Investigations Into Antioxidant Effects Of Rutin Supplementation On Neonatal Holstein Calves And Broilers

by

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Chapter 1

Literature Review

1.1 Introduction

Halliwell et al. (1995) describes an antioxidant as any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate.[1] These processes, which occur automatically and are therefore referred to as autoxidation (lipid peroxidation), are free radical chain reactions and are facilitated by a number of factors.[2] Free radicals are substances that possess a single unpaired electron that can damage tissues. These reactive oxygen species (ROS) play an important role in the body's immune response, redox regulation of gene transcription, and cell signaling.[3] However, the resulting cascade of ROS may be detrimental to tissue. Under normal conditions, antioxidant systems disrupt this cascade of ROS by trapping reactive substrate radicals and peroxide radicals before they can react with oxygen or another substrate.[2] The endogenous antioxidative defense system, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione (GSH) and others, allows the elimination of excess ROS including, among others superoxide anions ($O2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), alkoxyl radicals ($RO^{\bullet}$) and peroxyradicals ($ROO^{\bullet}$).[4] Without exogenous antioxidants, such as vitamin C, vitamin E, carotenoids, and polyphenols, these endogenous antioxidant systems would not be complete, as they play an essential role in many antioxidant mechanisms in living organisms. A healthy body maintains an equilibrium of these oxidative and antioxidative processes. When this equilibrium is compromised, such as during pathological conditions, oxidative processes may increase, overwhelming antioxidant systems. This condition is commonly referred to as oxidative stress and can be harmful to surrounding

tissues and can lead to increased risk of developing an infection. Oxidative stress contributes to many pathological conditions and diseases, including cancer, neurological disorders, atherosclerosis, hypertension, ischemia/perfusion, diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, and asthma.[5]

Although all animals are equipped with an endogenous antioxidant system to fight the continuous production of free radicals by the metabolic activities of the body, there is a limit to the protection offered by this endogenous antioxidant system.[6] This limit is further compromised by factors such as consumption of a rancid diet, intake of mycotoxins, nutritional deficiency, pathogenic infection, weaning, and heat stress.[6] In addition, post-slaughter conditions elicit the loss of efficiency in the biological antioxidant system resulting in lipid deterioration in muscle tissues and consequent oxidative rancidity in meat products.[6–8]

Due to a multitude of published research studies demonstrating the positive effects of antioxidants on health and production of livestock,[6] the supplementation of dietary antioxidants in feed is gaining popularity all over the world. Previously, antioxidant substances were almost exclusively used in the livestock industry as feed additives to prolong the shelf life of feedstuffs based on their effect for preventing lipid peroxidation.[6] It has been discovered that in addition to increasing the shelf life of feed, dietary supplementation of vitamin E, and other antioxidants, to cattle, lamb, and poultry has been shown to improve meat quality.[9,10] Recent studies in cattle have also shown the positive effects antioxidant supplementation may have on oocyte and embryo quality and developmental competence under heat stress conditions.[5,11,20,12–19] An increasing number

of studies have also shown that dietary antioxidants can alleviate oxidative stress in livestock and improve the quality of animal products.[6]

The antioxidants used in animal feeds can be categorized into two categories: natural and synthetic.[6,21] Natural antioxidants are commonly found in feedstuffs, especially plant-based materials. Although these antioxidants are naturally occurring, only a few are used commercially in the livestock industry. Vitamin E, vitamin A, and vitamin C are the most significant natural antioxidants used in livestock feeds.[6,21] In addition to natural antioxidants, synthetic antioxidants are also commonly used in feed. The most common synthetic antioxidants used in the livestock industry are synthetic phenolic compounds such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ).[6,21] Other synthetic antioxidants used are nitrogenous compounds such as ethoxyquin (EQ), capsaicin, and vanillylamide, with EQ being the most efficacious.[6] In general, synthetic antioxidants are perceived as more effective than equal quantities of natural antioxidants and can better resist processing losses.[22] The US Food and Drug Administration has regulated synthetic antioxidant inclusion rates in livestock allowing a maximum inclusion rate of 150 ppm for EQ, and 200 ppm for BHA and BHT.[6] Natural antioxidants have less-stringent regulations set by the government and are perceived by consumers as more acceptable when supplemented in livestock diets.

