Effects of a Noni-Supplemented Diet on Intestinal Tight Junction Proteins and Stress Biomarkers in Heat-Stressed Broiler Chickens

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Effects of a Noni-Supplemented Diet on Intestinal Tight Junction Proteins and Stress Biomarkers in Heat-Stressed Broiler Chickens

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Spring 2020

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Abstract

Heat stress hampers gut health by impeding its ability to absorb nutrients which leads to a lower feed efficiency and sicker birds. Therefore, there is a critical need to identify mechanism-based strategies to alleviate these effects. Polynesian poultry farmers have been supplementing broiler feed with *Morinda Citrifolia* (Noni), a medicinal plant high in anti-oxidants, to address this problem. The purpose of the present study was to evaluate the effect of *Morinda Citrifolia* (Noni)-supplementation on the expression of tight junction proteins. Male Broilers (Cobb 500, 3 wks, n=480) were subjected to 2 environmental conditions (TN, 24°C vs. HS, 35°C, 1 wk exposure), and fed two diets (control vs. 0.2% Noni) in a 2x2 factorial design. Chickens received water and feed *ad libitum*. Functional *in vitro* studies were conducted using IPEC-J2 and primary chicken gut epithelial cells. Once cells reached 80% confluence, cells were exposed to HS conditions (45°C) for 2h and 30 min. Control cells were maintained at 37°C. The expression of target genes and proteins were determined by quantitative real-time PCR using $2^{-\Delta\Delta CT}$ method and Western blot, respectively. A significant increase ($P < 0.05$) in the expression of either heat-shock protein 70 or 90 was observed in both *in vivo* and *in vitro* studies to indicate stress status. One-week HS broilers experienced a downregulation of ZO-2 and occludin tight junction protein expression. Interestingly, HS broilers fed a Noni diet had upregulated ZO-1, ZO-2 gene expression, but both ZO-1 and ZO-2 protein expression were downregulated in the HS Noni-fed broilers. ZO-1 and ZO-2 are essential scaffolding proteins that are key components of tight junction proteins. They allow tight junction proteins to bind to specific location between cells to function as a permeability barrier. Their absence could be responsible for an increase in gut permeability. Noni’s effect on interleukin expression under TN and HS conditions was also measured in which there was a tendency (yet not significant) to decrease IL-18 gene expression.
and significant reduction in IL-10 gene expression in both TN and HS broilers fed a Noni diet. No decreases in tight junction protein expression was visualized via western blotting in HS IPEC-J2 cells. Nonetheless, there is clear fragmentation of Claudin 1, Occludin, and Claudin 5 tight junctions which was visualized via immunofluorescence staining in heat stressed cells. When quercetin was added into the media of IPEC-J2 cells, ZO-2 protein expression was severely downregulated in cells regardless of containing quercetin. In the primary gut epithelial cells, there were no changes in any protein or genes except for a downregulation in Cadherin gene expression in heat stressed cells. Further studies to confirm and expand the results obtained in this study as well as the further characterization of the primary gut epithelial cells deserves further investigation.

**Keywords:** Noni, Heat stress, tight junction proteins, chicken, intestinal epithelial cells, IPEC-J2 cells
Acknowledgments

This work has been made possible by the Bumpers College Undergraduate Research and Creative Award Grant to GT Jr. and the Arkansas Bioscience Institute Equipment Grant and funding from Auyrvet Ltd. to SD.

I would also like to the Center of Excellence for Poultry Science, the Honors College, and my committee members Dr. Sami Dridi, Dr. Charles Rosenkrans, and Dr. Nicholas Greene for motivating and supporting me in completing my honors thesis.

Special thanks to Joshua Flees, Elizabeth S. Greene, Dr. Narayan Rath, and Guillermo Isaias Tellez for assisting me complete my honors thesis.
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Chapter I. Introduction

By 2050, earth is predicted to house a staggering 9.6 billion people and the global demand for animal protein will rise with the population. Poultry offers an efficient source of animal protein that will help feed this growing population. However, efficient poultry production is being challenged by steadily increasing global average temperatures. In the last decade, the intensity and duration of heat waves has been steadily increasing (Howden et al., 2007) and the frequency and severity of these heat waves is only expected to increase (Thornton et al., 2014). In animals, high ambient temperatures have been documented to result in heat stress (HS) which can vary from heat-related discomfort and mild illness to multiple organ damage and even death.

Broiler (meat-type) chickens appear to be especially susceptible to HS as they were genetically selected for rapid growth rate, which produces large amounts of body heat, making them less adapted to living in high environmental temperature (Bonnet et al., 1997). In broiler production, chronic HS has been shown to result in broilers obtaining a lower body weight and reducing feed intake while simultaneously increasing their feed conversion ratio (Lu et al., 2007)(Lara and Rostagno, 2013). In the intestine, HS can lead to permeability of the intestinal epithelium in a process known as leaky gut syndrome by dysregulating the expression and localization of tight junction proteins (Quinteiro-Filho et al., 2010).

As of 2003, HS alone has been estimated to cost the U.S. livestock industry $1.69-$2.36 billion dollars of which the poultry industry accounts for $128-$165 million dollars a year and as global temperatures increase, this number is predicted to rise (St-Pierre et al., 2003). Heat stress poses a severe threat to humans, animals, the agricultural industry, and food security and sustainability. It is therefore necessary to identify effective nutritional strategies to reduce these detrimental effects. *Morinda citrifolia*,(Noni) is a tree in the coffee family, *Rubiaceae,* that extends
through Southeast Asia and Australasia, and has been used by Polynesians for over two millennia for its nutritional and therapeutic effects including antibacterial, antiviral, antifungal, antitumor, antihelmin, analgesic, hypotensive, anti-inflammatory, and immune enhancing effects (Wang et al., 2002) (Wang and Su, 2001). Noni fruit contains a number of phytochemicals, including ligans, oligo and polysaccharides, flavonoids, iridoids, fatty acids, scopoletin, catechin, beta-sitosterol, damnacanthal and alkaloids (Hirazumi and Furusawa, 1999).

