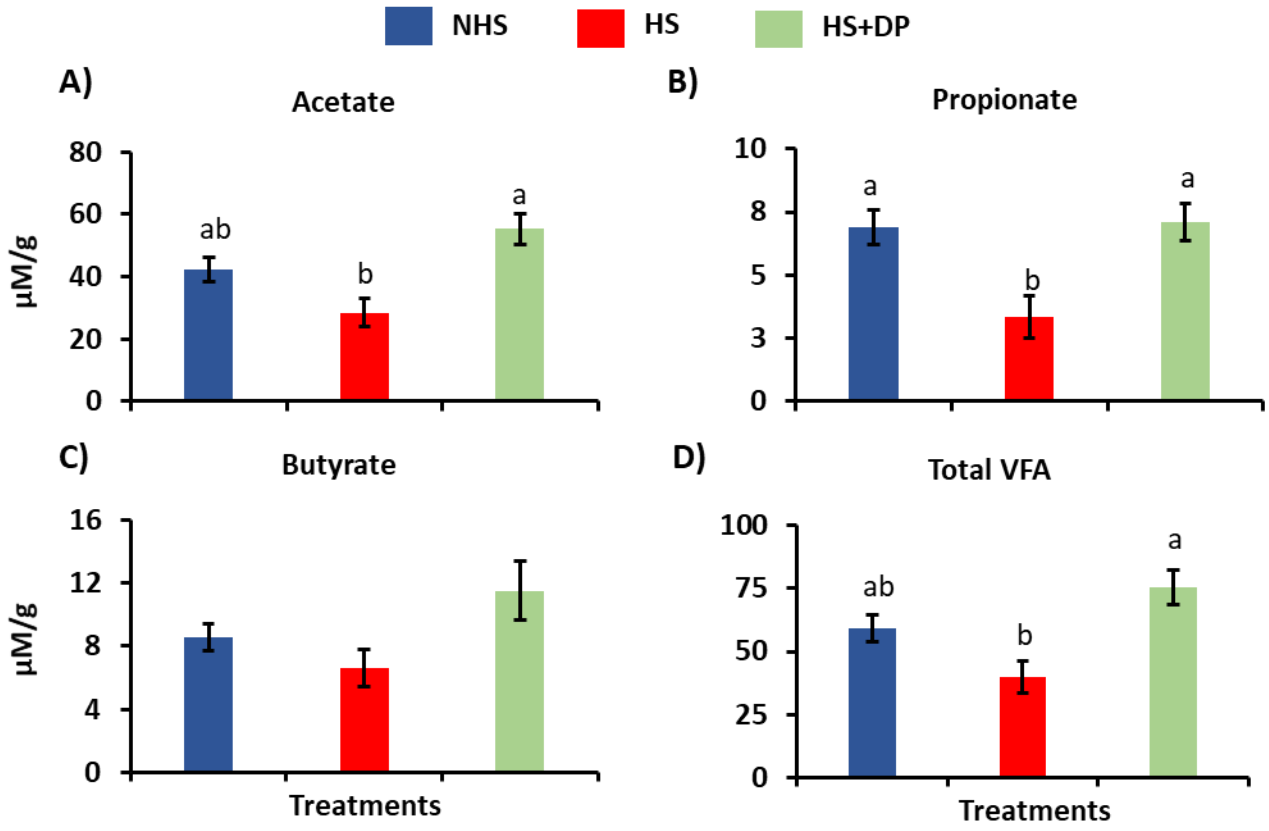


Figure 14 Effects of DP supplementation on the major volatile fatty acids in the cecal digesta of the heat-stressed broilers. A) Acetate, B) Propionate, C) Butyrate, and D) Total VFA. The effect of treatment was statistically different at * $p<0.05$.

3.1.5 Cecal microbial composition

The composition of the cecal microbiota at the phylum level of NHS, HS, and HS+DP groups after removing OTUs with low abundance is shown in Figure 15. *Firmicutes* and *Bacteroidetes* were found to be major dominant phyla across the samples in the NHS (49% and 50%), HS (62% and 36%), and HS+DP (62% and 35%).

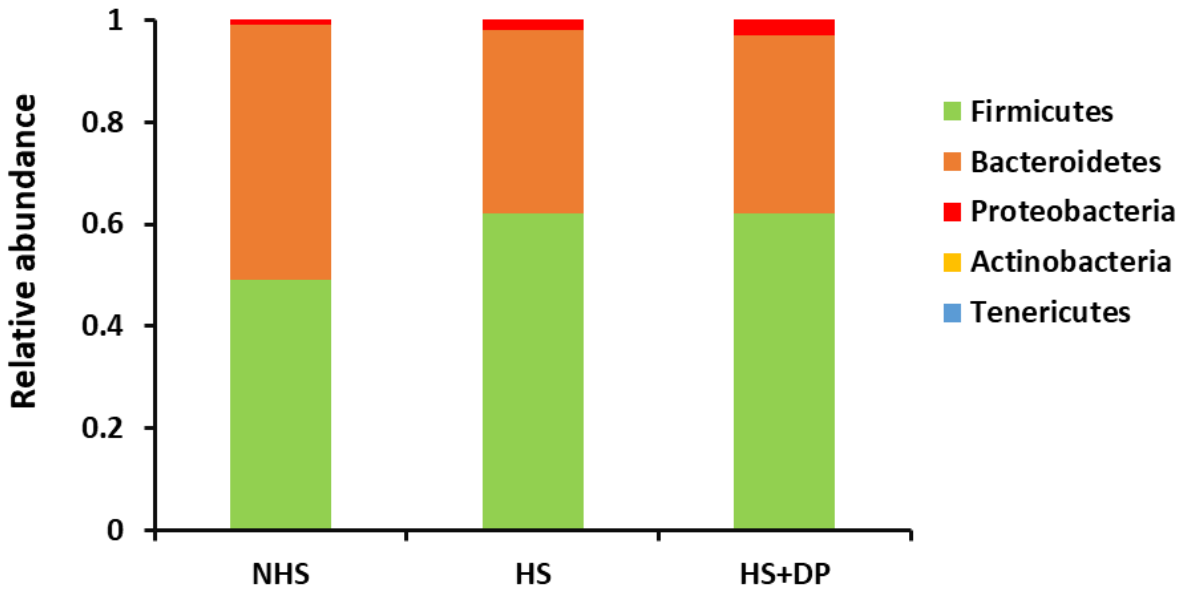


Figure 15 Effects of DP supplementation on the average relative abundance of the microbiota at the phylum level in the ceca of heat-stressed broilers.

At the class level (Figure 16), the cecal microbiota of the broiler birds in the NHS was dominated by the *Bacteroidia* (50.167%) followed by *Clostridia* (47.167%), while in HS and HS+DP groups were dominated by *Clostridia* (61.83% and 60.67%), followed by *Bacteroidia* (35.83% and 34.167%)

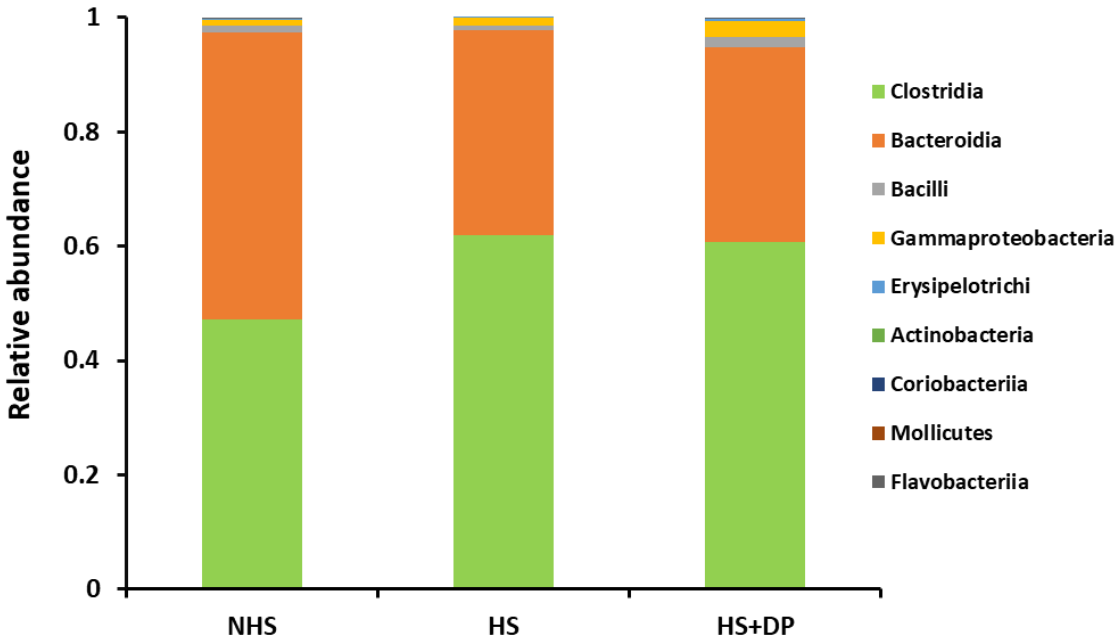
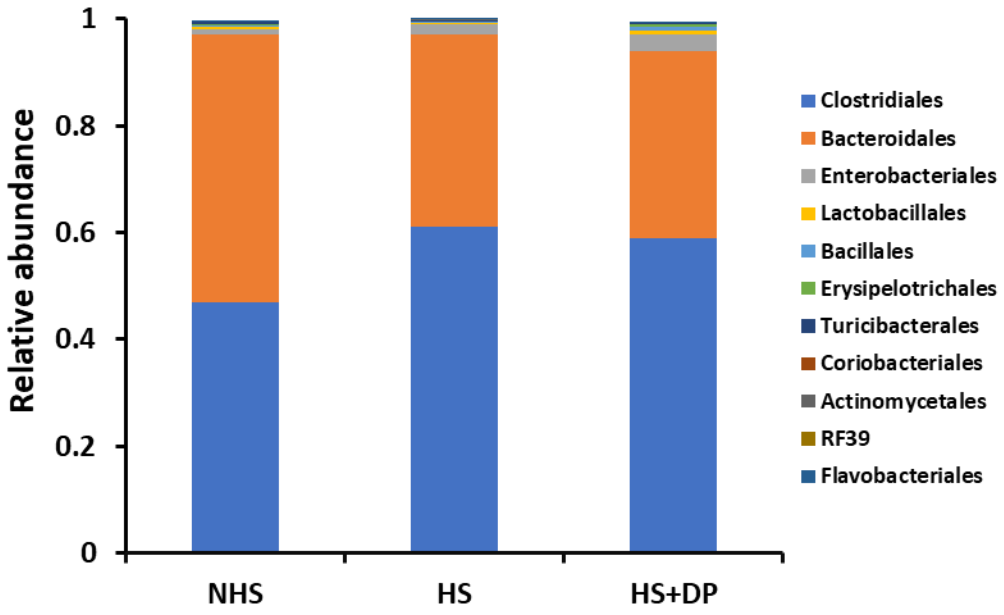


Figure 16 Effects of DP supplementation on the average relative abundance of the microbiota at the class level in the ceca of heat-stressed broilers.

At the order level (Figure 17), *Bacteroidales* was dominated in the NHS group followed by *Clostridiales*, whereas *Clostridiales* was predominant in HS and HS+DP group followed by *Bacteroidales*. Yet, the relative abundance of these dominant taxa was not significant across different groups. However, at the order level, *Bacillales* was found to be significantly abundant in the HS+DP group as compared to the HS group.

A)



B)

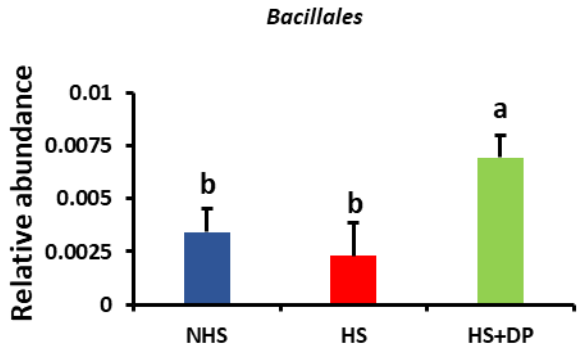
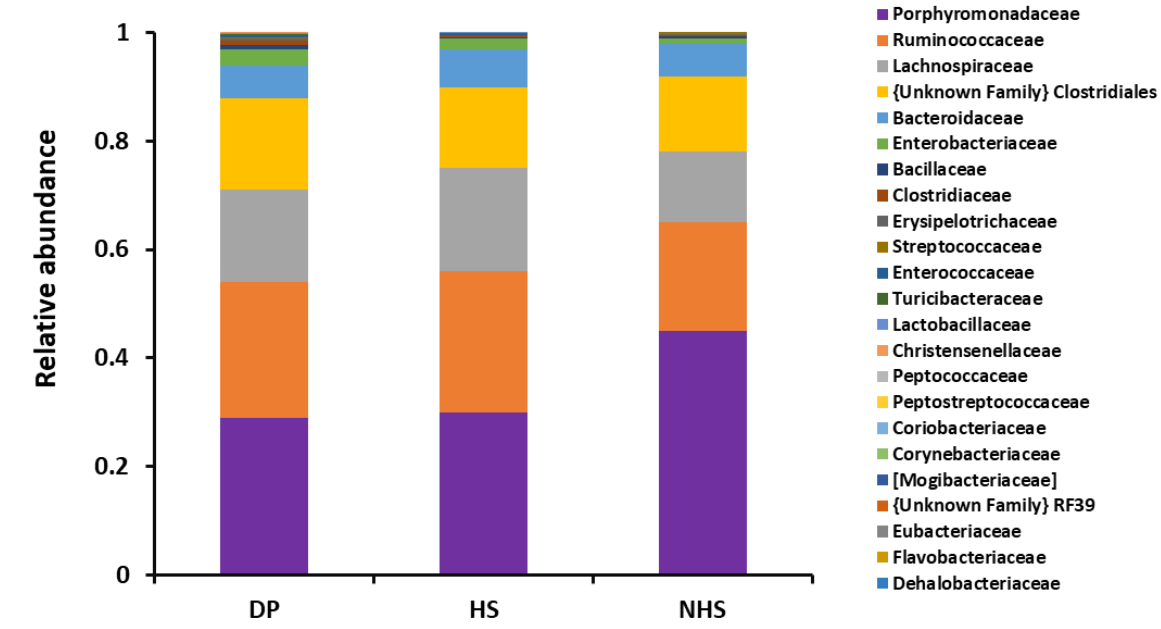


Figure 17 Effects of DP supplementation on the average relative abundance of the microbiota (A) and significantly abundance (B) microbiota at the order level in the ceca of heat-stressed broilers.

