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Mechanistic Understanding of Leaky Gut Syndrome in Heat Stressed Broiler Chickens

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Abstract

Broilers, meat-type chickens, are bred for high growth rate and meat yield. These chickens have specific challenges due to heat stress. The adverse effects that impact these chickens have lead us to study the mechanisms involved. The objective of this study is to evaluate the expression of genes involved in intestinal permeability of heat- stressed broiler chickens. All analyses are on previously collected tissues. A total of 600 one day old Cobb 500 male chicks were weighed and randomly assigned to 12 environmental chambers. Ambient temperature in the chambers was gradually decreased from 32˚C on d 1 to 24˚C at d 21. At 8am on d 21, the temperature was increased to 35˚C in 6 of the chambers to induce HS. All chambers reached 35°C within 15 minutes of temperature adjustment. Thermoneutral (TN) chambers were maintained at 24°C. After 2h of HS, the thermologger birds were humanely euthanized via cervical dislocation. The gut was dissected and a ~2cm portion of the duodenum, jejunum, and ileum were collected. These samples underwent RNA extraction and cDNA reverse transcription to prepare for quantification using real-time PCR. This is an additional study to evaluating what genes play a role in leaky gut syndrome in heat stress broiler chickens to further understand the genetic potential of regulating heat-stress. The results of this study showed a down-regulation of HSP 70 in the duodenum under heat-stress and mostly no significant change of HSP 90, HSP 60, HSP27, GRP75, Claudin, Occludin, and Zo-1.

Keywords: chicken, intestinal permeability, tight junctions, heat-shock proteins, gut barrier function

Introduction

Global temperatures have risen 1.4° Fahrenheit since 1880 and most of this increase has occurred since 1975 (1). Rapid warming of the earth is having impact on our livelihoods and livestock. Chickens especially, in comparison to other livestock, are more sensitive to heat stress (47). Chickens have a coverage of feathers, limited sweat glands, and high resting metabolic rate that make it challenging for them to adapt to higher temperature (42). Heat stress can have adverse effects on chickens such as behavioral (e.g. decreased time eating, increased time laying down) and physiological (e.g. increased panting, decreased growth rate, higher mortality) (13,43-44). Cooling facilities can be costly and hard to manage, particularly in parts of the world where energy and water needs are prioritized for human use, so many people have looked toward understanding physiological processes for other means of managing chickens' body temperature increase (41). One area of interest is the effect of heat stress on the gastrointestinal tract. It is known that the gastrointestinal tract is effects by heat stress (12), however the mechanisms involved are not well known.

The Poultry industry in the U.S. has immense economic, environmental, and social impact. Poultry alone provides 1,984,784 jobs in the country and generates about $495 billion in annual economic impact (2). Within the United States Meat Industry, chicken is the most produced meat, totaling to 9 billion chickens and 48 billion pounds processed in 2017 (3). Arkansas is one of the top three poultry processing states (2). Although poultry meat production supports the livelihoods and food security for billions of peoples worldwide, it is facing several challenges including heat stress which is one of the most challenging stressors. Such a large industry demands constant improvement and efficiency to maintain a high level of productivity. Therefore, mechanistic understanding of heat stress responses in poultry is of uppermost interest.

Literature Review

Heat Stress Impact on Broiler Chickens

Heat stress is one of the largest environmental stressors that broilers, a meat type chicken, face. There is a huge demand for chicken in the United States meat industry so the emphasis on weight gain is significant. It has been shown that weight gain is decreased in chickens that are raised in higher temperatures than those raised in normal temperatures (4). Chickens are especially sensitive to HS because of limited sweat glands, their coverage of feathers, and high resting metabolic rate (42). It is suggested that modern genetics in chickens promotes higher metabolic activity (41). It has even been shown that modern broilers with more rapid growth rates have a higher sensitivity to heat stress because of their high metabolic rates (5-6). The impact of body weight gain (BWG) has been shown to be more significant in the last 3 weeks of growth than in the initial first 4 weeks of growth (4, 6). The negative effects of heat stress range from behavioral and physiological to even immune response (13,43-44). In order to maintain thermoregulation, chickens will spend time panting and elevating their wings therefore, chickens under heat stress will spend less time eating, drinking, and being active (7, 13,43). Increase of panting also increases carbon dioxide levels in the body and decreases blood pH. (45). Chickens suffering from heat stress often have decreased growth and higher mortality (7).