Recently, our laboratories evaluated the effect of a Noni-enriched diet on hepatic heat shock protein and lipid metabolism-related genes in heat stressed broiler chickens. While Noni supplementation did not appear to effect growth performance, it did alter the hepatic expression of lipogenic proteins in a time and gene-specific manner. This suggests that Noni may be involved in modulating the stress response in chicken liver (Flees et al., 2017). As the liver and intestine are closely connected, we were interested in seeing if Noni altered the response of the intestine during heat stress. Hence, the purpose of the present study was to evaluate the in vitro and in vivo effect of Noni on the expression of tight junction proteins and other biomarkers involved in heat stress in broiler chickens.
Chapter II. Literature Review

*Modern Challenges of Heat Stress in Broiler Production*

As the planet continues to warm, the frequency of extreme weather events will increase and will have profound consequences on all living organisms, as well as on food production (Howden et al., 2007). Sixteen of the seventeen hottest years ever recorded have occurred since 2001 and climate trends are predicted to continue in an upward trend (Karl and Trenberth, 2003). As this upward trend continues, it will serve as a severe environmental stress factor on all forms of life (Melillo, et al., 1993; Roth and Deheyn, 2013). Poultry in particular appears to be very heat sensitive animals, due to lack of sweat glands and high metabolic activity (Abu-Dieyeh, 2006; Prieto and Campo, 2010). It is estimated that HS alone costs the U.S. broiler poultry industry $125-165 million dollars a year and this number is expected to climb (St-Pierre et al., 2003).

For broiler (meat-type) chickens, the external temperature for optimal performance is 18 to 22ºC (Borges et al., 2003). Under these conditions, the internal body temperature of a broiler is between 40.6°C-41.7°C. Nevertheless, when chickens are placed under HS conditions, their body temperature may be well above that; up to 45°C-47.2°C, which is the lethal limit (Mohanaselvan and Bhaskar, 2014). Heat stress or hyperthermia results from failed thermoregulation that occurs when animals produce or absorbs more temperature than it disperses (Lara and Rostagno, 2013). The adverse effects of HS can range from discomfort to multiple organ damage and, under severe stress, to death by spiraling hyperthermia. The Gut plays a vital role in nutrient absorption, digestion, and transport, yet it is very responsive and susceptible to HS. To reduce the body temperature, avian species have mechanisms to maintain homeostasis. These include convection, evaporative and radiant cooling through vasodilation and panting (Richards, 1970). However, as in mammals, hyperventilation creates a multitude of...
metabolic problems that impact the productivity of the birds such as lower egg shell quality, high mortality, significant increase in feed conversion, immunosuppression, bacterial translocation and leaky gut syndrome (Zeng et al., 2014; Huang et al., 2015; Baxter et al., 2017).

**Heat stress and gut health**

Under thermoneutral conditions, the gut is able to efficiently digest and absorb most nutrients through cell plasma membranes (transcellular transport) using specific receptors and energy expenditure. Epithelial cells in the intestine also serve as a barrier between then external environment and the internal body while allowing the absorption of water and digested nutrients (Elson and Cong, 2012; Salminen and Isolauri, 2006; Salzman, 2011). Any damage in this fragile epithelium, results in gut permeability and translocation of microorganisms to the portal vain leading to systemic infections and chronic inflammation (Ilan, 2012). Stress is known to have a significant impact on the gastrointestinal tract affecting gut motility, ion, fluid, and mucus secretion (Alverdy and Aoys, 1991; Collins and Bercik, 2009; Verbrugghe et al., 2011; Karavolos et al., 2013).

Furthermore, several studies indicate that both acute and chronic stress modify the gut and affect gut permeability by disruption of tight junction proteins (Maejima et al., 1984; Assimakopoulos et al., 2011a; Koh et al., 1996; Matter and Balda, 2007). Some of these alterations are associated with secretion of neurotransmitters and pro-inflammatory cytokines in the brain and in the gut which have profound effects on the gastric and intestinal physiologies (Lamprecht and Frauwallner, 2012; Bailey et al., 2011; Groschwitz and Hogan, 2009). Oxidative stress and free radicals also increases the disruption of the tight junctions, leading to changes in tyrosine kinase
and/or protein tyrosine-phosphatase activities, and modifying the phosphorylated state of junctional proteins (Sander et al., 2005). Intestinal epithelial cells adhere to each other through three distinct intercellular junctional complexes known as Desmosomes, Adheren Junctions (AJ), and Tight Junctions (TJ). Desmosomes are localized dense plaques that are connected to keratin filaments while AJ and TJ both consist of transcellular proteins (Cummins, 2012; Di Pierro et al., 2001). These proteins are connected intracellularly through adaptor proteins to the actin cytoskeleton (Assimakopoulos et al., 2011b). In contrast to transcellular transport, the transfer of molecules through the space between the cells across an epithelium (paracellular transport) is unmediated and passive down a concentration gradient, and this transport is regulated by the TJ (Hu et al., 2013). As multi-protein complexes, TJ not only hold cells together, but they form channels allowing the transport of substances across the epithelium (Awad et al., 2006).