Relative abundance at the family level revealed the dominance of *Porphyromonadaceae*, *Ruminococcaceae*, and *Lachnospiraceae* in the NHS, HS, and HS+DP groups (Figure 18). However, their abundance remained unchanged across different groups. At the family level, *Bacillaceae*, *Christensenellaceae*, and *Peptostreptococcaceae* were significantly enriched in the HS+DP group.

A)



B)

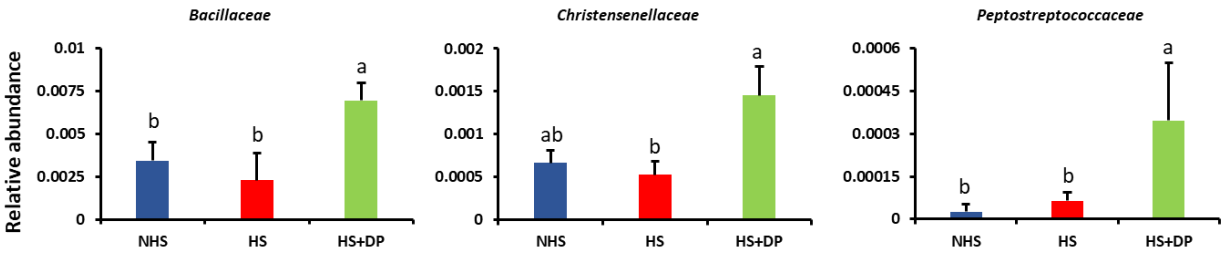
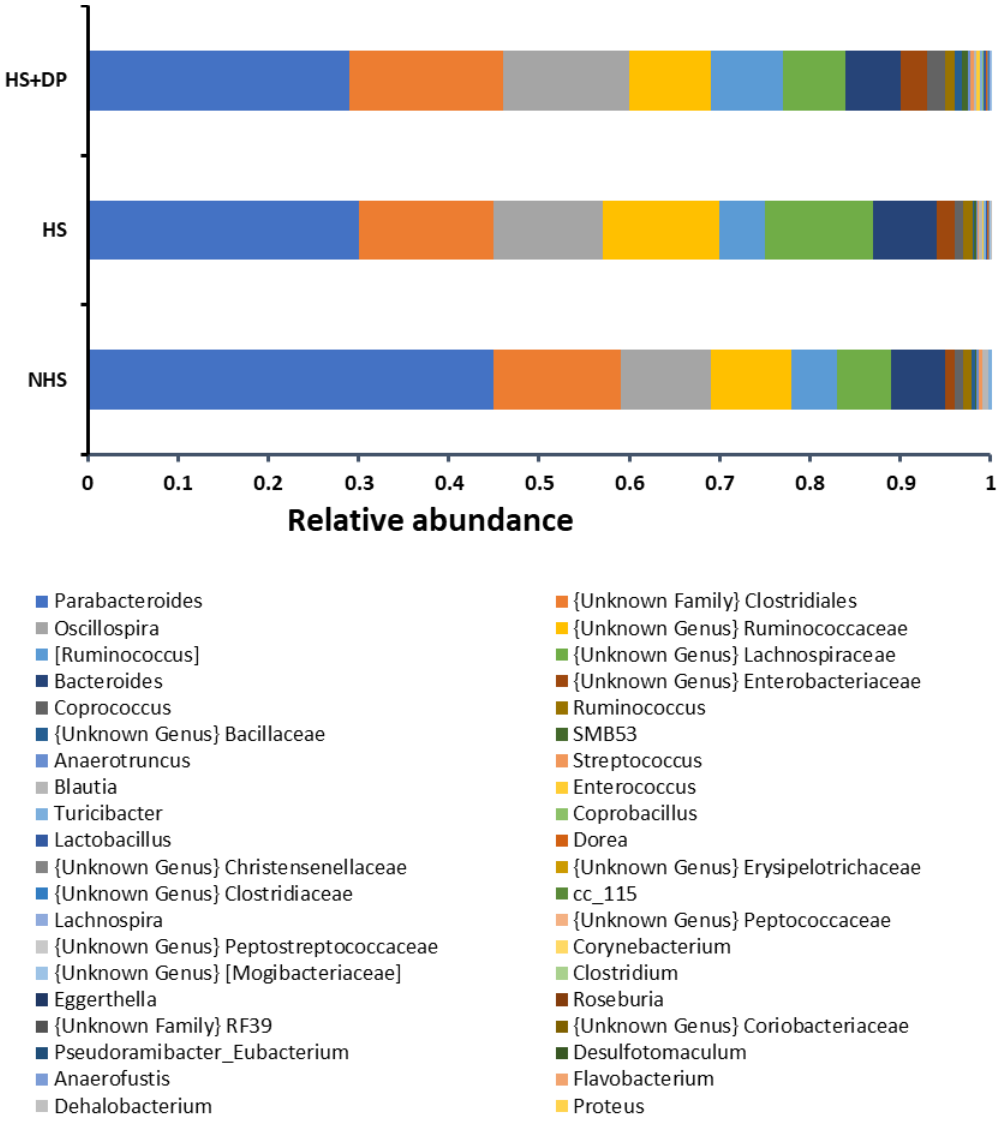


Figure 18 Effects of DP supplementation on the average relative abundance (A) and significantly abundance (B) microbiota at the family level in the ceca of heat-stressed broilers.

Taxon-based analysis at the genus level revealed that *Parabacteroides*, {Unknown Family} *Clostridiales*, and *Oscillospira* as the predominant genera (Figure 19). The relative abundance of these genera was not statistically different across different groups. However, on carrying out the ANOVA on different genera across treatment groups, the unknown genus of *Bacillaceae*, *Anaerotruncus*, unknown Genus of *Christensenellaceae*, and unknown Genus of *Peptostreptococcaceae* were significantly enriched in HS+DP group.

A)



B)

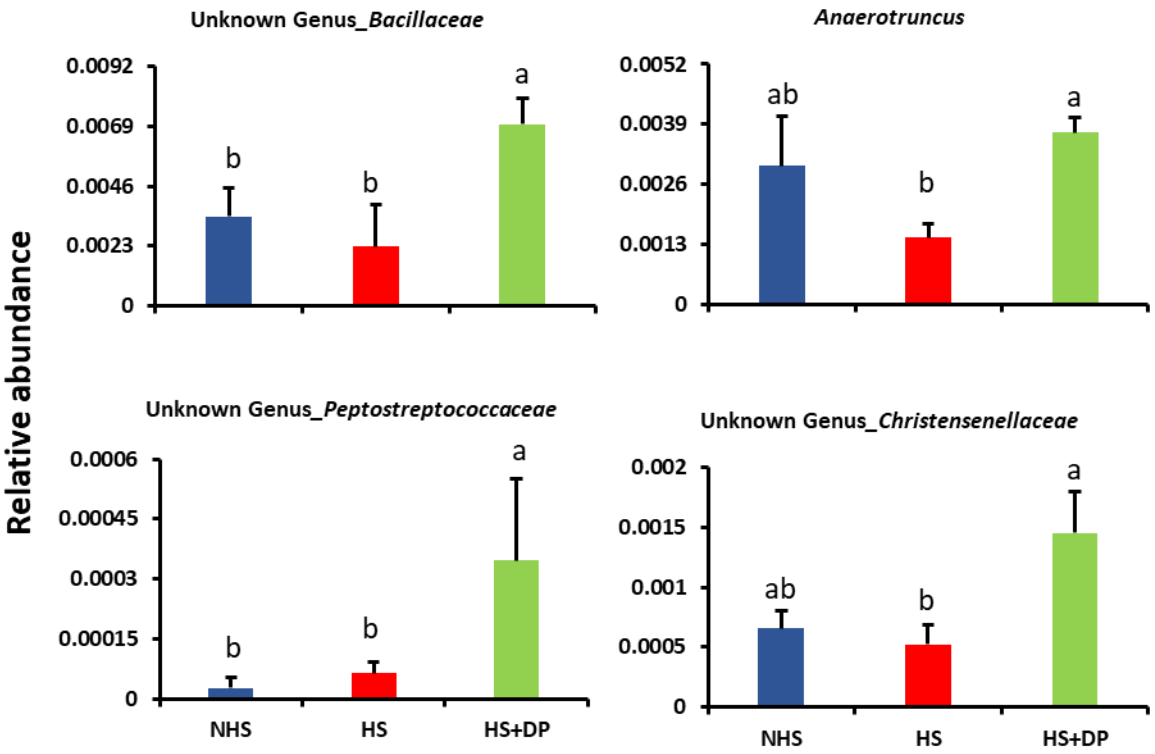


Figure 19 Effects of DP supplementation on the average relative abundance (A) and significantly abundance (B) microbiota at the genus level in the ceca of heat-stressed broilers.

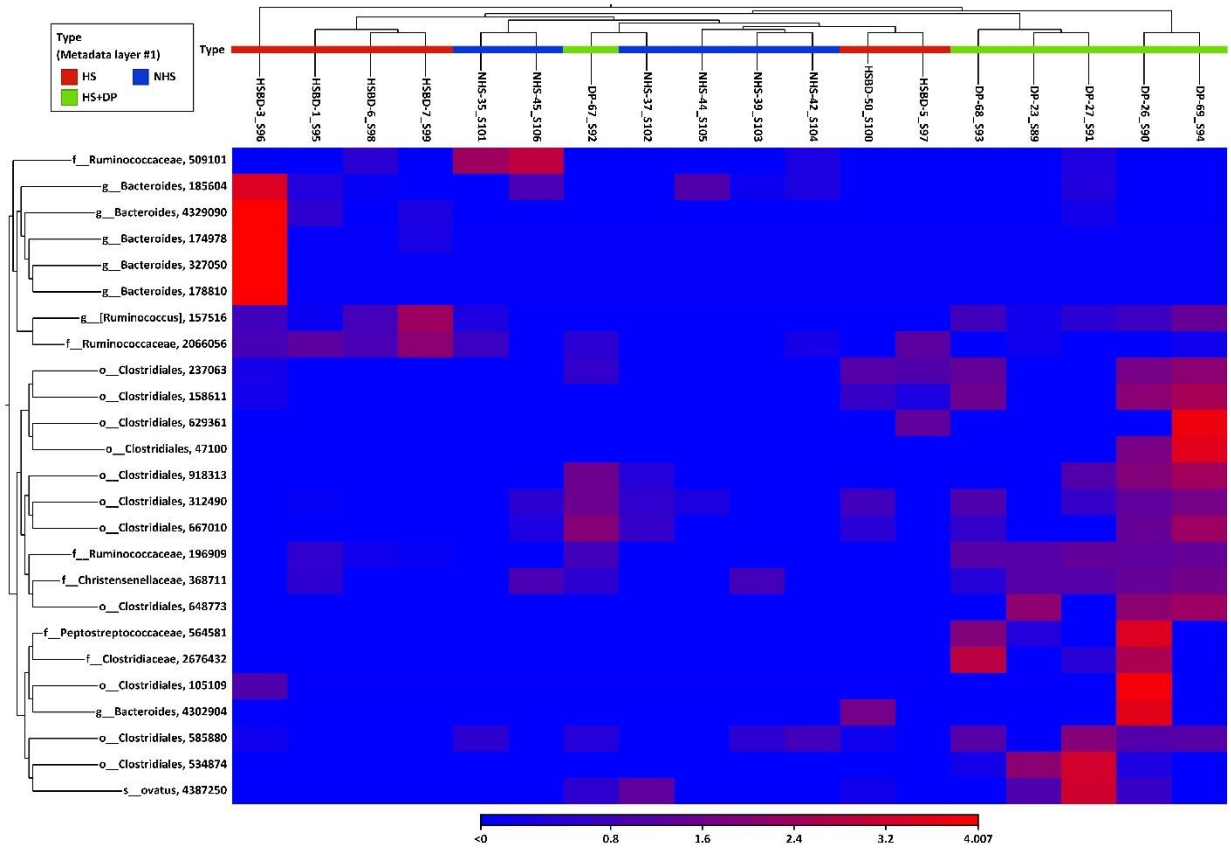
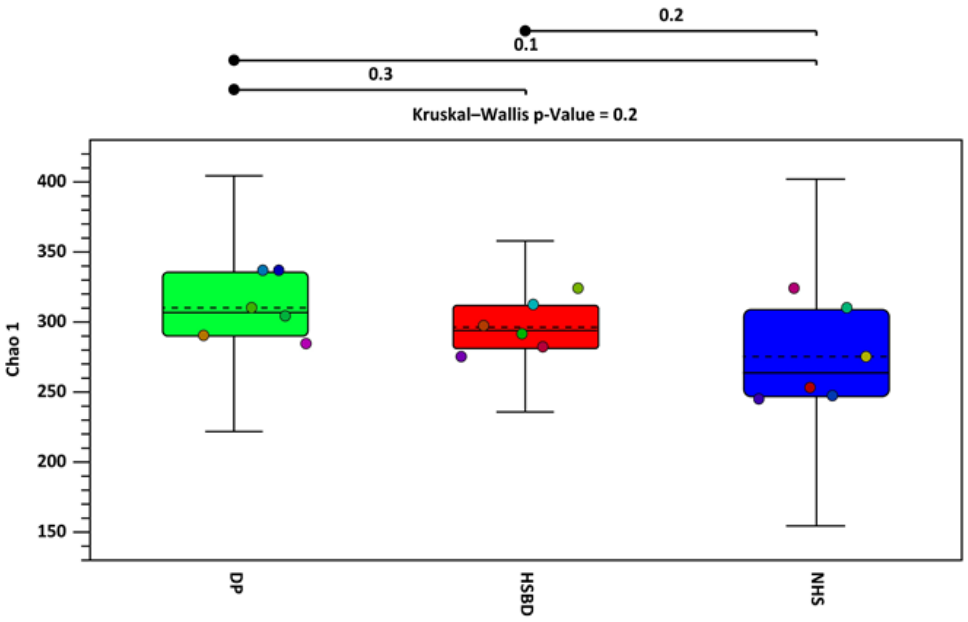


Figure 20 Heat map for the differential abundance taxa between treatment groups when supplemented with DP.