Heat Stress Impact on Intestinal health in Broiler Chickens

The intestinal epithelium has two important functions. One function is to serve as barrier to defend against bacteria, toxins, and other microorganisms. The other function is absorption of nutrients (18). Heat stress can have an effect on intestinal barrier function in broiler chickens. Heat stress can lead to decreased body weight, body weight gain, feed intake, and feed efficiency (9). Broilers subjected to heat stress exhibit shorter villus height and higher crypt depth than that

of a thermal neutral bird (10). Many studies use villus height to crypt depth ratio to determine if the gut is out of order. The villus and crypt are where cell apoptosis occurs to maintain cellular homeostasis (24-26). Heat stress can also affect intestinal microflora, with increases in *Salmonella*, *Escherichia coli*, and *Clostridium* colonization (10). Heat stress has a negative effect on the lining of the small intestine, resulting in a condition known as leaky gut, which is detrimental to bird wellbeing, growth, and health. This lining of the small intestine is made up of continuous cells that separate the gut from the external environment. This intestinal barrier, connected by tight junctions, allows for regulation of physiological processes as well as regulated physical diffusion (12). The barriers of the gut are all under neurohormonal control so they can be affected by certain stressors (23). Heat stress can disrupt these junctions and have adverse effects allowing toxins and infectious agents to "leak" in. Leaky gut may leave an animal exposed to various diseases (12). Leaky gut can lead to inflammation, food-sensitivity, skin-issues, and disruption of normal homeostasis (8).

Tight Junction Proteins

The gastrointestinal tract is extremely specialized for absorption and digestion. However, this means that it is a constant battle to keep diseases, toxins, and bacteria out of the body while still maintaining function. Tight junction (TJ) proteins serve as the link for the specialized epithelial cells that serve as a barrier against harmful agents (11, 15). Tight junction proteins are responsible for merging neighboring cell membrane together and closing the intercellular gap between the membranes (21). TJs form a semipermeable barrier which aids in the passage of ions and solutes while preventing the passage of toxins and foreign bodies. The seal that TJs make can involve proteins within the same membrane or involved proteins of neighboring cells (18). It is suggested that tight junction proteins are damaged when heat stress redirects blood from the

gastrointestinal tract to peripheral organs (20). The intestinal barrier integrity is depended upon tight junction proteins for they serve as the gatekeepers for paracellular transport of small molecules, ions, and water (22). ZO-1, a tight junction protein, belongs to a family of multidomain proteins. These proteins belong to a family of membrane-associated guanylate kinase homologs (MAGUKs). This involves them in specialized site such as synapses and epithelial tight junctions. Occludin, a transmembrane component has also demonstrated to contribute to epithelial tight junction barrier (17). Occludin is localized at tight junctions in endothelial and epithelial cells. Occludin is an integral membrane protein (30). Occludin is an adhesion molecule that has been shown to affect solute and ion permeability (31). Similarly, claudin is a transmembrane protein. The claudins are members of a four-membrane spanning protein family. They are in close approximately to occludin (32).

Heat Shock Proteins

Heat shock proteins are produced in response to stress. Expression of heat shock proteins is increased significantly in response to heat stress. Elevated HSP assist in survival by preventing misfolded proteins as well as breaking down denaturized proteins that were damaged due to high temperatures (14, 27). Heat stress has been shown to impair the intestinal integrity (17). Dokladny et al. (2006) noted that there is a significant increase of general heat shock protein expression (HSP 27, HSP 40, HSP 90, and HSP 70) in Caco-2 cells under heat stress. Heat stress has even been shown to induce more pronounced mRNA expression of HSP 70 and HSP 90 in the ileum than compared to the jejunum (11). In a jejunum specific study, HSP 70 and HSP 90 gene expression levels were upregulated under 72 hours of heat stress (14). HSP 70 was upregulated in acute heat stress in broilers (19). There was an increased gene expression in HSP 70 and HSP 90 in short-term heat stress (33). HSP 60 is a chaperonin homolog that is involved in