Interestingly, the molecular composition, ultrastructure, and function of TJ is regulated by intracellular proteins through a series of intracellular pathways that includes myosin light kinase (MLCK), mitogen-activated protein kinases (MAPK), protein kinase C (PKC) among others (He and Kogut, 2003). Occludin phosphorylation on Tyr, Ser and Thr is associated with disruption of TJ, hence, phosphorylation of occludin is involved in TJ permeability (Murakami et al., 2009). Any factors that affect the balance between protein kinases and protein phosphatases, such as HS or inflammation can affect gut permeability due to disruption of TJ (Qin et al., 2015; Muthusamy et al., 2014). In contrast, glycosylation of the TJ Junctional adhesion molecule-A (JAM-A) decreases gut permeability (Suzuki et al., 2009; Hirase et al., 2001). TJ regulate epithelial permeability and paracellular diffusion through 2 pathways, leak and pore (Murakami et al., 2012). The leak pathway allows transport of large non charged solutes while the pore pathway allows the transference of large charged molecules (Al-Sadi et al., 2008). As transmembrane
barrier proteins, TJ also function as a fence between the lumen and host (Turner et al., 1999). There are roughly 50 TJ proteins, which include the claudins, occludin, coxsackie viruse, adenovirus receptor (CAR), tricellulin, JAM’s, and scaffolding proteins. The primary role of scaffolding proteins is to regulate stand formation and placement of transmembrane proteins (Turner et al., 1997). Under thermoneutral conditions, paracellular junction are rigorously regulated (Di Pierro et al., 2001). However, under HS conditions, the TJ barrier becomes compromised and luminal substances leak into the blood stream, hence the term leaky gut (Bosenberg et al., 1988), a condition that induce chronic systemic inflammation which requires high resources of energy that impact negatively the performance of the animals. Alterations in gut permeability are associated with bacterial translocation (BT) in the portal and/or systemic circulation in several types of leaky gut syndromes leading to systemic inflammation (Maejima et al., 1984). Similarly, FITC-d is a large molecule (3-5 kDa) which does not usually pass through the intact gastrointestinal tract barrier. However, when there is a disruption of the tight junctions between epithelial cells, the molecule can enter circulation. The presence of gut permeability can be measured by elevated presence of FITC-d levels in the serum after oral administration is used as a (Baxter et al., 2017).

There are few studies that have examined the relationship between HS and intestinal integrity as well as the biological mechanisms associated with the negative effects of HS. In a recent study conducted by Flees et al. (2017), it was demonstrated that HS induces the hepatic expression of HSPs and stimulates de novo fatty acid synthesis in broilers which may explain the increased fat deposition observed during hot season. This study also showed that dietary supplementation of Morinda citrifolia (Noni) regulated the hepatic expression of lipogenic proteins in a time and
gene-specific manner, suggesting that the modulation of hepatic HSP expression by Noni might be involved in modulating the stress response in chicken liver (Flees et al., 2017).

**Noni, the “miracle plant”**

The great Greek physician Hippocrates, also known as ‘the father of medicine’ wrote “Let food be your medicine and let medicine be your food”. This concept applies to *Morinda citrifolia* L., commonly called Noni. The plant’s fruits, seeds, leaves, flowers and root have been used as home therapies by Polynesians for over 2000 years (Boonanantanasarn et al., 2012).

Over 150 nutraceuticals have been identified in Noni, such as scopoletin (Singh, 2007), octoanoic acid (Wang et al., 2002), amino acids, potassium, vitamin A, vitamin C (Swetal and Krishanamurthy, 2013), terpenoids(Nayak et al., 2009), alkaloids(Nayak et al., 2009), anthraquinones (Akihisa et al., 2007), β-sitosterol, flavone glycosides, linoleic acid, Alizarin, acubin, L-asperuloside, caproic acid, caprylic acid, and ursolic acid (Wang et al., 1999), rutin, and a putative proxerone (Potterat and Hamburger, 2007).

Noni is a commonly used medicinal plant in the Polynesian islands boasting over 40 known recorded remedies as an antibacterial (Atkinson, 1956); antiviral (Dixon et al., 1999); antifungal (Kumar et al., 2010); antitumor (Wang and Su, 2001)(Wang et al., 2009); anhelmin (Brito et al., 2009); alleviator of HS (Rajaei-Sharifabadi et al., 2017); analgesic (Basar et al., 2010); incecticide (Kovendan et al., 2012); wound healing (Nayak et al., 2009); bone mineralization (Boonanantanasarn et al., 2012); hypotensive (Abbott and Shimazu, 1985); anti-inflammatory (McKoy et al., 2002)(Akihisa et al., 2007); antioxidant (Su et al., 2005); anti-diabetic (Nerurkar et al., 2015); and immune enhancing effects (Palu et al., 2008). Noni has also been used during
famine periods due to its nutritional value, and for all this reasons is also known as “the miracle plant” (Dixon et al., 1999)(Singh, 2007). Noni is a plant from the coffee family Rubiaceae has many other common names: Canary wood (Australia); Fromager, murier indien (France); Indian mulberry (UK); Lada (Guam, Northern Mariana Islands); Mangal’wag (Yap); Kesengel, lel, ngel (Palau); Kikiri (Solomon Islands); Kura (Fiji): Mora de la India (Spanin); Nen (Marshall Islands, Chuuk); Non (Kiribati); Noni (Hawaii); Nono (Cook Islands, Tahiti); Nnu (Niue, Samoa, Tonga, ‘Uvea/ Futuna); Weipwul (Pohnpei) (Wang et al., 2002).