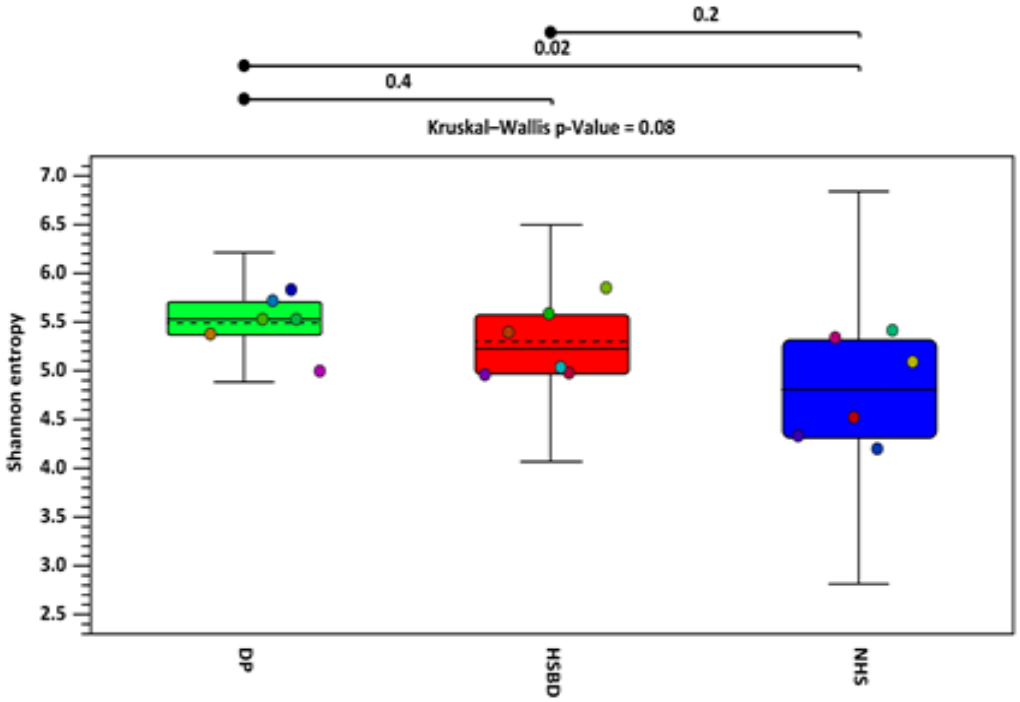
3.1.6 Alpha diversity of cecal microbiota

Alpha diversity measures the variance (diversity) within a sample and was measured by Chao1, Shannon, and Simpson index in this study. Chao1 measures the species richness, while Simpson measures the dominance or evenness. Shannon index, on the other hand, considers both the species richness and the evenness in the community. In this study, Shannon and Simpson's index was significantly increased in the HS+DP group as compared to the NHS group. However, there was no significant difference between treatments for Chao1 (Figure 21).

A)



B)



C)

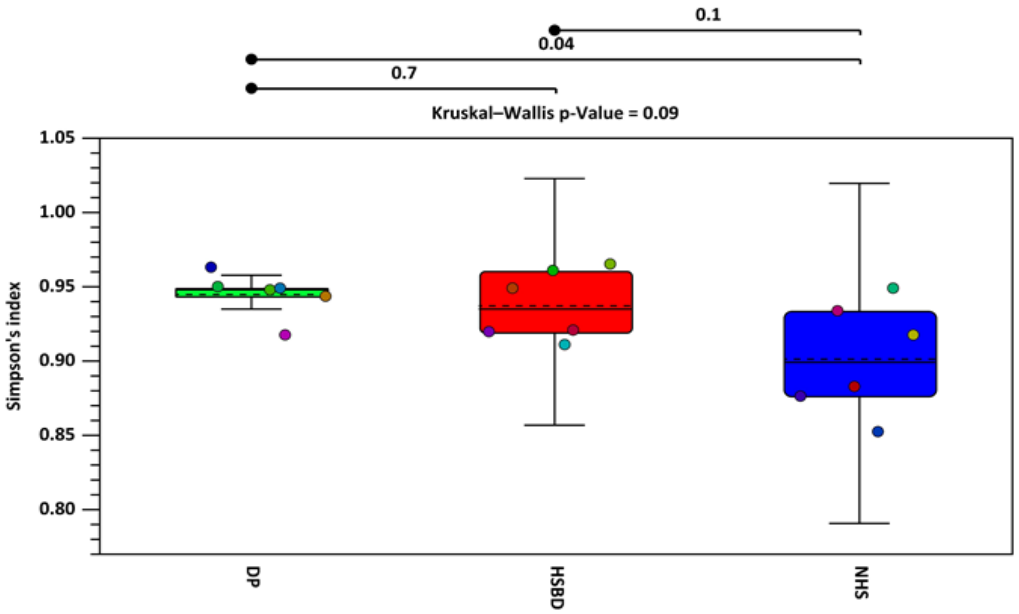
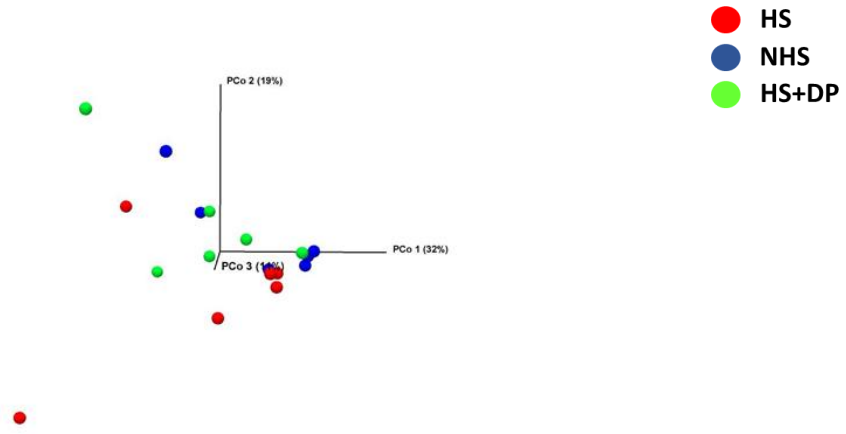


Figure 21 Effect of DP supplementation on microbial alpha diversity in heat-stressed broilers. A) chao1, B) Shannon entropy and C) Simpson's index.

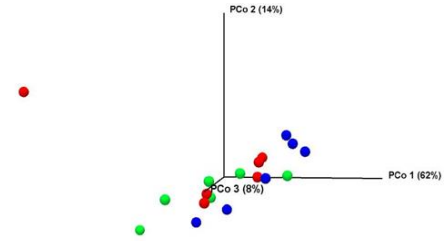
3.1.7 Beta diversity of cecal microbiota

Beta diversity measures the difference in the microbial composition between different environments (treatment groups) and was determined by using Bray-Curtis, unweighted UniFrac, and weighted UniFrac (Figure 22). The principal coordinate analysis (PCoA) was used to observe these different measures of beta diversity, and PERMANOVA analysis was carried out to determine the significance between the treatment groups. The unweighted UniFrac based PCoA showed that the cecal microbiota of the HS group was separate from the microbiota of other groups PERMANOVA analysis also confirmed the separation was significant between HS+DP, HS, and NHS group (p-value = 0.00181).

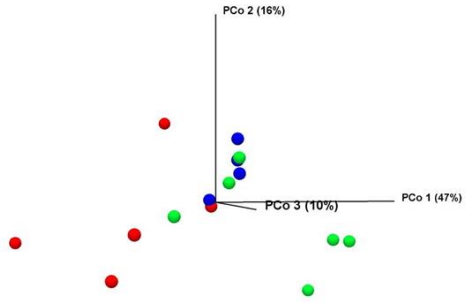
A)



B)



C)



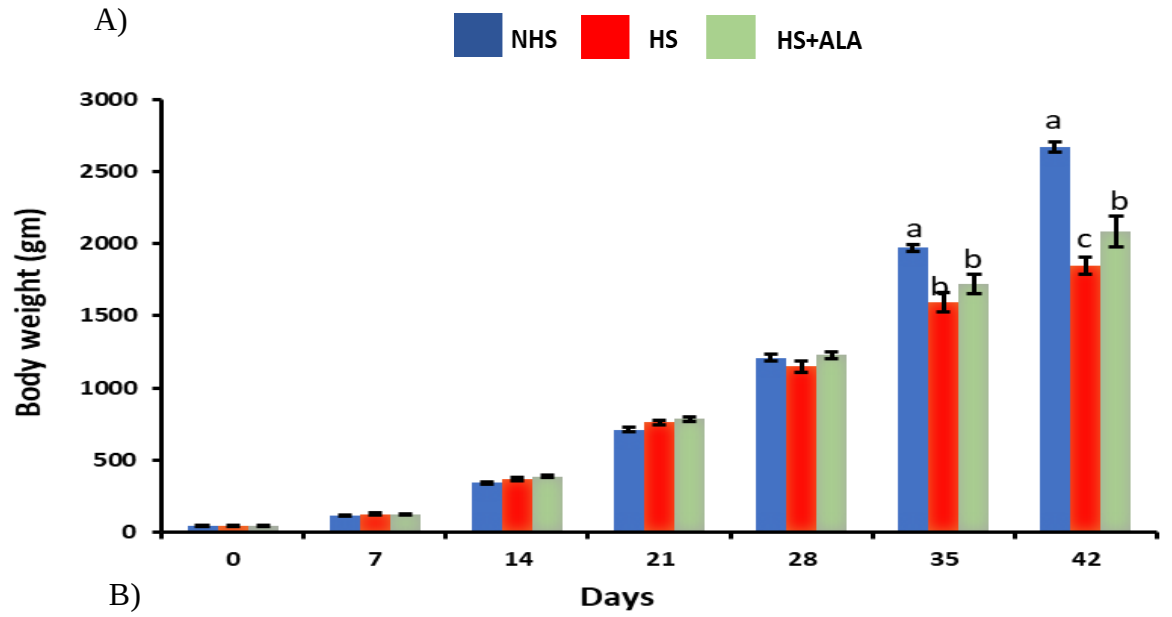
p-value = 0.00181

Figure 22 Effects of DP supplementation on microbial beta diversity in heat-stressed broilers. A) Bray-Curtis, B) Weighted UniFrac and C) Unweighted UniFrac

3.2 Effects of alpha-lipoic acid supplementation on the heat-stressed broilers

3.2.1 Growth performance

The effect of ALA supplementation on the growth performance of heat-stressed broiler birds is shown in Figure 23. Supplementation of the ALA was found to significantly improve the final body weight in the heat-stressed broiler birds. There was no significant difference in the body weight between the treatment groups until 28 days. However, on day 35, body weight was significantly decreased in the HS group and dietary supplementation of ALA in the heat-stressed broiler birds could not significantly improve the body weight. Nevertheless, on d 42, dietary supplementation of the ALA significantly improved the body weight in the heat-stressed broiler birds. During the heat stress period (21-42 d), the ADFI was significantly lowered in both HS and HS+ALA groups as compared to the NHS group. The ADG, on the other hand, was found to be significantly lowered in the HS group as compared to the NHS group, and dietary supplementation of ALA significantly improved ADG in heat-stressed broiler birds during the heat stress period (21-42 d). FCR was significantly high in the HS group and the ALA group as compared to the NHS group. Supplementation of the ALA was able to numerically lower the FCR in the heat-stressed broiler birds.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NHS
HS
HS+ALA
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2500
2000
1500
1000
500
0
0
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21
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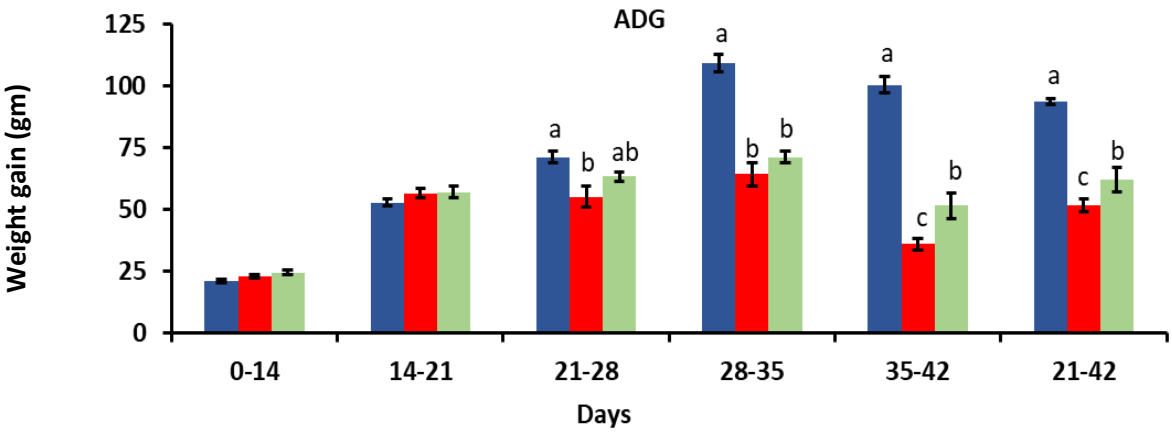
35
42
Days
a
a
b
b
a
b
c
b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ADG
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75
50
25
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14-21
21-28
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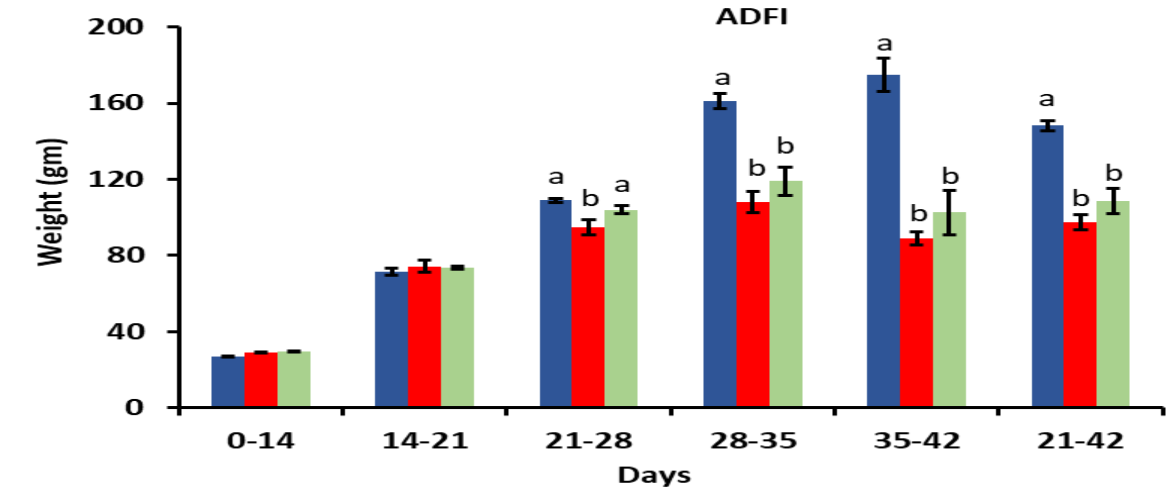
b
b
a
c
b
a
c
b