the folding of polypeptide chains. It has been found in the small intestinal mucosa, however, it has shown to have no ability to protect against acetic-acid perfusion-induced mucosal damage (46). Although, HSP60 is a mediator of the gastro-intestinal immune system (34). HSP 60 has been shown to bind with GRP75 (37). HSP 27 is a member of the heat shock protein family and not only responds to heat stress but oxidative stress and chemical stress. This protein acts as a protein chaperone and is involved in the inhibition of apoptosis as well as actin cytoskeletal remodeling (36). GRP75 is a glucose-regulated protein that is responsive to heat shock in avian species (28) unlike in mammalian species (40). GRP75 is located mostly in the mitochondria and is involved in mitochondrial protein import, folding, and degradation. Further understanding of GRP75 expression in heat stressed broiler chickens could lead to new mechanistic understandings of heat stress regulation. Studies have shown upregulation of GRP75 protein expression in liver and muscle tissues (28).

Materials and Methods

All analyses are on previously collected tissues. All animal care and procedures were approved by the Institutional Animal Care and Use Committee at the University of Arkansas. A total of 600 one day old Cobb 500 by-product male chicks were weighed and randomly assigned to 12 environmental chambers. Each chamber was divided into 2 floor pens covered with fresh shavings and equipped with separate feeders and drinkers. Birds had *ad libitum* access to feed and water throughout the trial. Ambient temperature in the chambers was gradually decreased from 32˚C on d 1 to 24˚C at d 21. At 8am on d 21, the temperature was increased to 35ºC in 6 of the chambers to induce HS. All chambers reached 35°C within 15 minutes of temperature adjustment. Thermoneutral (TN) chambers were maintained at 24°C. Temperature and humidity were continuously recorded in each chamber. Before the onset of HS, 2 birds per pen were

randomly selected and equipped with a Thermochron temperature logger (iButton, Embedded Data Systems, KY) for continuous monitoring of core temperature. After 2h of HS, the thermologger birds were humanely euthanized via cervical dislocation. The gut was dissected and a ~2cm portion of the duodenum, jejunum, and ileum were snap frozen in liquid nitrogen and kept at -80˚C until further processing and analysis.

RNA Extraction

Total RNA was extracted from chickens by Trizol reagent (Life Technologies) according to the manufacturer recommendations. This involved taking a small tissue sample from the small intestines and homogenizing the sample using a bullet blender. Samples are left to sit for 5 minutes in room temperature and 200uL of chloroform was added and mixed well. Sample was then centrifuged at 12,000g for 15 minutes at 4˚C. Top colorless aqueous phase was then transferred to a new 1.5mL tube. 500 ul of isopropanol was added and allowed to incubate at room temperature for 10 minutes. RNA was precipitated by centrifugation at 12,000g for 10 minutes at 4˚C. The supernatant was decanted and the RNA pellet washed with 500uL-1mL of DEPC-EtOH. DEPC is Diethyl pyrocarbonate which is used to inactive RNase enzymes. This was then centrifuged at 12,000g for 3 minutes at 4˚C, after which supernatant was again decanted and a kimwipe was used to remove excess EtOH. The RNA was resuspended in 25 uL of DEPC– H2O. RNA was quantified using the Take3 plate of the SynergyHTX microplate reader Samples were stored at -80˚C until further analysis.

cDNA Reverse Transcription

RNA was reverse transcribed using qScript cDNA SuperMix Synthesis Kit. Samples were combined with reagents qScript cDNA SuperMix (5X), RNA template, and RNase/DNase-free water in 0.2 – mL microtubes sitting on ice. primers for HSP 70, HSP 90, HSP 60, HSP 27,

GRP 75. Ribosomal 18S (GenBank accession no. AF173612, 515 bp): forward, 5′-TCCCCTCCCGTTACTTGGAT-3′ and reverse, 5′-GCGCTCGTCGGCATGTA-3′ was used as a housekeeping gene.