Noni is a small evergreen tree or shrub 3–10 m in height at maturity. The plant sometimes supports itself on other plants as a liana. There is much variation in overall plant form, fruit size, leaf size and morphology, palatability, odor of ripe fruit, and number of seeds per fruit (Nelson, 2003).

**Objectives**

The objectives of this study were to define the mechanisms employed by HS to alter the broiler intestinal barrier using both *in vivo* and *in vitro models* and to determine the effect of Noni in ameliorating gut health of heat-stressed broilers.
Chapter III. MATERIAL AND METHODS

Animal source, diets, and experimental design

Four hundred and eighty one-day old by-product male Cobb-Vantress broiler chickens (Fayetteville, AR, USA) were randomly sorted into 12 environmentally controlled chambers, each consisting of two pens. Each pen consisted of 20 birds and contained a feeder and water line. Control and experimental diets were formulated to approximate the nutritional requirements of broiler chickens as recommended by the National Research Council (1994), and adjusted to breeder’s recommendations (Cobb-Vantress Inc. 2015). No antibiotics were added to the diet (Table 1). Noni (0.2 %) in starter (1-21 d) and grower (22-29 d) diet was added to the experimental diets and mixed thoroughly in a graded sequence to specified concentration. The birds were given diets and water ad libitum. The chambers started at a temperature of 32°C and were slowly reduced till 24°C at day 21. Chambers were kept at a relative humidity of 55 ± 5%. Both temperature and relative humidity were recorded throughout the course of the experiment. On day 22, birds were placed assigned to either thermoneutral (TN), 24°C or heat stress (HS), 35°C conditions for 1 week. At this time they were fed two grower diets in a 2 x 2 factorial design. Internal body temperatures were monitored utilizing a Thermochron temperature logger (iButton, DS1922L, Maxim, CA). After 1 week of TN or chronic HS conditions, birds were euthanized via cervical dislocation and intestine samples were harvested. Intestine samples were extracted and immediately snap frozen in liquid nitrogen and then stored at -80°C for later gene and protein expression analysis. All experimental procedures involving animals used in this study were conducted in accordance with the recommendations in the guide for the care and use of laboratory animals of the National Institutes of Health and the protocol was reviewed and approved by the University of Arkansas Animal Care and Use Committee.
Cell Cultures

**IPEC-J2.** The intestinal porcine enterocytes cell line (IPEC-J2) are non-transformed immortalized cells commonly used as an *in vitro* model for the small intestine. Cells were cultured in DMEM (DMEM/ Ham’s F-12 [1:1]) supplemented with 5ng/mL human epidermal growth factor (hEGF), 1% insulin-transferrin-selenium (ITS), and 1% penicillin/streptomycin. Once in media, cells were seeded in a plastic tissue culture flask grown at 37°C, 5% CO2. They were passaged once, grown to confluence and then the experiment was underway. Once IPEC-J2 cells reached 80-90% confluency, cells were exposed to one of two environmental conditions (37°C vs. 45°C) and two treatments (50 μM quercetin, QCT vs. control) for 2 ½ hours. For cells inoculated with Quercetin (QCT), the dose was selected based on previous studies (Flees et al., 2017).

Primary Epithelial Gut Cells. Ten one-day-old broiler Cobb 500 chicks were used to cultivate these cells as they are easy to work with and have had no previous access to food reducing the chance of contamination from feed. These chicks were humanely euthanized via decapitation and blood was drained. Birds were sprayed down with 70% ethanol and placed on surgical bench. Duodenum was harvested and washed using 1mL of sterile Phosphate-buffered saline (PBS). The organs were then placed into sterile petri dishes containing oxygenated Dulbecco’s Modified Eagle Medium (DMEM). Organs were then moved to a biosafety cabinet and cleaned of fat and connective tissue. Once cleaned, organs were then moved into a second petri dish containing DMEM. Once all organs were cleaned and in the second petri dish, the cells of the duodenum were expunged by using to sets of forceps to squeezes the contents out. The cells were then placed into a third petri dish also containing DMEM. The cells and media were
pipetted and split into two 15mL conical tubes and spun down for 10 minutes at 300g. The media was then decanted and washed with DMEM and spun down again. The media was then removed and 5mL of collagenase were added. The cells and collagenase were incubated at 37°C, 5% CO₂ for 1 hour. The collagenase was removed, and the cells were again washed in media and spun down. After the removal of the supernatant, 10mL of trypsin were added and incubated at 37°C for 15 minutes with constant agitation. Cells were then spun down and the supernatant was removed. The cells were then resuspended in complete media and filtered. Cells were then spun down again decanted and then resuspended in complete media and then plated and kept at 37°C 5% CO₂. The complete media consists of DMEM, high glucose (4.5 g/L), with L-glutamate and sodium pyruvate supplemented with 10% FBS, EpiCGS (5mL/500mL media), and 1% penicillin/streptomycin. Once primary gut cells reached 80-90% confluency, cells were exposed to one of two environmental conditions (37°C vs. 45°C) for 2 ½ hours.