C)
ADFI
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120
80
40
0
0-14
14-21
21-28
28-35
35-42
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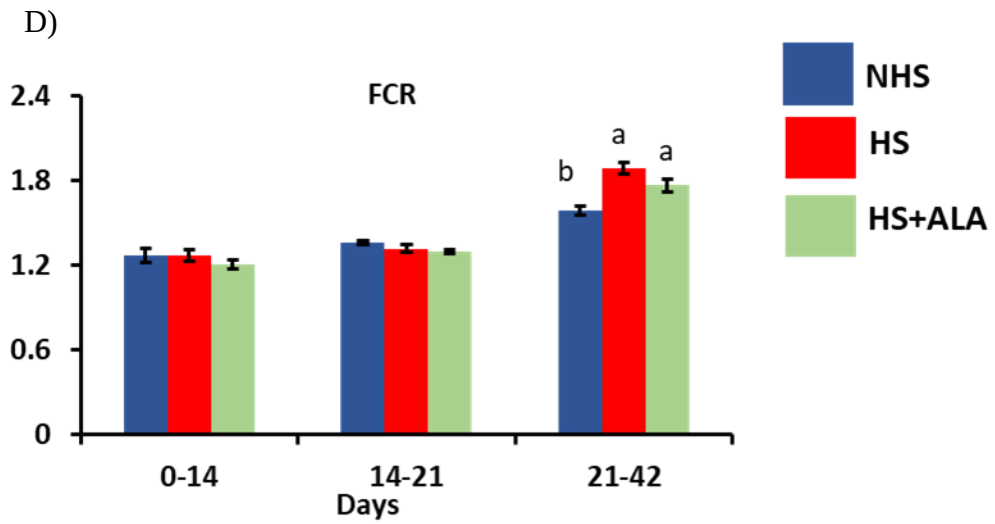


Figure 23 Effects of ALA supplementation on the growth performance of heat-stressed broilers. A) Bodyweight, B) ADG, C) ADFI, and D) FCR. Data presented as the mean ± SEM. The effect of treatment was statistically different at * $p<0.05$.

3.2.2 Effects of ALA on the intestinal gene expression

3.2.2.A Expression of heat shock protein-related genes

The effect of ALA supplementation on the mRNA expression of heat shock protein-related genes (*HSF1*, *HSF3*, *HSP70*, and *HSP90*) is shown in Figure 24. The mRNA expression of *HSF1*, *HSF3*, and *HSP70* remains unchanged across the treatment groups. Heat stress significantly lowered the mRNA expression of *HSP90* as compared to the control group (NHS group). At the same time, the supplementation of ALA significantly increased the mRNA expression of *HSP90* in heat-stressed broiler birds.

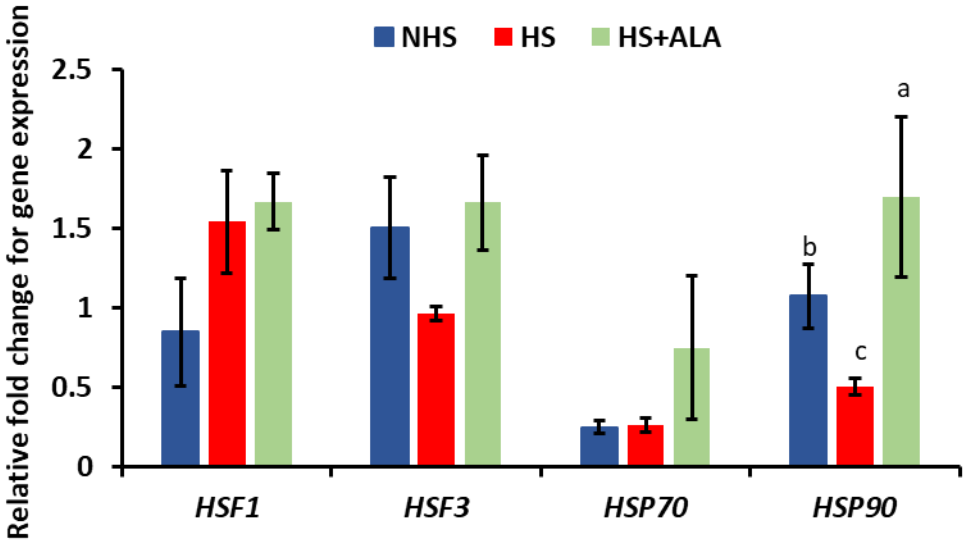


Figure 24 Effects of ALA supplementation on the expression of heat shock protein-related genes.

3.2.2.B Expression of antioxidant related genes

The effect of ALA supplementation on the mRNA expression of the antioxidant related genes (*SOD1, SOD2, PRDX1, TXN, GPX1, GPX3,* and *NRF2*) in heat-stressed broiler birds are shown in Figure 25. The mRNA expression of the *SOD2* and *PRDX1* was significantly lowered in the HS group as compared to the NHS group, while ALA supplementation significantly improved their expressions in heat-stressed broiler birds. Supplementation of ALA in the heat-stressed broiler birds significantly increased the mRNA expression of *GPX3* as compared to the NHS group. The mRNA expression of the *SOD1, TXN, GPX1,* and *NRF2* remains unchanged between the treatment groups.

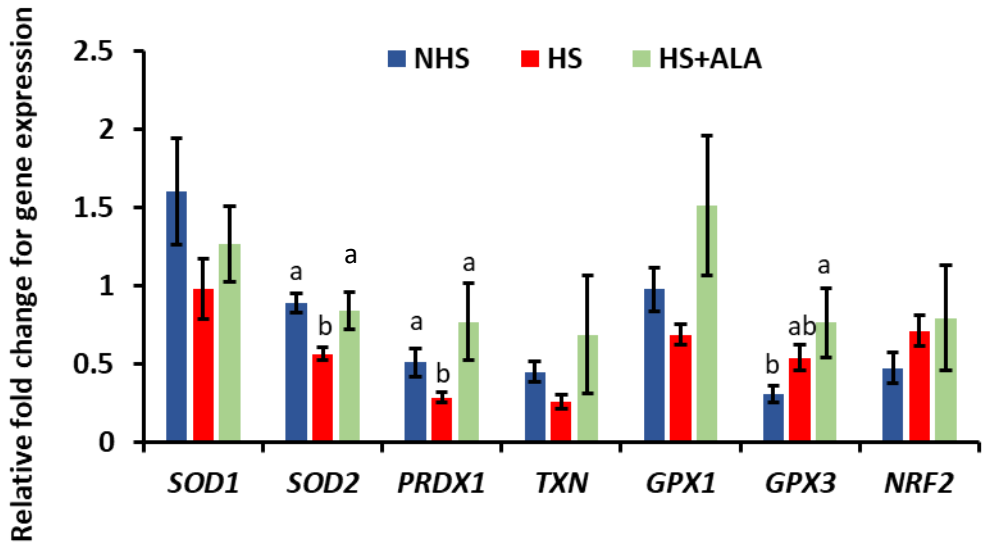


Figure 25 Effects of ALA supplementation on the expression of heat shock protein-related genes.

3.2.2.C Expression of tight-junction related genes

The mRNA expression of the tight-junction related genes (*OCLN* and *CLDN1*) between different treatment groups, i.e., NHS, HS, and HS+ALA, is shown in Figure 26. The heat stress significantly decreased the mRNA expression of *OCLN* in heat-stressed broiler birds as compared to the NHS group. In contrast, the supplementation of ALA significantly increased the expression of *OCLN* in heat-stressed birds. The mRNA expression of CLDN1 remains unchanged between treatment groups.

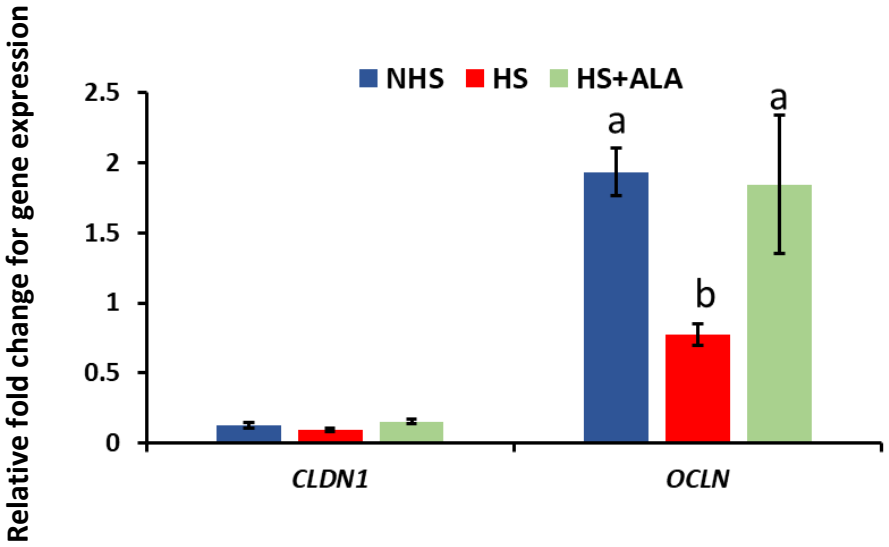


Figure 26 Effects of ALA supplementation on the expression of tight-junction related genes.

3.2.2.D Expression of immune-related genes

The mRNA expression of the immune-related genes (*IL4* and *MUC2*) between different treatment groups is shown in Figure 27. Supplementing ALA in the heat-stressed broiler birds significantly increased the expression of *MUC2* in heat-stressed broiler birds, as compared to the heat-stressed broiler birds provided with just basal diet (HS group). The mRNA expression of the *IL4* remained unchanged between the treatment groups.

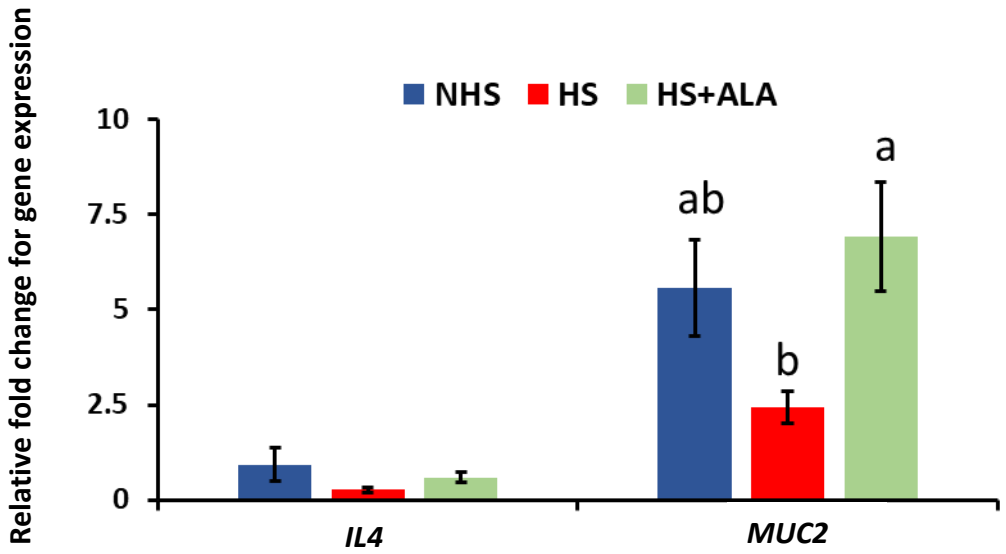


Figure 27 Effects of ALA supplementation on the expression of immune-related genes.

3.2.3 Ileum histomorphology

The effect of heat stress and ALA supplementation on gut histomorphology is shown in Figure 28. Villus height (VH) and Villus height to crypt depth ratio (VH/CD) were significantly lowered in the HS group as compared NHS group while supplementing ALA in heat-stressed broiler birds were found to improve these parameters. Villus surface area significantly decreased in the heat-stressed birds. However, dietary supplementation of ALA did not improve the surface area in heat-stressed birds. The crypt depth (CD) remained unchanged across the treatment groups.

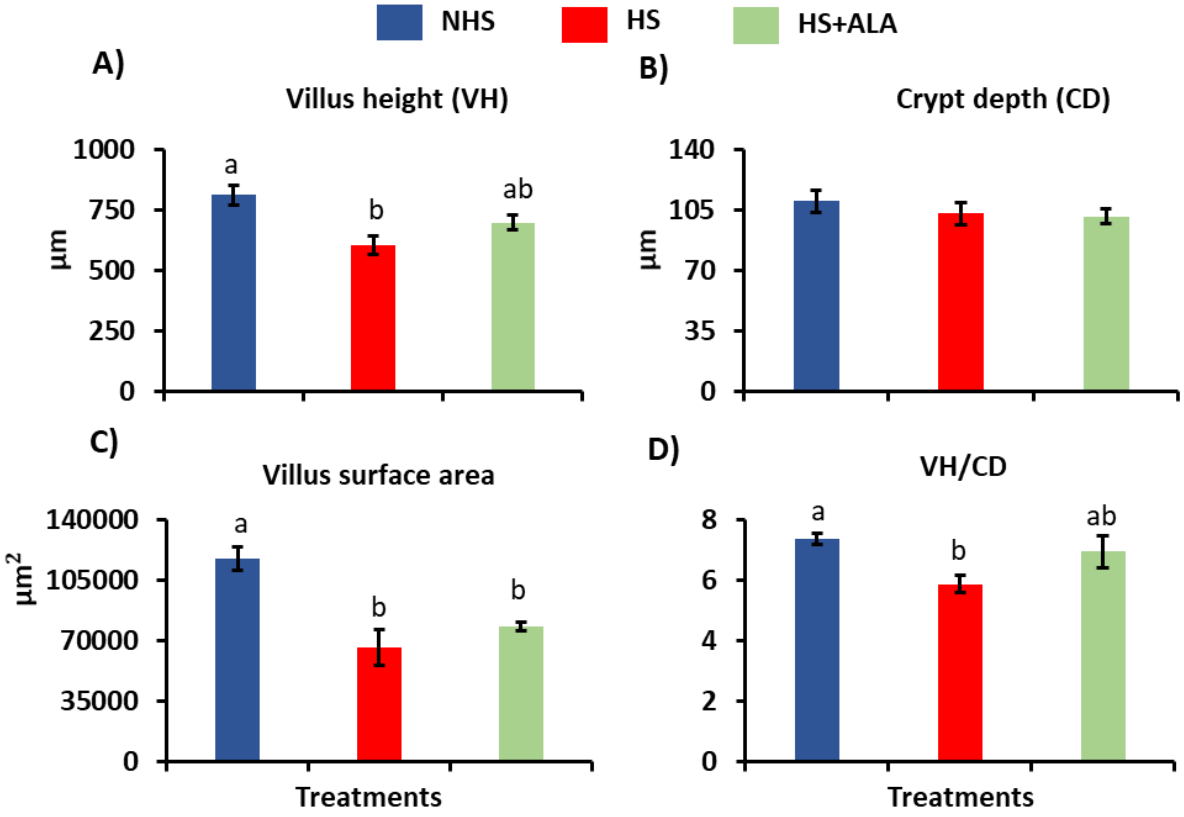


Figure 28 Effects of ALA supplementation on the ileum histomorphology of the heat-stressed broilers. A) Villus height (VH), B) Crypt depth (CD), C) Villus surface area, D) Villus height (VH): Crypt depth (CD). The effect of treatment was statistically different at * $p<0.05$.