Real-time PCR

The cDNA was amplified by quantitative real-time PCR with Power SYBR green Master Mix. SYBR green was binded to the double stranded cDNA and emitted green fluorescence to determine how much of the target genes were replicated. Primers (forward and reverse) were added so that it only quantifies the target genes. The volume of reagents varied on how many samples were given for the 96-well plate. The volume for Sybergreen was 10 uL* (#Samples+1) * 2. For forward and Reverse primes the volume added was 1 uL*(#Samples+1)*2 for each. Finally H2O added volume was 8uL*(#Sampled+1)*2. The qPCR cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of a two-step amplification program (95°C for 15 s and 58°C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the Sequence Detection system to exclude contamination with unspecific PCR products. Relative expressions of target genes were determined by the $2^{-\Delta\Delta Ct}$ method (48).

Statistical Analyses

Relative expressions of target genes were analyzed using Student t-test via GraphPad Prism v. 6.00 for Windows, GraphPad Software (La Jolla, CA). Differences in gene expression means were significant at $P < 0.05$.

Results and Discussion

The results of the relative gene expression are divided into two groups, heat-shock proteins and tight junction proteins. Heat shock proteins as well as tight-junction proteins were variably expressed in the three different tissue samples, duodenum, jejunum, and ileum.

HSP70 gene expression was down-regulated in the duodenum and although not significant I saw a trend of down-regulation in the ileum and jejunum due to heat stress (Figure 1) (Duodenum p=0.012, Ileum p=0.196, Jejunum p=0.208). Doklandny et al. (2006) saw a significant increase of protein expression in HSP 70 in Caco-2 cells (15). In another study that tested the mRNA expression of HSP70 under heat stress in chickens, there was an upregulation of mRNA expression of HSP 70 in the jejunum and ileum but no significant effects of mRNA levels were observed in the duodenum (11). In a jejunum specific study, gene expression of HSP 70 was upregulated under 72 hours of stress in heat stressed broiler chickens (14). HSP 70 protein expression in the jejunum was upregulated in acute heat stress in broilers (19). There was an increased gene expression in HSP 70 in short-term heat stress (33). Under a chronic heat study in broiler chickens, HSP 70 in liver tissue showed a significant up regulation in mRNA expression but a down-regulation in protein expression (35). HSP70, under a study done with porcine IPEC-J2 cells, HS significantly up-regulated mRNA expression and protein levels (38). There are notable difference in results of this study and comparable studies possibly due to differences in developmental phase of the chickens and heat-stress protocols. Experiments that resulti in upregulation of HSP 70 often involve birds that were in different developmental phases such as 32 or 37 days old (19,33) vs 21 days old in this experiment, which could help explain the differences in results. This suggests that heat-stress has a different effect on birds in different developmental stages. Experiments involving similar age birds or younger, such as 15 days old

(11), usually treated the experimental group for a longer duration. An experiment done with 21-day old chicks, similarly to this study, was instead heat-stressed until day 35. This suggests that longer term heat-stress could have a larger impact on birds. However, this study observed it was only protein expression that was upregulated and not mRNA expression would could implicate differences in expression on that gene level vs. protein level (35).

In HSP 90, I observed no significant difference in RNA expression. (Duodenum p=0.446, Ileum p=0.320, Jejunum p=0.549) (Figure 2). Doklandny et al. (2006) saws a significant increase of protein expression in HSP 90 in Caco-2 cells (15). In another study with chickens under heat stress the mRNA expression of HSP90 was significantly up-regulated in the jejunum and ileum but no significant effects of mRNA levels were observed in the duodenum (11). In a jejunum specific study, gene expression of HSP 90 in 11 and 13 day old Arbor Acres male broiler chickens was upregulated under 24 hours and 72 hours of heat stress in heat stressed broiler chickens (14). There was an increased gene expression in HSP 90 in short-term heat stress (33). Under a chronic heat study in broiler chickens, HSP 90 in liver tissue showed no significant difference in mRNA expression and protein expression (35). Other studies may have yielded different results because of different tissue types as well as different heat stress treatments. An experiment done that observed upregulation of gene expression in acute heat conditions differed in what day heat-stress was induced, day 32, (33). This study used 21 days old broiler which could explain the differences in results. This suggests that heat-stress has a different effect on birds in different developmental stages. Experiments involving similar age birds or younger, such as 15 days old (11), usually treated the experimental group for a longer deration. An