Cells found in the body of mammals are exposed to a wide variety of oxygen concentrations. This range of concentrations experienced by cells have been researched and described by researchers as normoxia, physioxia, and hypoxia. The term “normoxia” denotes atmospheric oxygen content, “physioxia” signifies biologically sufficient oxygen

concentration in tissues, and "hypoxia" that represents oxygen concentrations less than normal, indicating an oxygen deficit.[23,24] Different organs of the body experience varying types of oxygen levels. The cells of the small intestine are a great example of physioxia, due to the varying concentrations of oxygen found in the lumen (2%), villus tip (3%), and small intestinal wall (8%).[6] In addition, an oxygen concentration of around seven percent has been discovered at the serosal side of the small bowel.[23,24] Although this is lower than atmospheric oxygen concentration, it does not mean the intestines are experiencing hypoxic stress. It has also been found that the intestines experience daily fluctuations in oxygen concentration, namely higher blood and oxygen flow after intake of food versus a fasting state.[26] Although the small intestine has a relatively low concentration of oxygen compared to that of the atmosphere, this does not mean the intestines cannot be impacted by hypoxia. In fact, since the intestines are a mucosal organ supported by a rich and complex underlying vasculature, the intestines and epithelial cells that create the protective barrier are susceptible to damage caused by diminished blood flow due to hypoxia.[27] Hypoxic stress commonly occurs in tissues, such as the small intestine, when an infection and/or inflammation arises in that tissue.[7,8] This "hypoxic stress" occurs when neutrophils and macrophages are recruited in response to the infection. The innate immune cells and invading pathogens, increase the demand for oxygen of the infected tissue eventually exceeding the supply of oxygen to the tissue by the body resulting in a hypoxic state.[23] Innate immune cells such as neutrophils are inherently important to the body's defense against inflammation and infection of tissues. However, polymorphonuclear neutrophils (PMNs) recruited in response to an infected tissue rapidly produce reactive oxygen species and immensely increase oxygen demand by almost 50-

fold in the inflamed tissue, contributing to the decreasing oxygen supply of the tissue.[28,29] Studies have shown that chronic inflammation can contribute to decreased blood and oxygen supply to that tissue, which may result in abnormal vasculature and contribute to the pathogenesis of chronic inflammation.[30,31] Pathological vascular remodeling plays a pivotal role in the progression of a variety of diseases and conditions associated with ischemia, hypoxia, and inflammation, such as hypertension, atherosclerosis, restenosis, vascular insufficiency, and neoplasia.[32] As previously mentioned, chronic inflammation of the gastrointestinal tract due to chronic hypoxia may result in an increase in vascular remodeling which may interfere with and cause disruptions in the functionality of the epithelial barrier of the intestine.[32,33] Tight junctions (TJ) also play a vital role in intestinal permeability by keeping the intestinal physical barrier intact.[24,34–36] Damage to the TJ components has been shown to increase the permeability of epithelial cells due to hypoxia-induced disorganization of the cytoskeletal network by disrupting F-actin filaments and by excessive cleavage of α-spectrin, an apical protein, that binds to actin cytoskeleton and sodium transport proteins.[24,36] In another study using mice, it was found that the mucosal layer itself is highly susceptible to oxidative stress, and therefore reasonable that oxidative stress caused by hypoxia damages the mucosal layer making the intestinal wall more susceptible to autodigestion, oxidative stress, or permeability to endotoxins.[37,38]

1.2 Quercetin

1.2.1 Health benefits

Numerous studies have demonstrated that quercetin exerts systemic, coronary and pulmonary artery vasodilation, and antiaggregant effects *in vitro* and reduces blood

pressure, oxidative status, and end-organ damage in animal models of hypertension.[39–43] It has also been discovered that quercetin prevented morphological and functional changes in the heart, vessels and kidney, while increasing production of reactive oxygen species associated with hypertension using a rat model.[41] Other studies have demonstrated quercetin has other biological effects such as anti-inflammatory[44] and anticancer[45] activities through the activation and inactivation of various enzymes, including intracellular signaling molecules.[46] In another study, it was reported that a 48-hr exposure to quercetin enhanced intestinal barrier function through increasing claudin-4 expression in human intestinal Caco-2 monolayers.[47]

1.2.2 Uses in food animal

Flavonoids, such as quercetin, are widely distributed in higher plants that are ingested by food animals as part of their regular diet, albeit in relatively small concentrations in feed plants.[48] Due to their health-promoting properties, these compounds are often used as nutritional supplements in humans and animals, including livestock.[49–53] Public concern over the use of pharmaceuticals in livestock is also mounting, leading to alternative methods of improving animal health, such as flavonoids, becoming more widespread.[53–55] As a result, numerous feed additives for cattle containing flavonoids have become commercially available.[53]