3.2.4 Volatile fatty acids

The effect of ALA supplementation on the major volatile fatty acids in the cecal digesta of the heat-stressed broiler birds are shown in Figure 29. The amount of propionate was significantly decreased in the HS groups as compared to the NHS groups. At the same time, dietary supplementation of the ALA significantly increased propionate amount in the cecal digesta. The amount of acetate was significantly decreased in the HS group as compared to the NHS group, while supplementation of ALA in heat-stressed broiler birds significantly increased its amount. Supplementation of the ALA in the heat-stressed broiler birds also significantly increased the concentration of butyrate as compared to the other two groups. Overall, total volatile fatty acids were significantly lower in the HS group as compared to the NHS group, and dietary supplementation of ALA was able to increase the total concentration of the total VFA significantly.

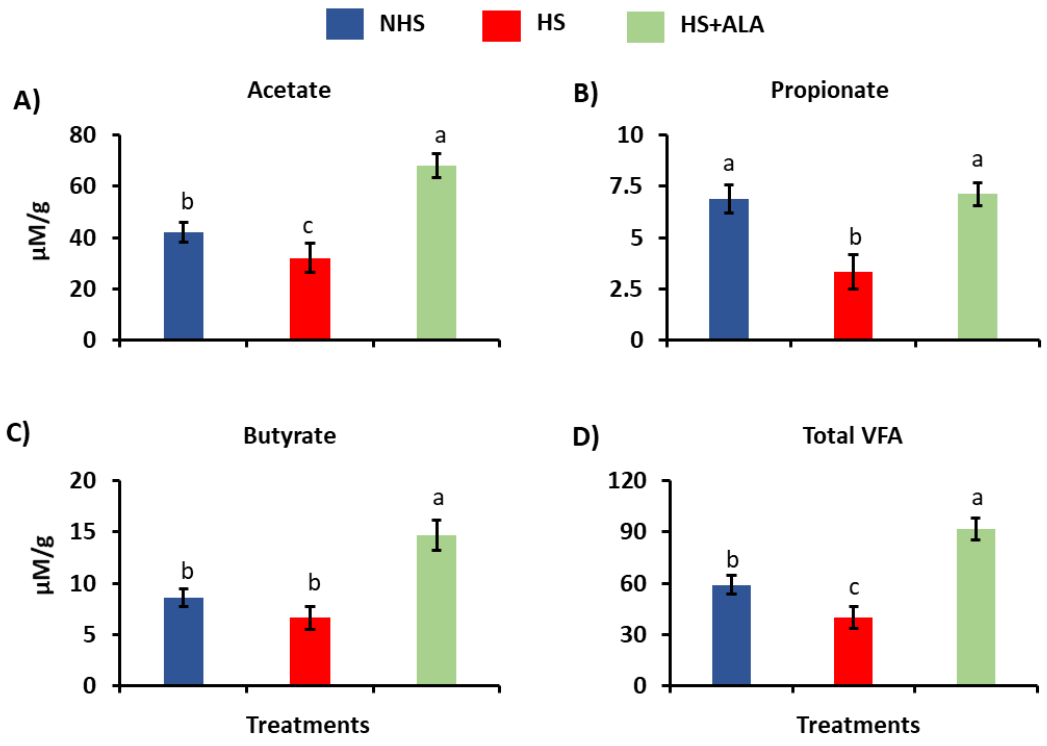


Figure 29 Effects of ALA supplementation on the major volatile fatty acids in the cecal digesta of the heat-stressed broilers. A) Acetate, B) Propionate, C) Butyrate, and D) Total VFA. The effect of treatment was statistically different at * $p<0.05$.

3.2.5 Cecal microbial composition

At the phylum level, HS and HS+ALA groups were dominated by *Firmicutes* (63%, 63%), followed by *Bacteroidetes* (36%,35%) while NHS group have an almost equal abundance of *Firmicutes* (49%) and *Bacteroidetes* (50%) (Figure 30).

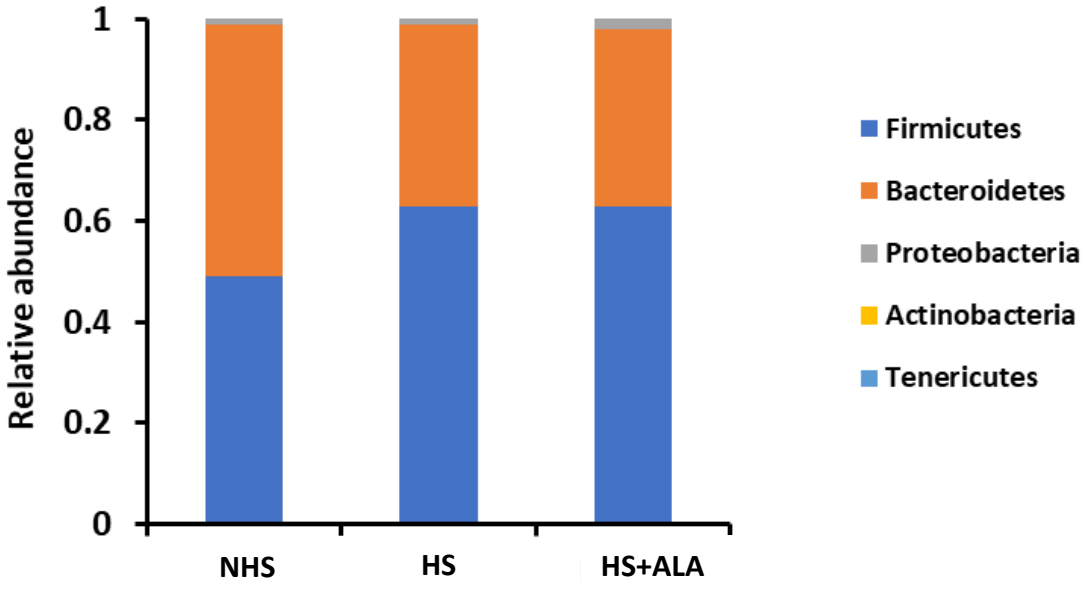


Figure 30 Effects of ALA supplementation on the average relative abundance of the microbiota at the phylum level in the ceca of heat-stressed broilers.

Taxon-based analysis at the class level revealed that *Clostridia* and *Bacteroidia* were the dominant class across different treatment groups. However, these dominant taxa weren't statistically different across treatment groups (Figure 31).

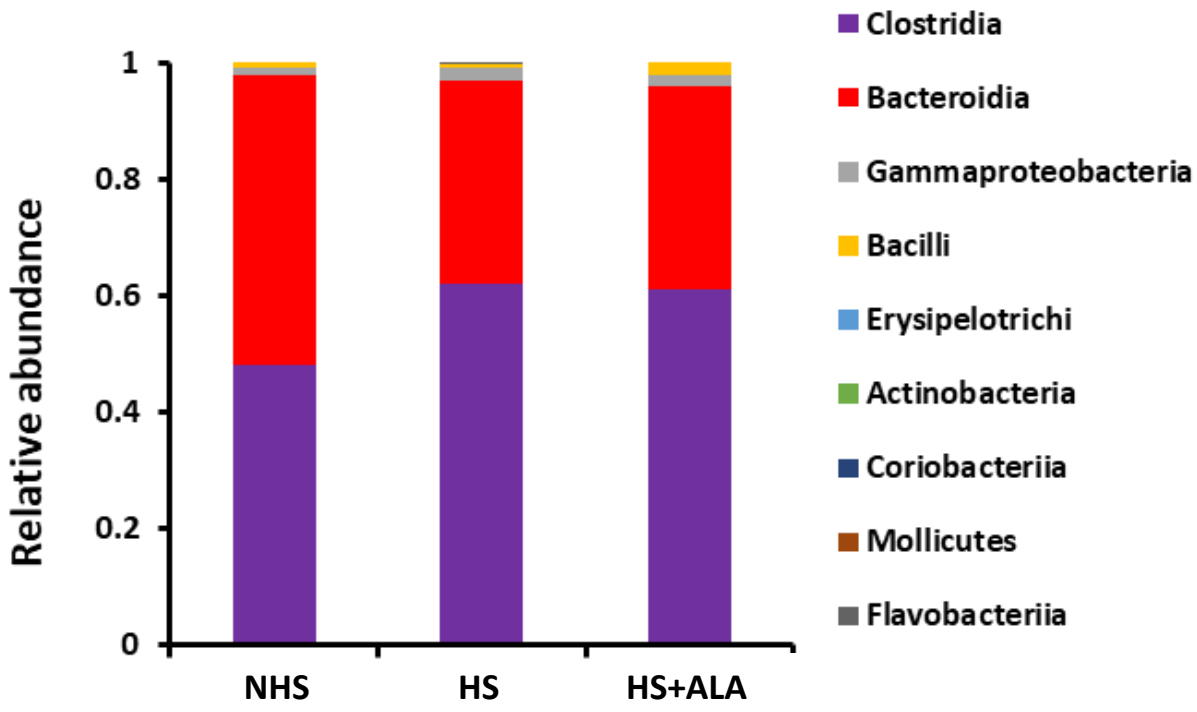
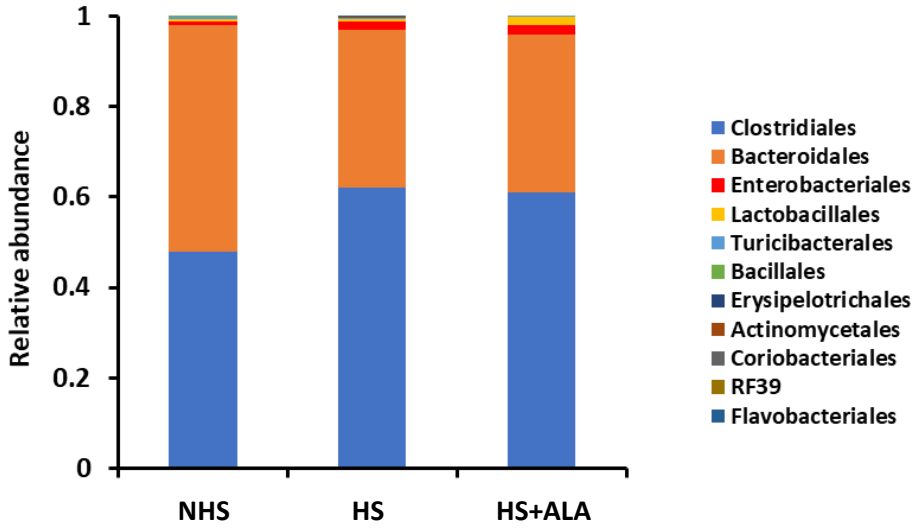


Figure 31 Effects of ALA supplementation on the average relative abundance of the microbiota at the class level in the ceca of heat-stressed broilers.

At the order level, *Clostridales* and *Bacteroidales* were dominate in different treatment groups-NHS (48%, 50%), HS (62%, 35%) and HS+ALA (61%, 35%). Yet, these dominant orders were not statistically significant across the treatment groups. However, supplementation of ALA was found to be significantly increased in *Lactobacillales* in heat-stressed birds (Figure 32).

A)



B)

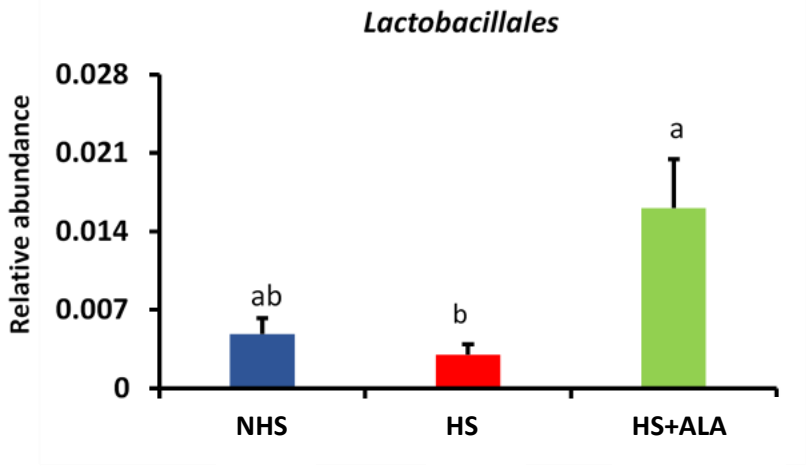
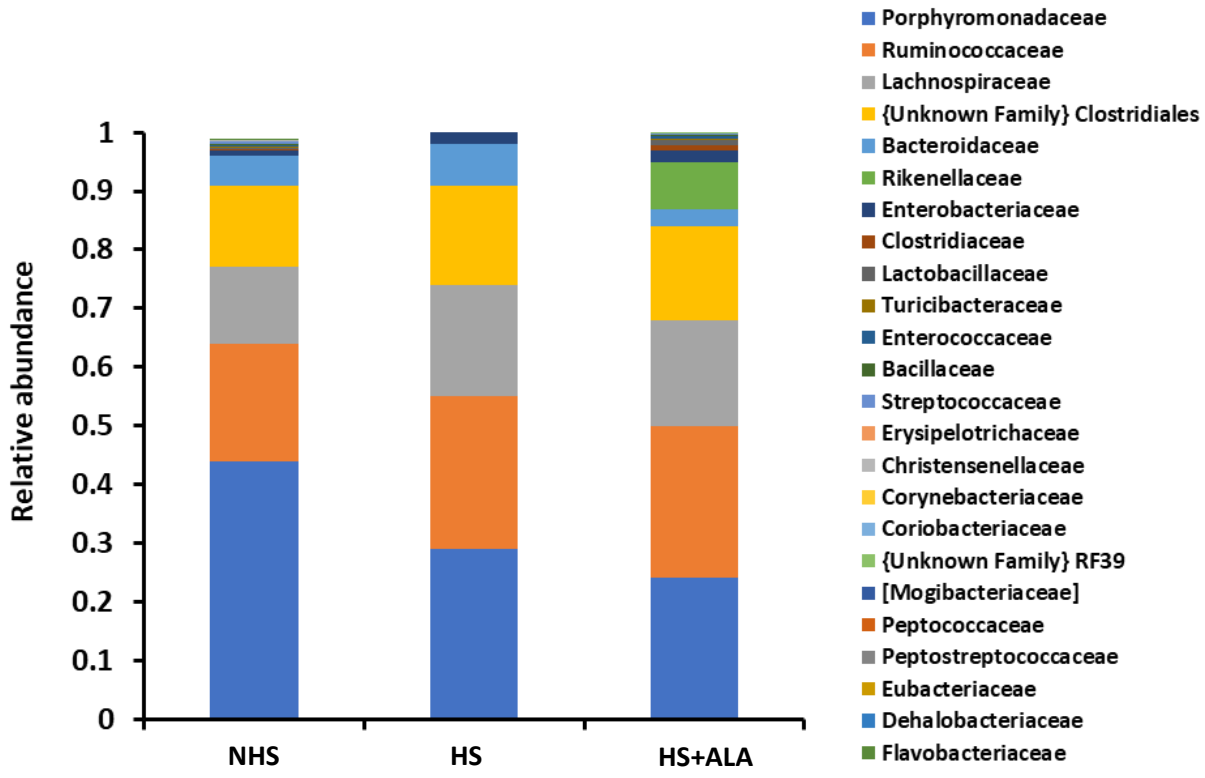


Figure 32 Effect of ALA supplementation on the average relative abundance of the microbiota at the order level (A) and significantly abundant microbiota at the order level (B) in the ceca of heat-stressed broilers.