experiment done with 21-day old chicks, similarly to this study, was instead heat-stressed until day 35. This suggests that longer term heat-stress could have a larger impact on birds (35).

HSP 60 gene expression was not significantly changed but there may be a trend toward upregulation (p=0.252) in the jejunum. HSP60 has been shown to bind with GRP75 and may play a role in various inflammatory actions (37).

In HSP 27 I observed no significant difference in RNA expression (Duodenum p=0.595 Jejunum p=0.264, Ileum p=0.587) (Figure 3). Doklandny et al. (2006) saw a significant increase in protein expression of HSP 27 (15). Under a chronic heat study in broiler chickens, HSP 27 showed a significant up regulation in mRNA expression (35). An experiment done with 21-day old chicks, similarly to this study, was instead heat-stressed until day 35. This suggests that longer term heat-stress could have a larger impact on birds (35).

In GRP 75 I observed no significant difference in RNA expression (Duodenum p=0.432, Ileum p=0.490, Jejunum p=0.545) (Figure 4). In another study done in our lab, we studied GRP75 expression in broiler chicken hypothalamus, liver, and muscle tissue under acute (2 –h) heat stress (35˚C). This study showed unchanged protein and mRNA levels in the hypothalamus and upregulation of GRP75 protein expression but not mRNA expression in the liver and muscle. It was noted that GRP75 mRNA and protein levels did not correlate (28). GRP75 might be regulated at translational and posttranslational levels (29).

In Claudin, although not significant, I saw a trend to upregulation (duodenum p=0.222, ileum p=0.187) in both the duodenum and ileum (Figure 5). Uerlings et al. (2018) noticed no affected mRNA levels of Claudin within 72 h of thermal stress in broiler chickens. Claudin-1, under a study done with porcine IPEC-J2 cells, HS significantly down-regulated mRNA expression and protein levels (38). Another study conducted with IPEC-J2 cells resulted in a downregulated expression of Claudin under 41˚C exposure for 24h (39). Other studies may have yielded different results because of different tissue types as well as different heat stress treatments.

In Occludin, I observed no significant difference in RNA expression (p=0.383) in duodenum and (p=0.138) in the ileum (Figure 6). A study conducted with IPEC-J2 cells resulted in a downregulated expression of Occludin under 41˚C exposure for 24h (39). In another study there was an increase in occluding protein expression due to heat stress in Caco-2 cells. This study showed that it may be protein specific because heat exposure caused a decrease in ZO-1 and did not affect claduin-3 (31). Another study by Dokladny et al. (2008) showed an increased expression of occludin protein in HS exposure. Uerlings et al. (2018) noticed a decrease in the mRNA levels of Occludin within 72 h of thermal stress in broiler chickens. Other studies may have yielded different results because of different tissue types as well as different heat stress treatments.

In Zo-1 I observed no significant difference in RNA expression (p=0.461) in the duodenum and (p=0.5291) in the ileum (Figure 7). Uerlings et al. (2018) noticed decrease in the mRNA levels of Zo-1within 72 h of thermal stress in heat stress broiler chickens. Zo-1, under a study done with porcine IPEC-J2 cells, HS significantly down-regulated mRNA expression and protein

levels (38). Another study conducted with IPEC-J2 cells resulted in a downregulated expression of Zo-1 under 41˚C exposure for 24h (39). My results with Zo-1 are consistent with other studies and show and decrease in mRNA expression of Zo-1 under acute heat stress. Other studies may have yielded different results because of different tissue types as well as different heat stress treatments.