**RNA Isolation, Reverse Transcription (RT), and Quantitative Real-Time PCR**

Total RNA was isolated from Intestine samples and primary gut cells using Trizol® reagent (ThermoFisher Scientific, Rockford, IL) as directed by the manufacturer. We evaluated RNA integrity and quality by using 1% agarose gel electrophoresis. The purity and concentrations of the RNA were assayed by Take 3 Micro-Volume Plate using Synergy HT multi-mode micro plate reader (BioTek, Winooski, VT). RNA samples were then treated with DNase and reverse transcribed (1 μg) using qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD) as reported in Rajaei-Sharifabadi H. et al. (2017). Real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) was also conducted as indicated in Rajaei-Sharifabadi H. et al. (2017) utilizing 5 μg of 10X diluted cDNA, 0.5 μM of each forward and reverse primer, and
SYBR Green Master Mix (ThermoFisher Scientific, Rockford, IL) to amplify the expression of target genes. The qPCR cycling conditions were adapted from Lassiter et al. (2015). The relative expression of target genes was established by the $2^{-\Delta\Delta C_{T}}$ method (Schmittgen and Livak, 2008). Ribosomal 18s was used as a housekeeping gene while the TN control diet group was utilized as a calibrator.

**Protein Isolation and Western Blot Analysis**

Intestine tissue, primary gut cells, and IPEC-J2 cells were isolated as exemplified by Lassiter et al. (2015). Once isolates were created, their total protein concentrations were detected utilizing Synergy HT multi-mode microplate reader (BioTek, Winooski, VT) and a Bradford assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin utilized as a standard. Once total Protein samples were quantified (30 μg for cells, 70 μg for tissues), they were run on NuPAGE 4-12% Bis-Tris Gels (Life Technologies, Waltham, MA) and underwent Western blotting as described in (Nguyen et al., 2015) and subjected to immune blotting. Pre-stained molecular weight marker (Precision Plus Protein Dual Color) was used as a standard (BioRad, Hercules, CA). The following polyclonal antibodies were used: goat anti-claudin 1 (Santa Cruz Biotechnology, Dallas, TX), rabbit anti-glycosylated and phosphorylated claudin 5 (Santa Cruz Biotechnology, Dallas, TX), rabbit anti- HSP 60 (Santa Cruz Biotechnology, Dallas, TX), mouse anti-HSP 70 (ThermoFisher Scientific, Waltham, MA), rabbit anti-HSP 90 (ThermoFisher Scientific, Waltham, MA), rabbit anti-occludin (Santa Cruz Biotechnology, Dallas, TX), rabbit anti-TAZ (Abcam, Cambridge, MA), rabbit anti-ZO-1 (Abcam, Cambridge, MA), and rabbit anti-ZO-2 (ThermoFisher Scientific, Waltham, MA), and rabbit anti-GAPDH as a housekeeping protein (Santa Cruz Biotechnology, Dallas, TX). Secondary antibodies were used at a 1:5000 dilution for
1 hour at room temperature. The signal was visualized by enhanced chemiluminescence (ECL plus; GE Healthcare Bio-Sciences, Buckinghamshire, UK) and captured by FluorChem M MultiFluor System (Proteinsimple, Santa Clara, CA). Image Acquisition and Analysis were performed by AlphaView software (Version 3.4.0, 1993–2011, Proteinsimple, Santa Clara, CA).

**Immunofluorescence Staining**

Immunofluorescence staining was performed as previously described in (Dridi et al., 2012). IPEC-J2 cells were grown in chamber slides until cells reached 80-90% confluency. Cells were thereafter fixed with methanol for 20 min at −20°C. And then blocked with serum-free protein block (Dako, Carpinteria, CA) for 1 hour at room temperature. Cells were subsequently incubated with primary antibodies (1:200, in Antibody Diluent, Dako, Carpinteria, CA) overnight at 4°C. The signal was visualized with DyLight 488- or 590-conjugated secondary antibody (Thermo Fisher Scientific, Grand Island, NY). Slides were cover slipped with a Vectashield with DAPI (Vector Laboratories, Burlingame, CA), and images were acquired and analyzed using Zeiss Imager M2 and AxioVision software (Carl Zeiss Microscopy).

**Statistical Analysis**

*In vivo* and *in vitro* results involving Noni/QCT were analyzed by two-factor ANOVA with diet (Control vs. Noni) and environmental conditions (TN vs. HS). *In vivo* and *in vitro* results involving only changes in temperature were analyzed by one-way ANOVA, with environmental conditions (TN vs. HS) being the variables. Significant differences among the means were determined by Tukey's multiple-range test at P<0.05 using GraphPad Prism version 6.00 for
RESULTS

1- Heat stress modulates the expression of HSPs, tight junction proteins, and inflammatory cytokines in broiler gut

As depicted in Figure 1, HS exposure for 1 week significantly upregulates protein expression of HSP70 (Figure 1-b) indicating a stress status in the chicken gut. Concomitant with these changes, the expression of occludin, and ZO-2 was downregulated in the gut of heat-stressed compared to TN birds (Figure 1-b). There was no detected change in the protein expression of ZO-1, HSP 90, Phosphorylated Claudin 5, Glycosylated Claudin 5, or TAZ (Figure 1-c). Heat-stressed broilers experienced no significant difference in mRNA abundance of Claudin 1, Occludin, HSP 70, ZO-1, or ZO-2.

Figure 2 summarizes the effects of a Noni-supplemented diet on the gene expression for Interleukins in broiler chickens. Under both thermos neutral and HS conditions, broilers fed a noni-supplemented underwent a significant reduction in IL-10 mRNA production. However, no significant changes were observed on IL-18 under thermos neutral or HS conditions (Figure 6 a and b).
2- Heat stress modulates the expression of HSPs and tight junction proteins in IPEG-J2 and chicken primary gut cells

Figures 2 shows the results of the effect of HS on expression of tight junction and heat shock proteins in IPEC-J2 cells. IPEC-J2 cells that were heat stressed for 2.5 hours were observed to have a significant upregulation of HSP 90 protein expression (Figure 2-b). No significant differences were observed in HSP 70, HSP 60, Occludin, or TAZ protein (Figure 2-b). However, immunofluorescence staining of IPEC-J2 revealed a fragmentation of Claudin 1, Occludin, and Claudin 5 under heat stressed. The immunofluorescence for ZO-1 was not properly visualized (Figure 2-c).