The relative abundance of the dominant family is shown in Figure 33. *Porphyromonadaceae, Ruminococcaceae, Lachnospiraceae,* and *unknown Family of Clostridiales* were the dominant family found across the treatment groups. These dominant families, however, were not significantly abundant among groups. *Lactobacillaceae* and *Peptostreptococcaceae* were significantly enriched in the HS+ALA group.

A)



B)

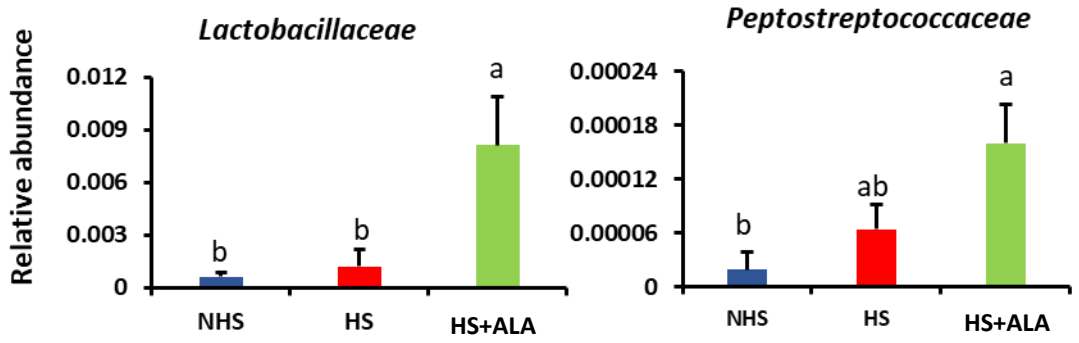
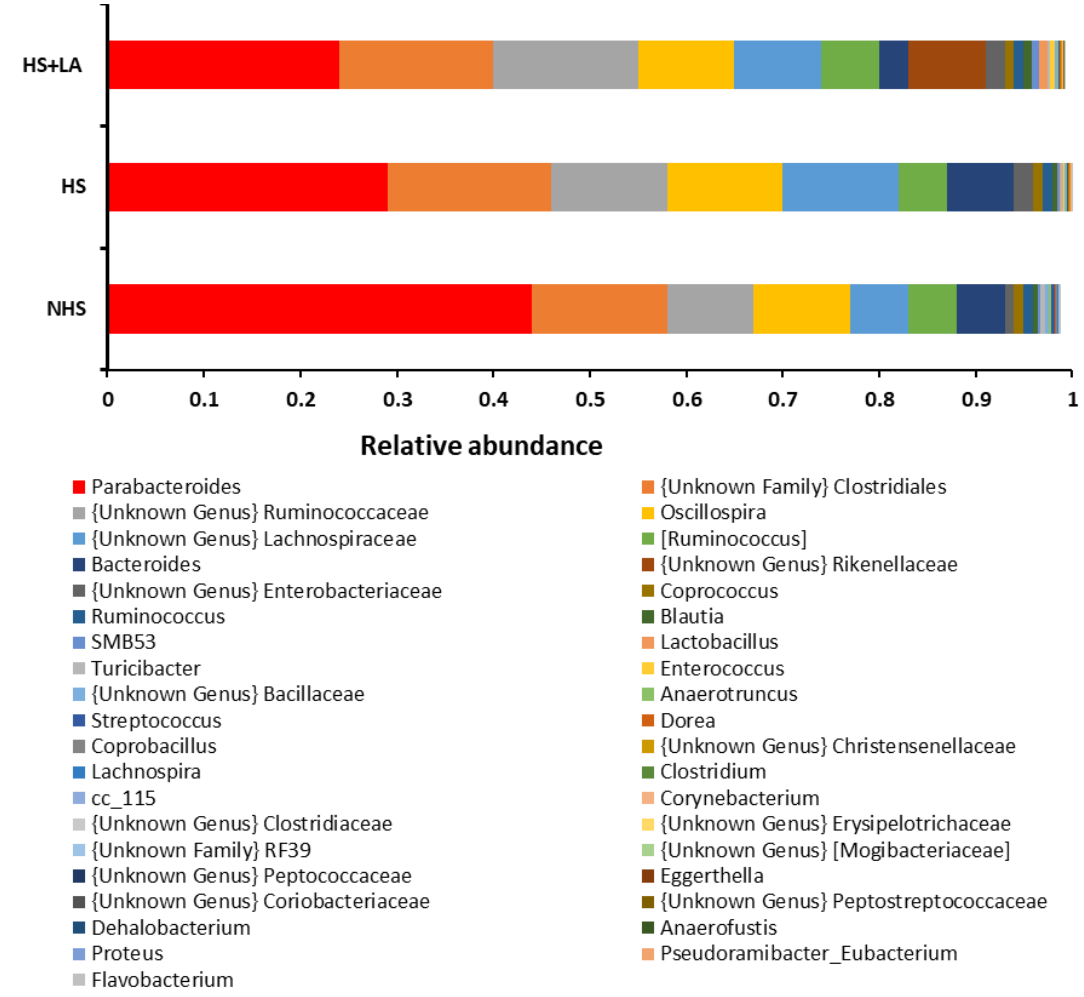


Figure 33 Effects of ALA supplementation on the average relative abundance of the microbiota at the family level (A) and Significantly abundant microbiota at the family (B) in the ceca of heat-stressed broilers.

The major dominant genus among the groups is shown in Figure 33. *Parabacteroides* was the most dominant genus across all groups- HS (29%), HS+ALA (24%), and NHS (44%), followed by *unknown family_Clostridales*. Although the relative abundance of *Parabacteroides* was drastically reduced in the heat-stressed birds, it was not significantly different among treatments. Dietary supplementation of the ALA significantly enriched the *Lactobacillus* and unknown genus of *Peptostreptococcaceae* in the heat-stressed birds (Figure 34).

A)



B)

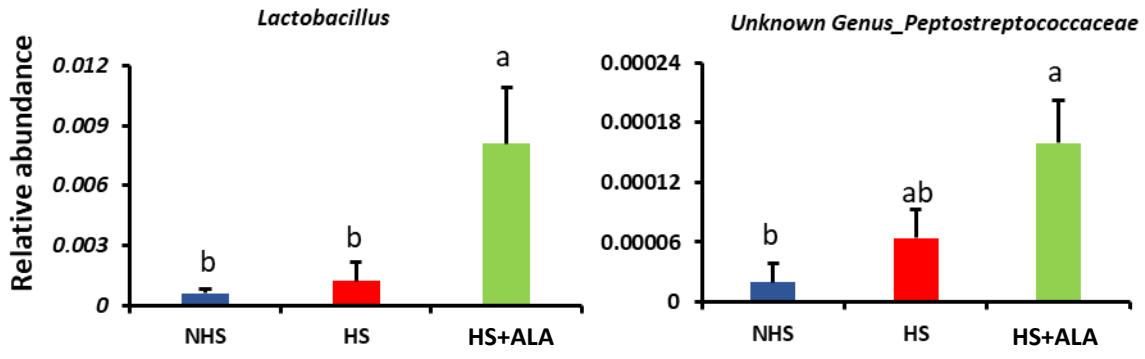


Figure 34 Effects of ALA supplementation on the average relative abundance (A) and significantly abundance (B) microbiota at the genus level in the ceca of heat-stressed broilers.

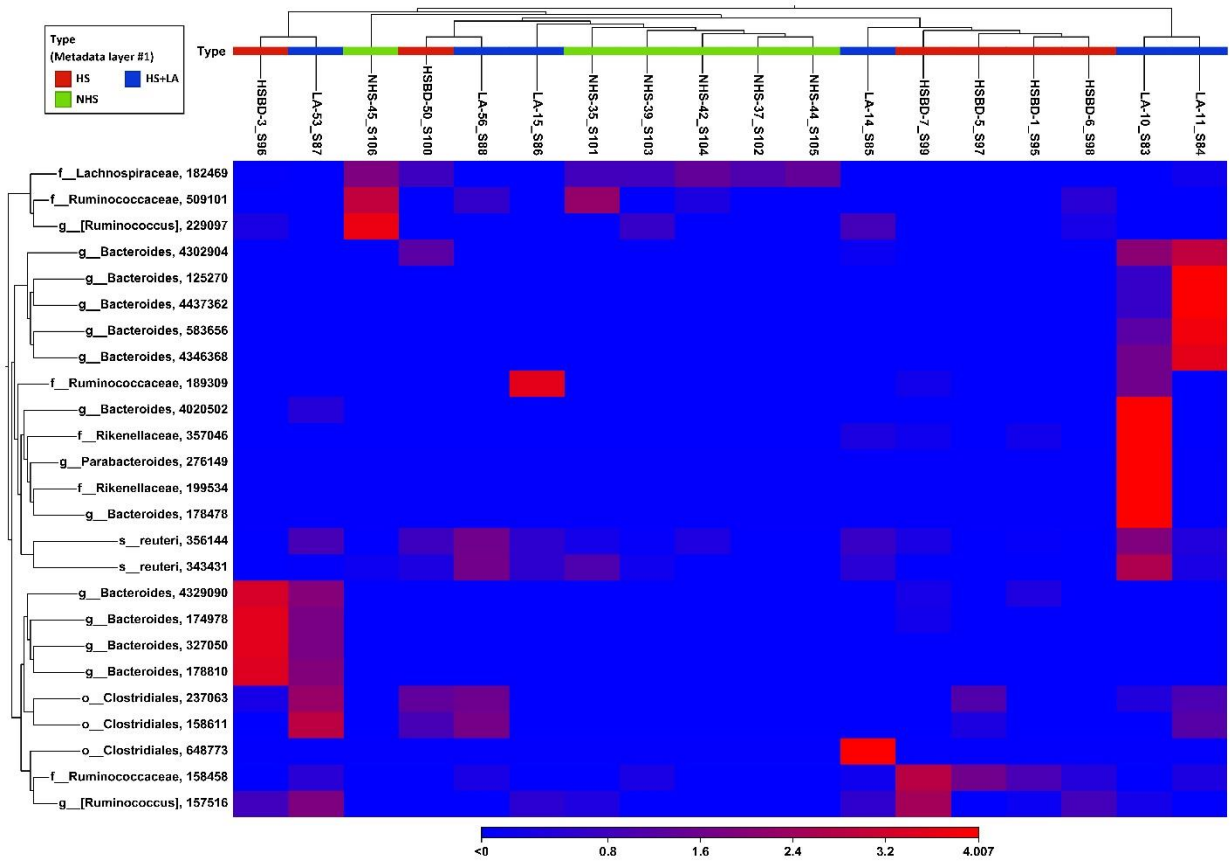


Figure 35 Heat map for the differential abundant taxa between treatment groups when supplemented with ALA.

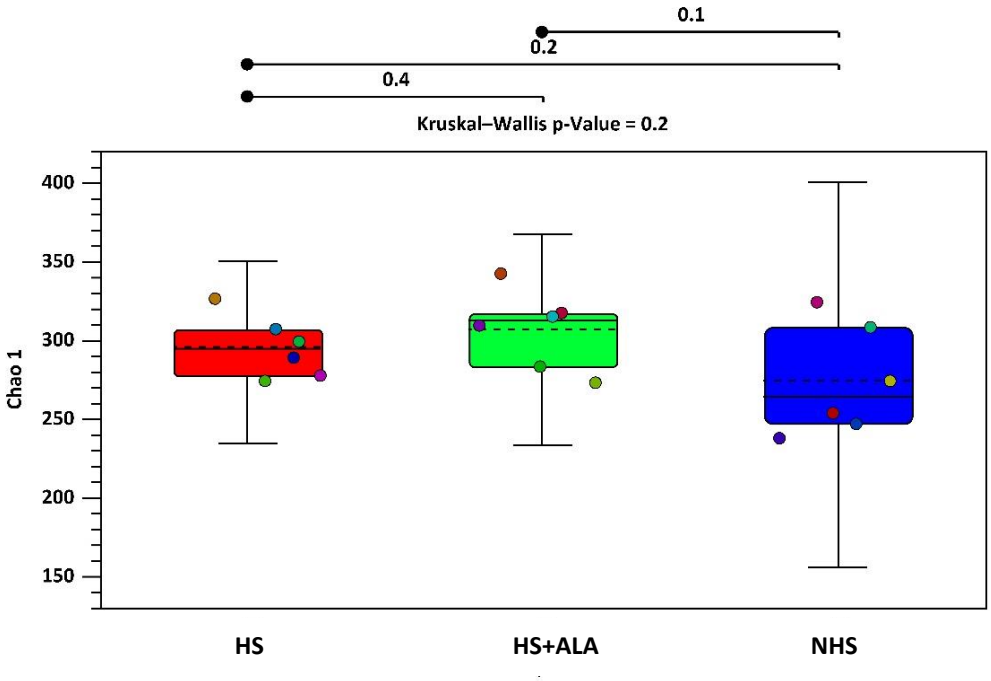
3.2.6 Alpha diversity of cecal microbiota

An index reflecting species diversity richness and diversity (Shannon entropy) was found to be significantly higher in HS+ALA as compared to NHS. In contrast, no significance was found between groups even when using different indexes of alpha diversity i.e., Chao1 and Simpson's index (Figure 36).