Summary

The results of this study showed a down-regulation of HSP 70 in the duodenum under heat-stress and no significant change of HSP 90, HSP 60, HSP27, GRP75, Claudin, Occludin, and Zo-1. In HSP 70, although not significant, I saw a trend of down-regulation in the ileum and jejunum. In HSP 60, although not significant, I saw a trend of upregulation of gene expression in the jejunum. In Claudin, although not significant, I saw a trend to upregulation in both the duodenum and ileum. In comparison to other studies there were a few experimental differences that could account for the differing of results. For example, many different studies used birds in different developmental phases. The different developmental phases in birds may suggest that birds are affected by heat-stress differently depending on what developmental stage they are in. Another experimental difference in other studies is the difference of heat-stress duration that the experimental group was exposed to. Additionally, many other studies heat-stressed their birds for longer or even induced chronic heat-stress as opposed to an acute heat stress such as this study. This may suggest that birds during this duration of heat-stress (~2 hrs) are not yet producing heat-shock proteins in response to heat stress.

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Figures

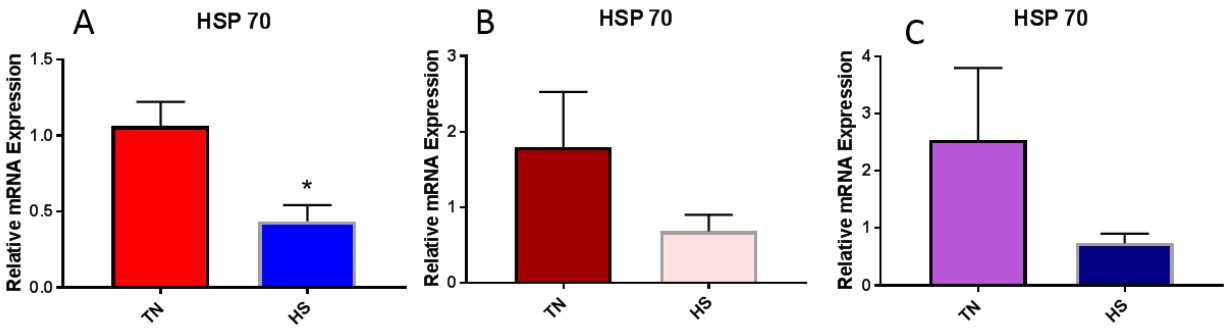

Fig. 1 The effects of heat stress on HSP 70 in broiler chickens. A. qPCR analysis in duodenum (p=0.0124). B. qPCR analysis in ileum (p=0.1961). C. qPCR analysis in jejunum (p=0.2077).

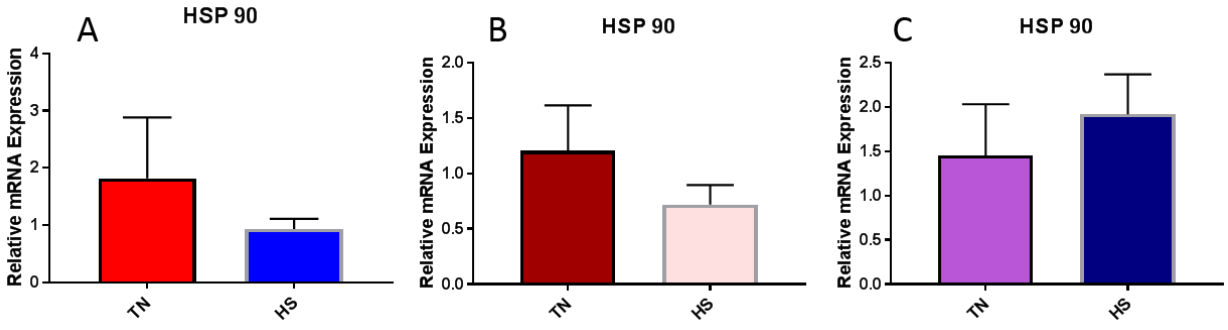

Fig. 2 The effects of heat stress on HSP 90 in broiler chickens. A. qPCR analysis in duodenum (p=0.4463). B. qPCR analysis in ileum (p=0.3199). C. qPCR analysis in jejunum (p=0.5492).