The results of the effect of HS on the expression of several genes and proteins in primary gut cells are summarized in Figure 3. There was no detected change in the protein expression of occludin, phosphorylated claudin 5, glycosylated claudin 5, or claudin 1 (Figure 3-b). No changes were detected also in the mRNA expression of Claudin 1, Calprotectin, Lipocalin, Occludin, ZO-2, Muc2, Vil 1, Catenin, ZO-3, GJA-1, PAT-J, or JAM-1 (Figure 3-c and d). However, there was a significant reduction (P<0.05) in the relative mRNA expression of Cadherin in cells subjected to HS (Figure 3-d). An immunofluorescence of the primary gut epithelial cells revealed prevalent presence of claudin 1, claudin 5, occludin, and ZO-1 in the primary gut cells.

3- Effect of Noni supplementation on gut integrity in heat-stressed broilers

Figure 4 summarizes the effect of Noni supplementation on expression of tight junction and heat shock proteins in heat stressed broilers. Under TN conditions, supplementation of Noni, significantly upregulated the mRNA abundance of ZO-1, ZO-2, ZO-3, and YAP-1 when
compared against TN control diet-fed broilers (Figure 4-c, d, e, and h). Gene expression for ZO-1, ZO-2, and ZO-3 was also found to be upregulated in broilers fed a Noni diet under HS conditions when compared to broilers fed a standard control diet under HS conditions (Figure 4-c, d, and e). YAP-1 gene expression was also upregulated in broilers fed a control diet under HS conditions when compared to broilers fed a control diet at TN conditions (Figure 4-h).

Nonetheless, protein expression for ZO-1 was downregulated in HS Noni fed birds when compared to their TN control diet-fed counterpart and it was also downregulated in broilers fed a Noni diet under HS conditions when compared to broilers given a noni diet under TN conditions (Figure 4-c). ZO-2 was also downregulated in broilers fed a noni diet under HS conditions when compared against broilers fed a control diet at HS conditions and broilers fed a Noni diet at TN conditions (Figure 4-d). Protein expression for phosphorylated claudin 5 was upregulated in broilers given a supplemented diet under HS conditions when compared to broilers given a control diet under HS conditions (Figure 4-b). No significant changes in Occludin, Claudin 1, or HSP 90 mRNA expression occurred between any group (Figure 4-f, g, and i). Similarly, no significant changes in protein expression occurred between Glycosylated Claudin 5, Occludin, Claudin 1, HSP 90, or TAZ (Figure 4-b).

4- Effect of quercetin on tight junction protein in heat-stressed IPEG-J2 cells

The results of the effect of QCT in thermoneutral and heat stressed IPEC-J2 cells are shown in Figure 5. Noni contains several known active compound one of them being QCT, a well characterized compound. Therefore, this study was used to study the effects of QCT on IPEC-J2 cells. IPEC-J2 cells subjected to HS regardless of being treated with QCT supplemented media experienced a significant decrease in ZO-2 protein expression when compared to the their
thermoneutral counterpart (Figure 5-c). No significant changes in ZO-1, occludin, or glycosylated claudin 5 were detected.

**DISCUSSION**

Heat stress adversely affects growth performance and inflicts heavy economic losses to the poultry industry. Hence, new alternative mechanism-based strategies to alleviate the negative effects induced by HS are needed. The tropic medicinal plant, *Morinda citrifolia* (known as Noni), is being used in livestock nutrition, though the literature is limited and conflicting for its impact on growth performance. Recently, our laboratory has evaluated the effect of Noni on feeding and drinking behavior as well as on the hypothalamic expression of stress and metabolic-related genes in broiler chickens exposed to acute HS (Rajaei-Sharifabadi et al., 2017). At molecular levels under HS conditions, Noni supplementation down regulated the hypothalamic expression of HSP90 and its related transcription factors HSF1, 2, and 4, increased orexin mRNA levels, and decreased the phosphorylation levels of AMPKα1/2Thr172 and mTORSer2481, suggesting that Noni supplementation might modulate HS response in broilers through central orexin-AMPK-mTOR pathways (Rajaei-Sharifabadi et al., 2017). In another study, although Noni-enriched diet on heat stressed broiler chickens did not affect growth performance, Noni supplementation regulated the hepatic expression of lipogenic proteins in a time and gene-specific manner, suggesting that Noni might be involved in modulating the stress response in chicken liver (Flees et al., 2017).