Quercetin, and other flavonoids, are commonly used as feed supplements in food animals due to their antioxidative capabilities *in vitro*,[56–58] as well as their health promoting properties.[53] Supplementation of quercetin in goats has been shown to improve performance and suppress hepatic inflammation.[59] Quercetin has also been found to increase the activities of antioxidant enzymes in the tissue of pigs,[60] act as an efficient

dietary supplement to alleviate transport stress in finishing pigs,[61] and enhance the bioavailability of moxidectin in lambs.[62] In dairy cattle it has been demonstrated that quercetin has positive effects on performance,[63] glucose metabolism and milk composition,[64] and liver health.[65] There are also some *in vitro* studies that revealed the potential of using pure flavonoids or plants rich in flavonoids to reduce methane production without compromising the fermentation process.[66–68]

1.2.3 Studies done in food animal

A substantial amount of studies on quercetin have been conducted using monogastric species and cell culture experiments.[56] Therefore, the supplementation of quercetin in both monogastric and ruminant food animals is low, with relatively few studies conducted using food animals, especially ruminants, as models. However, using research conducted with monogastric species as evidence, it can be hypothesized that flavonoids, if present in circulation, might have similar effects in ruminants.[56] A number of studies have been conducted investigating the bioavailability of quercetin from its aglycone and its glucorhamnoside rutin in dairy cattle.[53,56,66] These studies have found that, in contrast to monogastric species, quercetin as its glucorhamnoside rutin is more bioavailable than quercetin aglycone.[53,56,66] In a different study, researchers investigated the bioavailability of quercetin after oral administration of quercetin aglycone or rutin in neonatal calves. In this study it was found that quercetin aglycone is more bioavailable than rutin because neonatal calves due not have functional rumens and are therefore functionally monogastrics.[69] In addition, it was found that feeding quercetin to newborn calves does not impair glucose metabolism but affects postprandial glucose uptake and splanchnic glucose oxidation.[49]

Swine models have also been utilized to examine quercetins bioavailability and effects on body weight loss, carcass characteristics, meat quality and antioxidant status under transportation stress. In one study, it was found that pigs fed quercetin were heavier, had increased average daily gain, decreased drip loss at 24-hr postmortem from the *Longissimus thoracis et lumborum*, and also lower ROS and thiobarbituric acid reactive substances (TBARS) levels in serum, muscle, and liver.[61] Another study examining the bioavailability of quercetin in pigs, discovered rutin had the lowest relative bioavailability, and quercetin aglycone is absorbed from the upper small intestine.[70] These findings are in agreement with other studies in other species.

The effect of dietary quercetin supplementation has also been investigated using broilers. In one study, researchers investigated the effects of quercetin on P-gp expression levels and functional activity in chicken. This study found that quercetin can alter expression levels and functional activity of P-gp when simultaneously administered with P-gp substrate enrofloxacin.[71] Another study concluded that dietary supplementation of quercetin may prolong the shelf-life of poultry meat by reducing the rate of lipid oxidation, and increasing relative heart weight, potentially contributing to improved health of the broilers during production.[72]

Small ruminants such as goats and lambs have also been used as models to investigate quercetin. Post-ruminal quercetin was given to goats fed a high grain diet to see if the flavonoid could provide anti-inflammatory benefits and have positive effects on laminitis. This study revealed that abomasal supplementation of quercetin does result in improved performance and anti-inflammation of the liver and hoof at the mRNA level.[59]

It is also reported that co-administration of quercetin to lambs resulted in a significant increase in the bioavailability of the anthelminthic moxidectin.[62]

1.2.4 Health benefits in food and other animals

Quercetin has been shown to have numerous health benefits in livestock as well as other animals such as mice,[73] rats,[74] rabbits,[75] dogs,[76] and horses.[77] Mice and rat models are commonly used in experiments, therefore a great deal is known about the health benefits quercetin has on these animals. For example, quercetin has been found to be partially protective in a rat model of pulmonary arterial hypertension due to its effects on lowering pulmonary arterial pressure, right ventricular hypertrophy, and vascular remodeling.[40] In addition, rutin has been shown to reverse or prevent metabolic changes such as glucose tolerance, changes in hepatic and cardiovascular structure and function, and reversed oxidative stress and inflammation in the liver and heart in a rat model.[74] Quercetin has also been shown to have health benefits in mice. One study demonstrated rutin effectively attenuated lipopolysaccharide(LPS)-induced acute lung injury by inhibiting histopathological changes and infiltration of leukocytes in the lung.[73]