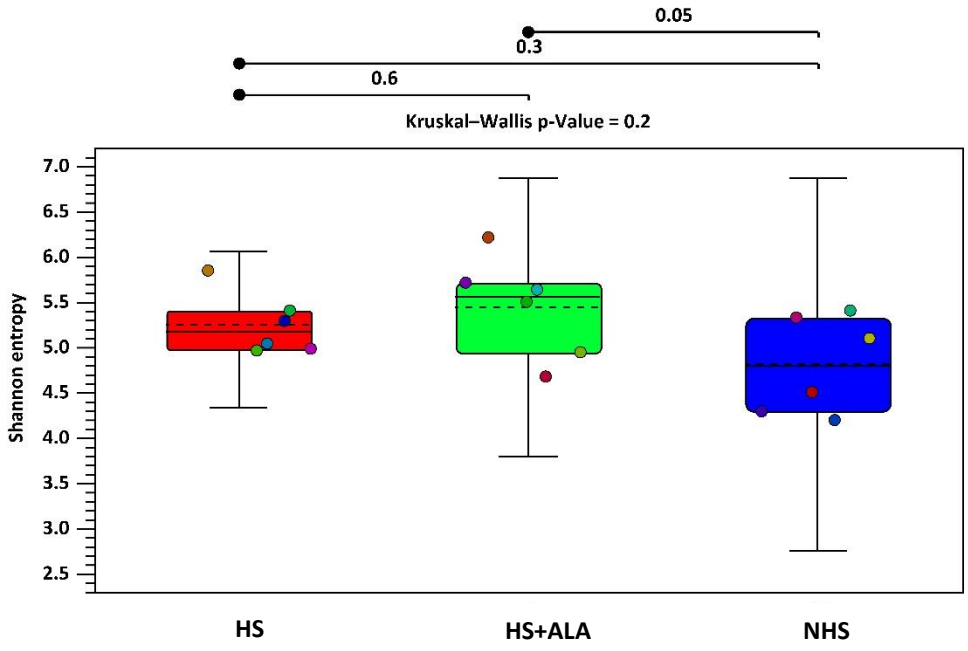
3.2.7 Beta diversity of cecal microbiota

Beta diversity in this study was measured by using Bray-Curtis, weighted UniFrac, and unweighted UniFrac, where we found that samples for HS+ALA were separated from the other two groups in PCoA for Bray-Curtis and unweighted UniFraac (Figure 37). Further, PERMANOVA analysis was carried out to check the statistical significance between different groups, where we found Bray-Curtis and unweighted UniFrac to be significant.

A)


0.1
0.2
0.4
Kruskal–Wallis p-Value = 0.2
Chao 1
400
350
300
250
200
150
HS
HS+ALA
NHS

B)


0.05
0.3
0.6
Kruskal–Wallis p-Value = 0.2
Shannon entropy
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
HS
HS+ALA
NHS

C)

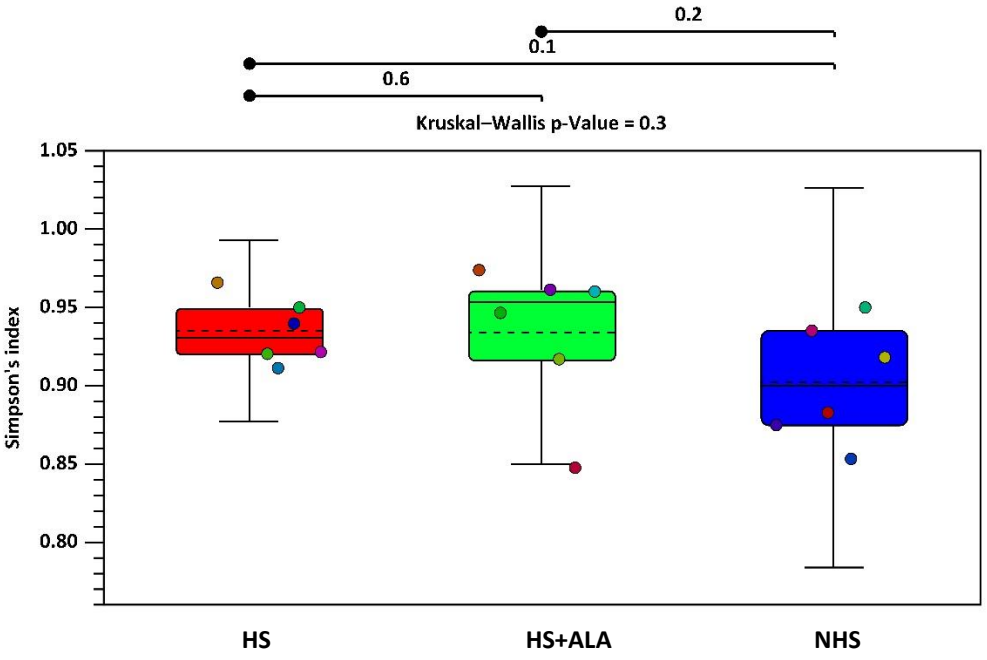
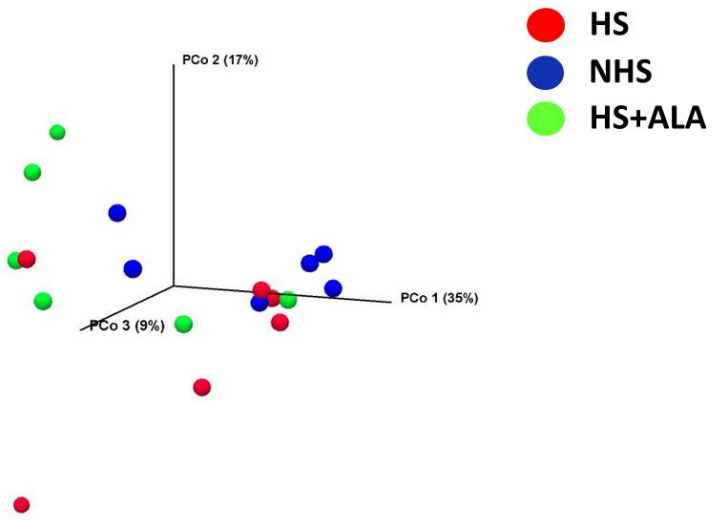


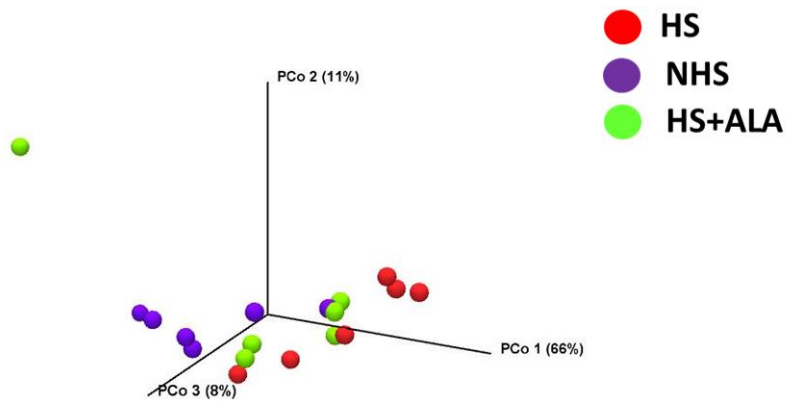
Figure 36 Effects of ALA supplementation on microbial alpha diversity in heat-stressed broilers. A) chao1, B) Shannon entropy and C) Simpson's index.

A)



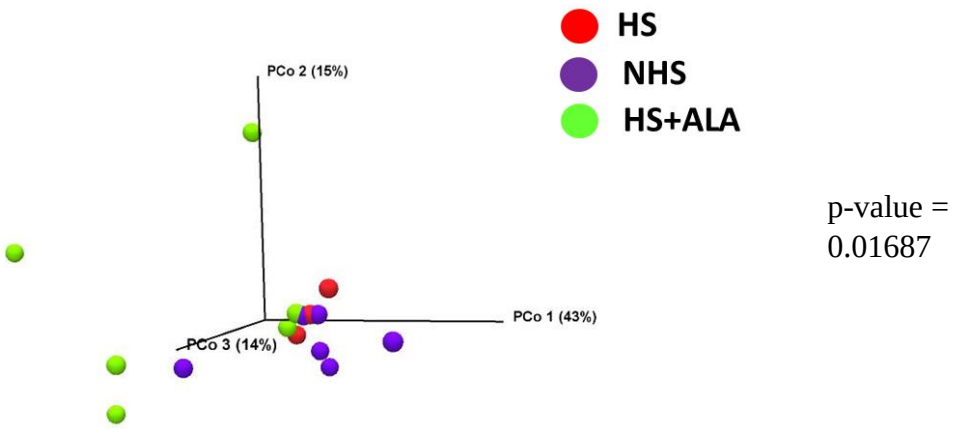
p-value = 0.03991

B)



p-value = 0.08413

C)



p-value = 0.01687

Figure 37 Effects of ALA supplementation on the microbial beta diversity in heat-stressed broilers. A) Bray-Curtis, B) Weighted UniFrac and C) Unweighted UniFrac

CHAPTER 4: DISCUSSION

Various compounds have been fortified in the poultry feed to attenuate the negative effect of heat stress in the poultry. Yet, there has been a necessity of an effective compound that can effectively be used in the poultry industry. Thus, in this study, we used two compounds: dried plum and alpha-lipoic acid and evaluated the mitigatory effects of these compounds in heat-stressed broiler birds.

4.1 Mitigatory effects of dried plum on heat-stressed broilers

In this study, we found that heat stress significantly decreased body weight, ADFI, and ADG, while significantly reduced feed efficiency. These findings are in accord with the previous studies done in the heat-stressed birds. Feed intake in the poultry has been found to reduce by 5% for every 1°C rise in the temperature range of 32-38°C (Kumari et al., 2018). The major reason for this decline in production performance is mainly due to many physiological changes during the stress condition (Pearce et al., 2013). Among them, oxidative stress induced by heat stress is considered as the major factor for the decline in production performance (Luo et al., 2018). Previous studies in heat-stressed birds have found that supplementing vitamins, antioxidants, and phytochemicals were able to improve oxidative stress conditions and improve production performance (Lin et al., 2006). The dried plum (DP) that we used in this study is a good source of polyphenols, antioxidants, vitamins, and minerals. Improvement in the production performance that we observed while fortifying 2.5% DP in feed can be attributed to the antioxidant, polyphenols, vitamins, and minerals present in it. Similarly, we also found significantly lowered FCR in heat-stressed broiler birds supplemented with DP, which is due to better feed consumption along with improved body weight.

As DP was able to improve the growth performance in heat-stressed broiler birds, we further delineated the underlying mechanism behind the mitigatory effect of DP. For that, we took

a systematic approach and considered the different physiological parameters in the gastrointestinal tract as it is considered the main target of heat stress (Lambert, 2009). Ileum, the terminal part of the small intestine, is associated with the absorption of most of the nutrients in the poultry and is more prone to heat stress in chicken (Varasteh et al., 2015). Therefore, in this study, at first, we analyzed different groups of genes in the ileum of the small intestine.

In response to the elevated temperature, the cell possesses two major kinds of protective mechanisms to maintain normal cellular function: firstly, by the production of the heat shock proteins, and secondly, by increasing the production of the antioxidant inside the cell. Heat shock protein (HSPs) are the group of proteins that are produced by the cell under stress condition. These proteins are transcriptionally regulated by heat shock factors (HSFs) (Åkerfelt et al., 2010). Both the HSP70 and HSP90 acts as chaperons, ensure proper folding of the proteins, and have cytoprotective action (Wegele et al., 2004). Therefore, in this study, the expression of *HSP70, HSP90, HSF1*, and *HSF3* was analyzed in the ileum of heat-stressed broiler birds.

Previous studies have reported that HSFs and HSPs are up-regulated in the intestine during the time of acute stress (Varasteh et al., 2015). In this study, however, the chicken subjected to the heat stress has significantly lower expression of *HSF3* and *HSP90*, while the expression of *HSF1* and *HSF70* were not changed as compared to normal birds. The significant lower expression of the *HSP90* may be due to the exhaustion of the protective mechanism inside the cells or decline in expression in chronic stress. Interestingly, the expression of *HSF1*, *HSF3*, *HSF70*, and *HSP90* were increased in heat-stressed birds supplemented with DP. The increased expressions of *HSP70* and *HSP90* are likely due to the increased expression of *HSF1* and *HSF3*. It is reported that HSF1 is found to induce HSP70 (Inouye et al., 2003), and HSF3 is found to promote the expression of all HSPs in chicken (Tanabe et al., 1998). Additionally, increased expression of

HSP70 is also found to increase broilers digestive enzyme activity (Hao et al., 2012), and promote the production of glutathione (GSH), superoxide dismutase (SOD), and total antioxidant capacity (TAOC) (Gu et al., 2012).

In response to the oxidative stress elicited by heat stress in poultry, NRF2-a redox-sensitive nuclear transcriptional factor- is translocated in the nucleus, where it binds in the promotor region of the antioxidant response element in the DNA, leading to the production of different antioxidants which are involved in the detoxication and elimination of reactive oxidants. To better understand the antioxidant status of the heat-stressed birds, the different antioxidant-related genes such as superoxide dismutase 1 *(SOD1),* superoxide dismutase 2 *(SOD2),* glutathione peroxidase 1 *(GPX1),* glutathione peroxidase 3 *(GPX3),* peroxiredoxin 1 *(PRDX1),* thioredoxin *(TXN),* and nuclear factor erythroid 2–related factor 2 *(NRF2)* were analyzed. SOD1 and SOD2 are isoforms of SOD. SOD1, cytosolic Cu/ZnSOD, is mainly localized in the cytoplasm, mitochondrial intermembrane spaces, nucleus, lysosomes, and peroxisomes whereas SOD2 is mitochondrial manganese (Mn) containing enzymes (Fukai and Ushio-Fukai, 2011). Superoxide radical is the predominant free radicals produced inside the cell (Singal et al., 1998). SODs dismutase the superoxide radical into hydrogen peroxide and thus is considered the main element of the first level of antioxidant defense in the cells (Surai, 2016). Similarly, GPX1 and GPX3 are the selenium-dependent forms of glutathione in avian species. GPX1 is mainly localized in the cytoplasm and mitochondria, while GPX3 is most abundant in the plasma. GPXs catalyzes the reduction of hydroperoxides and H_2O_2 by glutathione (Surai et al., 2018). PRDX1 is a member of the peroxiredoxins family, which used thioredoxin to reduce H_2O_2, hydroperoxides, and proximities to balance ROS inside the cell (Neumann et al., 2003). TXN is a key member of the thioredoxin system-important antioxidant system in defense against oxidative stress in the cell.