Noni is a plant rich in antioxidants with a long history of being used as a medicinal plant in the Polynesian islands. Today, farmers are choosing to supplement their livestock feed with Noni to
enhance growth and overall health (Singh, 2007). However, in the scope of TJ integrity under HS conditions, no studies have been conducted to evaluate the effect of Noni. In the present study, while Noni increased the gene expression of ZO-1 and ZO-2 in broilers subjected to HS conditions, protein expression for both ZO-1 and ZO-2 is decreased in those same broilers given a noni diet under HS conditions. It is important to note that ZO-2 protein expression did decrease regardless of being supplemented with noni. The role of ZO-1 and ZO-2 scaffolding proteins is to hold the tight junctions in certain locations to prevent permeability (Lu et al., 2014). When the amount of scaffolding proteins decreases, TJ’s are no longer stabilized to certain crucial locations between cells and begin to fragmentize (Liao et al., 2008). This fragmentation of TJ between cells, which is evident in the IPEC-J2 immunofluorescence, can be inferred to disrupt the intestinal barrier and cause increase gut permeability and induce leaky gut syndrome as has been previously reported (Assimakopoulos et al., 2011b). Noni does however appear to increase phosphorylated claudin 5 protein expression under HS conditions which is that activated form of claudin (Yamamoto et al., 2008);(Ishizaki et al., 2003). Yet as stated before though there might be an increase in the phosphorylated claudin 5, the decrease in ZO-1 and ZO-2 scaffolding proteins means that these proteins are not being properly held in place (Ballard et al., 1995).

Tight junctions have conserved mechanisms in various organisms (Stumpf et al., 2013). Though the IPEC-J2 cells exhibited similar tendencies as broilers in expressing TJ’s, making them a suitable cell model for chicken yet they are not the most optimal model for chicken as these cells come from mammalian neonatal piglet cells. Our work in developing a primary cell culture is to create a more representative model of the intestines of chickens. As seen in their immunofluorescence, these cells express claudin 1, claudin 5, ZO-1, and occludin all of which are present in the intestines of broilers (Figure 3-e). These cells appear to have the same
tendencies in TJ protein expression as those found in broiler gut tissue samples under TN and HS conditions.

Though Noni does lead to a decrease in ZO-1 and ZO-2, its high antioxidant levels are evident as shown by it tendency (yet not significant) to decrease IL-18 expression in both TN and HS conditions. It was also interesting to observe that under both thermoneutral and HS conditions, broilers fed a noni-supplemented diet showed a significant reduction in IL-10 mRNA production. These findings are unexpected considering the anti-inflammatory effects that Noni has on have been demonstrated in other species by several investigators (Singh, 2007; Swetal and Krishnamurthy, 2013). Nevertheless, Noni has been also capable of stimulating the release of several mediators from murine effector cells including TNF-α, IFN-γ, IL-12 and nitric oxide (Wang et al., 2002; Liu et al., 2001), which are pro-inflammatory cytokines and compounds with negative effects on TJs proteins (Awad et al., 2017; Piche, 2014; Lu et al., 2014). Further studies to confirm and expand the results obtained in this study as well as the further characterization of the primary gut epithelial cells deserves further investigation.
REFERENCES


Atkinson, N. 1956. ANTIBACTERIAL SUBSTANCES FROM FLOWERING PLANTS. Australian Journal of Experimental Biology & Medical Science 34.


Table 1. Ingredient composition and nutrient content of broiler chicken corn-soybean based diets used in all experiments on as-is basis with or without 0.2 % Noni

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<td>Phosphorus (%)</td>
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*a* Inclusion of 10⁶ spores/g of feed mixed with calcium carbonate.

*b* Vitamin premix supplied the following per kg: vitamin A, 20,000 IU; vitamin D3, 6,000 IU; vitamin E, 75 IU; vitamin K3, 6.0 mg; thiamine, 3.0 mg; riboflavin, 8.0 mg; pantothenic acid, 18 mg; niacin, 60 mg; pyridoxine, 5 mg; folic acid, 2 mg; biotin, 0.2 mg; cyanocobalamin, 16 µg; and ascorbic acid, 200 mg (Nutra Blend LLC, Neosho, MO 64850).

*c* Mineral premix supplied the following per kg: manganese, 120 mg; zinc, 100 mg; iron, 120 mg; copper, 10 to 15 mg; iodine, 0.7 mg; selenium, 0.4 mg; and cobalt, 0.2 mg (Nutra Blend LLC, Neosho, MO 64850).

*d* Ethoxyquin.
Table 2. Oligonucleotide real-time qPCR primer

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*a Accession number refer to Genbank (NCBI). GJA-1, Gap Junction Protein Alpha 1; HSP 70, Heat Shock Protein 70; HSP 90, Heat Shock Protein 90; IL-10, Interleukin 10; IL-18, Interleukin 18 JAM-A, Junction Adhesion molecule A; Muc-2, Mucin 2; PATJ, InaD- like protein; VIL1, Vilin-1; YAP-1, Yes Associated Protein 1; ZO-1, Zonula occludens-1 ZO-2, Zonula occludens-2; ZO-3, Zonula occludens-3.
Figure 1.

**WB**

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**PCR**

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Target Protein Level/GAPDH, AU

Control
Heat Stress
Figure 2.

**a**

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**b**

Bar chart comparing the target protein levels (GAPDH, AU) under Control and Heat Stress conditions. The chart shows HSP 90, HSP 70, HSP 60, Occludin, and TAZ.

**c**

Images showing immunofluorescence analysis under Control and Heat Stress conditions, highlighting Claudin 1, Occludin, Claudin 5, ZO-1, and Merge.
Figure 3.

(a) Control and Heat Stress

- **Occludin**: KDa 63
- **Gly/P-Claudin 5**: 35, 23
- **Claudin 1**: 22
- **GAPDH**: 27

(b) Target Protein Level/GAPDH, AU

- **Occludin**
- **Gly-Claudin 5**
- **P-Claudin 5**
- **Claudin 1**

(c) mRNA

- **Claudin 1**
- **Occludin**
- **ZO-1**
- **ZO-3**

- **Control**
- **Heat Stress**

(d) Target Protein Level/GAPDH, AU

- **Cadherin**
- **Catenin**
- **GJA-1**
- **JAM-A**
- **Muc 2**
- **PATJ**
- **Vil1**

- **Control**
- **Heat Stress**

(e) Claudin 1 and Claudin 5

- **ZO-1 and Occludin**
Figure 4.