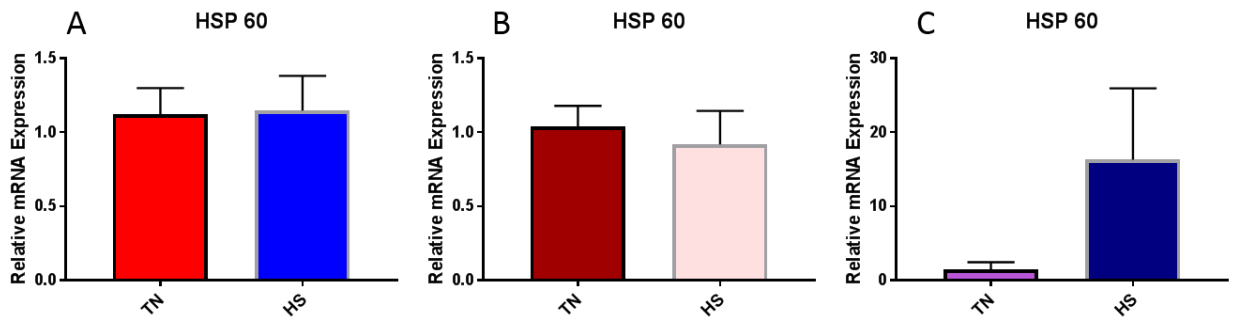

Fig. 3 The effects of heat stress on HSP 60 in broiler chickens. A. qPCR analysis in duodenum (p=0.9256). B. qPCR analysis in ileum (p=0.6697). C. qPCR analysis in jejunum (p=0.2521).

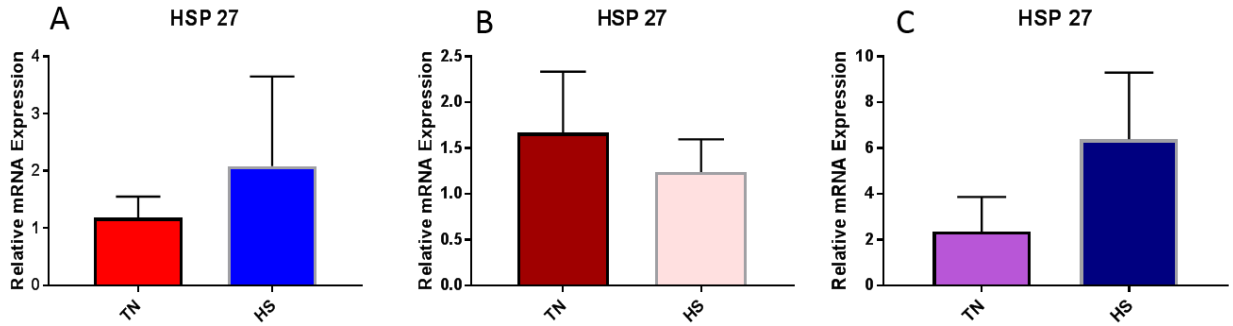

Fig. 4 The effects of heat stress on HSP 27 in broiler chickens. A. qPCR analysis in duodenum (p=0.5949). B. qPCR analysis in ileum (p=0.5874). C. qPCR analysis in jejunum(p=0.2636).