Moreover, thioredoxin is also involved in immune response, DNA and protein repairs, and cell death (Lu and Holmgren, 2014). In this study, the expression of *NRF2, SOD1, SOD2, GPX1, GPX3, TXN,* and *PRDXN1* were significantly improved in the heat-stressed broiler birds supplemented with the DP. Indeed, significant expression of the *NRF2* along with antioxidant (*SOD1, SOD2, GPX1*, and *GPX3*) indicated that DP was able to activate *NRF2* mediated antioxidants enzymes in the heat-stressed broiler birds. Previous experiments on phytochemicals have demonstrated the polyphenols play a vital role in the activation of the NRF2 mediated antioxidants enzymes (Zhang et al., 2018a; Hu et al., 2019). In agreement with those studies, polyphenols present in the DP possibly may have played a similar role resulting in the upregulation of antioxidant genes in heat-stressed birds, thus reducing ROS and lipid peroxidation within the cell.

Intestinal epithelial tight junctional barrier plays a vital role against paracellular penetration of the pathogenic bacteria, endotoxins, and feeds associated antigens (Anderson and Van Itallie, 1995; Dokladny et al., 2006; Moeser et al., 2007). Occludin (OCLN) and Claudin (CLDN) are the major transmembrane proteins that make up the tight junctions. Occludin help regulates paracellular permeability and plays a key role in cellular structure and barrier function. Claudins form the backbone of tight junctions and play a significant role in the tight junction ability to seal the paracellular space (Lee, 2015). During heat stress, peripheral circulation of the blood increased as a result blood flow in the epithelium in the intestine is reduced resulting in hypoxia which leads to the disruption of tight junction, reduced intestinal integrity and increases intestinal permeability which is found to increase the circulating endotoxins (Pearce et al., 2013). In this study, the expression of the *OCLN* and *CLDN1* was significantly increased in the ileum of heat-stressed broiler birds supplemented with DP. This result indicates that dietary DP was able to improve the

intestinal integrity in the heat-stressed birds, which may be attributed as one of the reasons for the improvement in the production performance in heat-stressed birds. This finding was in line with a similar experiment done by Shin et al. (2018), where they supplemented betaine in the heat-stressed laying hens. Flavonoids, polyphenol compounds, are found to exhibit promotive and protective effects on intestinal tight junction barrier functions (Suzuki and Hara, 2011). Enhanced expression of the tight junction in the heat stress in the DP supplemented birds may differ due to the flavonoids present in it.

Next, we analyzed the different immune-related genes in the ileum of heat-stressed broilers birds. The expression of *IL4* and *MUC2* was significantly increased by supplementing DP. Interleukin 4 (IL4) is a cytokine that plays a vital role in regulating the immune system and is found to play a major role in the differentiation of Th2 cells in response to antigens (Zhu, 2015). Moreover, IL4 also protects lymphoid cells from apoptosis. Besides, it also regulates proliferation, differentiation, and apoptosis in multiple cell types such as myeloid, mast, dendritic, endothelial, muscular, and neuronal cells (Zamorano et al., 2003). Thus, a significant expression of *IL4* indicates the enhanced immune response in heat-stressed broiler birds. Also, we observed enhanced expression of mucin 2 (*MUC2*) in DP supplemented broiler birds. Mucin is the major constituents of mucus layers and plays a pivotal role in protecting the gut from pathogens, acidic chyme, and digestive enzymes. Besides, they are also found to influence nutrient absorption and digestion (Montagne et al., 2004). Considering these facts, significant expression of *MUC2* has probably played a protective role against pathogens along with enhancing nutrient digestion in heat-stressed broilers.

High temperature has been related to the disruption of the intestinal morphology in the broilers. Several studies have shown that heat stress harms intestinal morphology in poultry,

resulting in decreased crypt depth (Burkholder et al., 2008; Sohail et al., 2012) villus height, and villus height ratio (Sohail et al., 2012; Yi et al., 2016). In accordance with those findings, the result of this study demonstrated that the morphology of the ileum was damaged due to epithelial shedding due to intestinal ischemia in the heat-stressed birds (Rivera et al., 2011). Although statistically not significant, supplementing DP was able to improve the villus height and villus height to crypt ratio. VFAs are found to exert tropic effects on the intestinal morphology (Wong et al., 2006). The beneficial effects of DP on the intestinal morphology may be exhibited due to increased colonization of the beneficial bacteria along with the production of higher amounts of short-chain fatty acids in heat-stressed broiler birds.

Volatile fatty acids (VFAs) are produced mainly by the fermentation of the dietary fibers in the cecum of the poultry. Acetate, propionate, and butyrate are the major volatile fatty acids produced in the cecum of the poultry. Interestingly, VFAs are found to play a vital role in the gut and immune homeostasis. Acetate, dominant VFA, is involved in the glycolytic pathway in the muscles; propionate is associated with gluconeogenesis in the liver, while the butyrate serves as a fuel for colonocytes (Jha et al., 2019). In this study, we found that the amount of acetate, propionate, and total VFAs was increased significantly in the heat-stressed birds supplemented with DP. In contrast, the amount of butyrate increased numerically. These improvements in the VFAs can be attributed to the high dietary fibers present in DP. Moreover, these results of VFAs help corroborate our finding of improved growth performance, ileum histomorphology, and expression of tight-junction genes.

Gut microbiota has been found to play a significant role in intestinal health. High temperature, on the other hand, has been associated with the dysbiosis of the cecal microbiota in poultry. Once we found that DP was able to improve the growth performance and other gut health

parameters in this study. We were interested in evaluating the effect of DP on heat-stressed broiler birds for which we performed 16S rRNA sequencing analysis of the cecal content.

This study shows that supplementation of DP was able to significantly improve bacterial richness and dominance of cecal microbiota in heat-stressed broiler birds, which may be attributed due to the high dietary fibers and polyphenols in the DP. Our results showed that heat stress in broilers produces a significant change in unweighted UniFrac measures of beta diversity, which was consistent with the previous study (Xing et al., 2019) and indicates that bacteria having relatively lower dominance were significantly different between the treatments.

Afterward, we determined significantly abundance taxa, where we found that at the order level - *Bacaillale*, at the family level - *Bacillaceae*, *Christensenellaceae*, *Peptostreptococcaceae*, and at the genus level - unknown geneus_*Bacillaceae*, *Anaerotruncus*, unknown Genus of *Christensenellaceae*, and unknown Genus of *Peptostreptococcaceae* were significantly enriched in the heat-stressed broiler birds supplement with the DP.

The family *Bacillaceae* belongs to phylum *Firmicutes.* In a study in drosophila larvae, the relative abundance of the *Bacillaceae* was found to be significantly lower in the cancerous larvae than one who without the tumor. Furthermore, they hypothesized *Bacillaecae* could eliminate cancer cells at the beginning of carcinogenesis, potentially by stimulating the immune system (Jacqueline et al., 2017). Additionally, *Bacillus* spp. member of *Bacillaceae* is found to display antimicrobial, antioxidant, and immune-modulatory activity in the host and has gained significant attention in the past decade as a potential probiotic (Elshaghabee et al., 2017). Considering these facts, we can conclude that significant enrichment of *Bacillaceae* in heat stress broiler birds has played a vital role in improving the immune status in the GI tract.

The family *Christensenellaceae* belongs to phylum *Firmicutes* and is known to play a vital role in human health (Waters and Ley, 2019). *Christensenellaceae* is considered one of the signature taxa of a healthy gut and are shown in conditions associated with inflammation (Mancabelli et al., 2017). Moreover, the significant abundance of the *Christensenellaceae* was found to have a negative correlation with the visceral fat mass (Beaumont et al., 2016) and is also negatively associated with the total cholesterol, and low-density cholesterol (bad cholesterol). Interestingly, a higher amount of abdominal fat is found in heat-stressed poultry (He et al., 2019b). Taken together, a significant abundance of the *Christensenellaceae* in the DP in this study demonstrates its beneficial effect in the gut. *Christensenellaceae* are associated with diets higher in fibers (Waters and Ley, 2019). Thus, we can speculate that the significant abundance of the *Christensenellaceae* in heat-stressed broiler birds supplemented with DP is due to the higher dietary fiber in it.

The family *Peptostreptococcaceae* belongs to phylum *Firmicutes* and is found as the normal commensal of the gut. They are reported to be higher in the gut of healthy rats than one with dysbiosis (Leng et al., 2016). This highlights its role in maintaining gut homeostasis. The *Peptostreptococcaceae* are involved in the production of VFAs from amino acids. Thus, the significant abundance of the *Peptostreptococcaceae* may also have involved in increasing the production of VFAs in the ileum of heat-stressed birds supplemented with DP (Kisuse et al., 2018).

Finally, at the genus level, *Anaerotruncus* was significantly enriched in heat-stressed birds supplemented with DP. Interestingly, *Anaerotruncus* is among one of the 17 strain of the intestinal bacteria that was found to stimulate the regulatory T cells and helped attenuate the inflammation in a mouse colitis model (Atarashi et al., 2013). Also, antibiotic-induced noninfectious colitis in humans is reported to be treated by the community of 17 intestinal bacteria that include *Anaerotruncus* by fecal transplantation (Satokari et al., 2014). Moreover, probiotic

supplementation during the recovery phase after antibiotic administration was found to suppress the growth of Shigella and Escherichia, while blooming *Anaerotruncus* species (Grazul et al., 2016). Considering these, the significant abundance of *Anaerotruncus* in our study highlights its potential role in attenuating inflammation and enhancing immunity in the intestine.

4.2 Mitigatory effects of alpha-lipoic acid on heat-stressed broilers

In this study, we found that heat stress significantly decreased body weight, ADFI, ADG, while significantly reduced feed efficiency. Dietary supplementing ALA was only able to significantly improve final body weight and ADG in the heat-stressed broiler birds. FCR, on the other hand, was numerically lowered while supplementing ALA.

Afterward, we carried out a similar experiment and observed the different gut health parameters as we did in our DP study. Firstly, we studied different groups of genes in the ileum of the heat-stressed broiler birds, i.e., heat shock-related, antioxidant related, tight junction related, and immune-related genes. For heat shock-related genes, we analyzed the expression of *HSF1, HSF3, HSP70*, and *HSP90*, where we found that supplementing ALA was only able to improve the expression of *HSP90* in heat-stressed broiler birds. Similarly, we then looked at the expression profile of different antioxidant related genes as we performed in the DP study. The result showed that only expression of *PRDX1, SOD2,* and *GPX3* was significantly increased by supplementing ALA. These antioxidants (*PRDX1, SOD2,* and *GPX3*) probably helped to scavenge the free radicals present inside the cells. Similarly, ALA was able to significantly improve the expression of a tight-junction gene (*OCLN*) and immune-related gene (*MUC2*). Dietary supplementation of ALA was also able to improve the villus height and villus height to crypt ratio. Interestingly, ALA supplementation significantly increased the amount of acetate, propionate, butyrate, and total VFA

in heat-stressed broiler birds. This improvement in the VFAs probably helped to improve the growth of heat-stressed broiler birds.

Finally, we analyzed cecal microbiota, where we found that supplementing ALA was able to significantly improve the relative abundance of *Lactobacillales* (order), *Lactobacillaceace* (family), *Peptostreptococcaceae* (family), *Lactobacillus*, and unknown genus of *Peptostreptococcaceae* in the heat-stressed broiler birds.

Peptostreptococcaceae belongs to phylum *Firmicutes* and are found to play a role in gut homeostasis. Besides, they are also found to produce VFAs in the intestine (Kisuse et al., 2018). *Lactobacillus* is the gram-positive bacteria that produce lactic acid, which helps to lower the pH and prevent the growth of pathogenic bacteria (He et al., 2019b). Thus, significant dominance of the *Lactobacillus* probably reduced the risk of pathogen amplification and invasion in heat-stressed birds supplement with ALA. Besides, *lactobacillus* is found to have the antioxidant capacity and can remove the ROS to mitigate the damage induced by oxidative stress (Xin et al., 2014). Moreover, *Lactobacillus* is also considered as a potent probiotic to improve gut health (Martín et al., 2019).

Improvement in the body weight and ADG in heat-stressed broilers supplemented with the ALA can be mainly attributed to the improvement in the gut microbiota and VFAs. More specifically, *Lactobacillus* and *Peptostreptococcaceae* might have played a vital role in the improvement of body weight and VFAs. As ALA is also both water and fat-soluble antioxidant and has higher bioavailability (Sohaib et al., 2017), it is speculated that ALA supplementation was effective in scavenging free radicals, resulting in the improvement of gut physiology.

4.3 Conclusion and future direction

In this study, we investigated the mitigatory effects of two different compounds (DP, and ALA) on the heat-stressed broiler birds by observing the growth performances and gut health parameters. Supplementation of 2.5% DP was identified as a novel strategy to mitigate heat stress in broiler birds. We found that fortification of DP was not only able to improve the growth performance but was also able to improve the expression of heat shock protein-related, antioxidant related, tight junction related and immune-related genes; improve villus height and villus height ratio; increased the concentration of VFAs; and also enriched the beneficial bacteria (*Bacillaceae, Christensenellaceae, Peptostreptococcaceae,* and *Anaerotruncus*) in heat-stressed broiler birds. These improvements can be attributed to the high dietary fibers, antioxidants, and polyphenols present in DP.

In the case of ALA, we were able to improve the final body weight and ADG, expression of *HSP90, PRDX1, GPX3, SOD2, MUC2,* and *OCLN*, villus height and villus height ratio, increase the concentration of VFAs and enriched the beneficial bacteria (*Peptostreptococcaceae* and *Lactobacillus*). While comparing mitigatory effects between two compounds, DP is more potent than ALA in this study.

This study identified the fortification of DP and ALA as an effective strategy to mitigate heat-stressed in broilers. Further, mitigatory effects of DP and ALA need to be evaluated in heat-stressed laying hens. Such research will give an idea about its beneficial effects in egg production. Besides, bioactive compounds in DP need to be identified, and different dosages of DP need to be tested to make its inclusion more economical for farmers.

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