(a) Western blot analysis showing the expression levels of Claudin 1, ZO-1, ZO-2, HSP 90, Occludin, TAZ, Gly/Claudin 5, and GAPDH in TN-C, TN-N, HS-C, and HS-N groups. The molecular weights are indicated in kDa.

(b) Bar graphs showing the mRNA levels of ZO-1, ZO-2, HSP 90, Occludin, TAZ, Gly-Claudin 5, P-Claudin 5, and Claudin 1 in TN-C, TN-N, HS-C, and HS-N groups. Significance levels are indicated by letters a and b.

(c) mRNA levels of ZO-1 in control and noni groups.

(d) mRNA levels of ZO-2 in control and noni groups.

(e) mRNA levels of ZO-3 in control and noni groups.

(f) mRNA levels of Occludin in control and noni groups.

(g) mRNA levels of Claudin 1 in control and noni groups.

(h) mRNA levels of HSP 90 in control and noni groups.

(i) mRNA levels of YAP-1 in control and noni groups.
Figure 5.

(a) Representative Western blots showing the expression levels of ZO-1, ZO-2, Occludin, Gly-Claudin 5, and GAPDH in TN-C, TN-Q, HS-C, and HS-Q groups. The molecular weights (kDa) of the proteins are indicated.

(b) Graph showing the expression levels of ZO-1/GAPDH in TN and HS groups.

(c) Graph showing the expression levels of ZO-2/GAPDH in TN and HS groups.

(d) Graph showing the expression levels of Occludin/GAPDH in TN and HS groups.

(e) Graph showing the expression levels of Gly-Claudin 5/GAPDH in TN and HS groups.
Figure 6.

(a) IL-18 mRNA levels in TN and HS. The graph shows the expression levels of IL-18 mRNA, comparing Control and Noni treatments in TN and HS conditions. The y-axis represents the IL-18 mRNA expression level, while the x-axis indicates the treatment groups (TN and HS).

(b) IL-10 mRNA levels in TN and HS. Similar to the IL-18 mRNA graph, this one compares Control and Noni treatments in TN and HS conditions. The y-axis represents the IL-10 mRNA expression level, and the x-axis indicates the treatment groups (TN and HS).
**Figure 1.** Effect of HS on expression of TJs and HSPs in broilers subjected to HS. Levels of ZO-1, ZO-2, HSP 90, HSP 70, occludin, TAZ, phosphorylated claudin 5 and glycosylated claudin 5 were determined by western blot (a) and their relative expression (b) was presented as a normalized ratio to housekeeping GAPDH protein. Data are represented as mean ± SEM (n=4/group). Relative expression of TJs (c) mRNA was measured by real-time RT-PCR. Data are represented as mean ± SEM (n=4/group). Treatment means without a common letter indicate significant difference at P < 0.05.

**Figure 2.** Effect of HS on expression of TJs and HSPs in IPEC-J2 cells. The relative expression of HSPs, occludin and TAZ were determined by western blot (a) and their relative expression (b) was presented as a normalized ratio to housekeeping GAPDH protein. Data are represented as mean ± SEM (n=3/group). Treatment means without a common letter indicate significant difference at P < 0.05. Immunofluorescence staining (c) shows that HS induces fragmentation of claudin 1, occludin, and claudin 5 in IPEC-J2 cells.

**Figure 3.** Effect of HS on expression of TJs in primary gut cells. Levels of occludin, glycosylated claudin 5, phosphorylated claudin 5, and claudin 1 were determined by western blot (a) and their relative expression (b) was presented as a normalized ratio to housekeeping GAPDH protein. Data are represented as mean ± SEM (n=2/group). Relative expression of TJs (c) cell adhesion molecules (cadherin and catenin), gia-1, jam-1 muc2, patj, vill (d) mRNA was measured by real-time RT-PCR. Data are represented as mean ± SEM (n=4/group). Treatment means without a common letter indicate significant difference at P < 0.05.

**Figure 4.** Effect of Noni supplementation on expression of TJs and HSPs in heat stressed broilers. Levels of ZO-1, ZO-2, HSP 90, occludin, TAZ, phosphorylated claudin 5 and glycosylated claudin 5, and claudin 1 were determined by western blot (a) and their relative expression (b) was presented as a normalized ratio to housekeeping GAPDH protein. Data are represented as mean ± SEM (n=2/group). Relative expression of TJs (c-g), HSP 90(h), and YAP-1 (i) mRNA was measured by real-time RT-PCR. Data are represented as mean ± SEM (n=4/group). Treatment means with an * indicate main effect of temperature. Treatment means without a common letter indicate significant difference at P < 0.05.

**Figure 5.** Effect of Quercetin in thermoneutral and heat stressed IPEC-J2 cells. Protein levels of ZO-1, ZO-2, occludin, and glycosylated claudin 5 were determined by western blot (a) and their relative expression (b-e) was presented as a normalized ratio to housekeeping GAPDH protein. Data are represented as mean ± SEM (n=2/group). Treatment means with an * indicate main effect of temperature at P < 0.05.

**Figure 6.** Effect of a Noni-supplemented diet on Interleukin production. Relative expression of IL-18 (a) and IL-10 (b) mRNA was measured by real-time RT-PCR. Data are represented as mean ± SEM (n=4/group). Treatment means with an * indicate main effect of diet at P < 0.05.