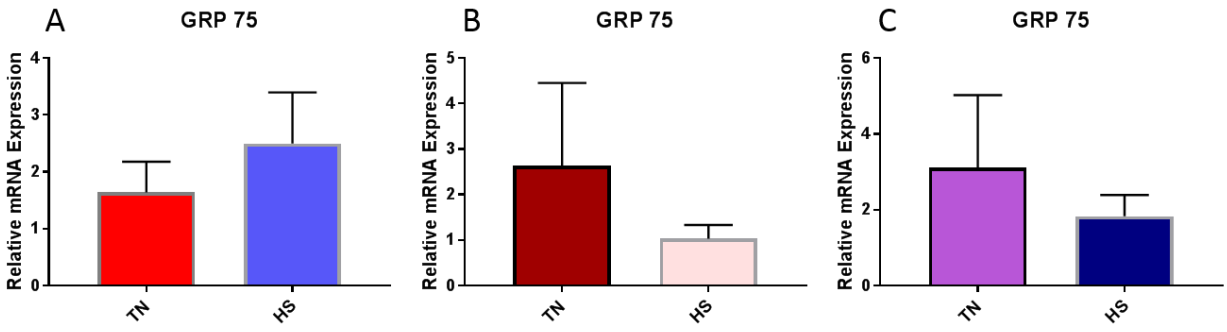

Fig. 5 The effects of heat stress on GRP75 in broiler chickens. A. qPCR analysis

in duodenum (p=0.4323). B. qPCR analysis in ileum (p=0.4903). C. qPCR analysis in jejunum (p=0.54510).

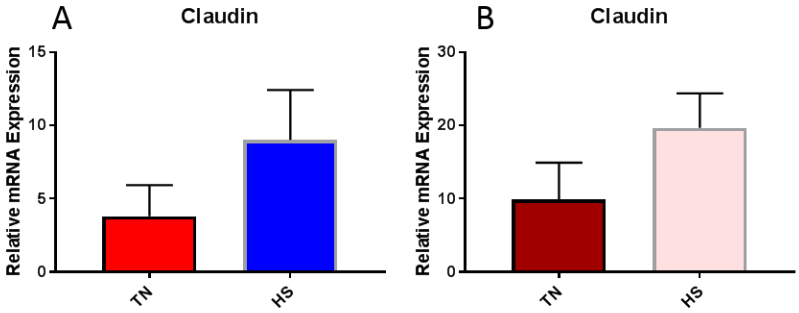

Fig. 6 The effects of heat stress on Claudin in broiler chickens. A. qPCR analysis

in duodenum (p=0.2221). B. qPCR analysis in ileum (p=0.1873).

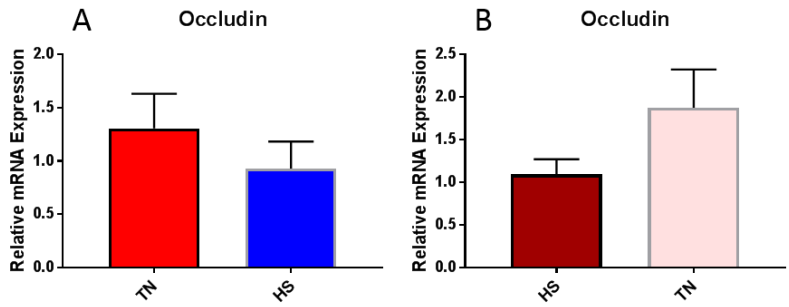

Fig. 7 The effects of heat stress on Occludin in broiler chickens. A. qPCR analysis in duodenum (p=0.3828). B. qPCR analysis in ileum (p=0.1377).

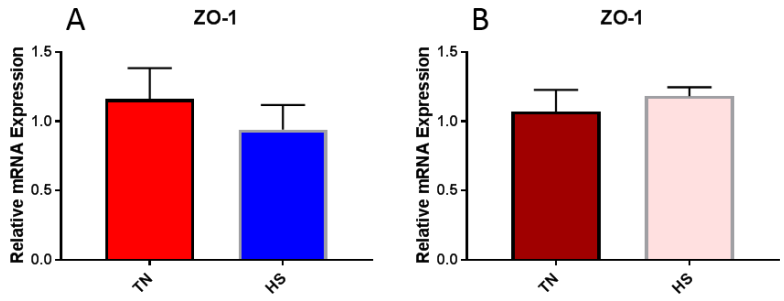

Fig. 8 The effects of heat stress on Zo-1 in broiler chickens. A. qPCR analysis in duodenum (p=0.4608). B. qPCR analysis in ileum (p=0.5291).