Rabbits, like rats and mice, are used as experimental models to examine the effect a treatment, such as quercetin, has on the animal. Therefore, the health benefits of quercetin found in rabbits are secondary to the information gathered during the experiment. Regardless, several studies have been conducted using rabbit models to examine the effect quercetin has on cardio protection, inflammation, and the bioavailability of certain drugs. As a result, it has been shown that quercetin does in fact inhibit myocardial ischemia-reperfusion-induced NOX2, iNOS, eNOS mRNA and protein expressions in rabbits and may be a novel antioxidant for cardioprotection.[78]

Quercetin modulates the deleterious inflammatory effects of a hypercholesterolemic diet in rabbits, suggesting its beneficial effect in decreasing inflammation in atherosclerotic progression and regression.[79] Quercetin also increases the bioavailability of the antihypertensive drug diltiazem in rabbits pretreated with quercetin.[75]

In horses, the health benefits of quercetin have been investigated to a lesser extent than that of rodents. However, there have been studies investigating quercetin's effects on systemic inflammation and its effectiveness on stallion sperm being freezable. It was found that since quercetin shows bioavailability in horses, it may be useful in controlling inflammatory disorders.[77,80]It appears as though a concentration of 0.01mM of quercetin seems to protect sperm motility during cryopreservation.[81] However, more research needs to be conducted to confirm these results.

1.3 Antioxidants and pulmonary hypertension

There are several antioxidants that have been shown to reduce pulmonary hypertension. Commonly used antioxidants include Vitamins A and C, L-arginine, flavonoids, and mitochondria targeted agents, Coenzyme Q10, acetyl-L-carnitine and alpha-lipoic acid.[82]

Vitamin A and its derivatives such as beta-carotene have been found to exhibit cardioprotective effects and a correlation with higher plasma levels to lower blood pressure in men.[83] Another vitamin A derivative, lycopene, has been shown to reduce blood pressure in patients with stage I hypertension.[84] However there are studies that have found beta-carotene and lycopene ineffective at reducing hypertension and can have adverse mitochondrial effects.[85,86]

Dietary intake of vitamin C, also known as ascorbic acid, has been shown to correlate inversely with hypertension[87,88] and induce modest reductions in blood pressure in both normotensive and hypertensive populations.[89–92] Vitamin C concentrates in local tissues an order of magnitude higher than that of plasma which allows it to effectively compete for superoxide and reduce thiols.[93,94]

L-arginine is an amino acid and main substrate for NO production from eNOS in a tetrahydrobiopterin dependent reaction.[95] Low cellular levels of L-arginine have been demonstrated in human hypertension.[96,97] This suggests that low levels of L-arginine may lead to reduced levels of bioavailable NO which could contribute to hypertension. Therefore, supplementation of L-arginine could reduce blood pressure by allowing for restoration of normal NO bioavailability.[82]

Mitochondria-related antioxidants such as coenzyme Q10 (CoQ), acetyl-L carnitine (ALCAR), and α-lipoic acid (LA) have all been shown to either have anti-hypertensive efficacy,[98] or reduce blood pressure in hypertensive models.[49,99–103] CoQ is a key component in the electron transport chain and may reduce mitochondrial superoxide production by increasing the efficiency of electron transfer from complex I and II down the mitochondrial electron transport chain.[104] It may also have an antioxidant effect on the plasma membrane by reducing lipid peroxidation.[105] It is thought that LA participation in mitochondrial-associated pathways, in cell signaling that may improve coupling of eNOS and anti-inflammatory actions are potential beneficial effects of LA supplementation.[106,107] The antioxidant mechanism for ALCAR supplementation appears to be secondary to reductions in mitochondrial ROS production.[108]

Flavonoids as well as polyphenols have also been found to reduce pulmonary hypertension. Flavonoid is a broad term that describes approximately 4,000[109] plant-derived compounds that share a common skeleton of phenylchromane.[110] These compounds are known for their antioxidant and chelating abilities[109] as well as their cardioprotective effects.[109,111] Quercetin is a well-known, well studied, and potent flavonoid that provides insight on the absorption and metabolism of other polyphenolic compounds.[109] Quercetin, when given chronically, has demonstrated antihypertensive effects in the most common rodent models of hypertension.[40,41,109,112,113] Rutin, a glucorhamnoside of quercetin, has also demonstrated antihypertensive effects in spontaneously hypertensive rats.[114] Curcumin is a polyphenol compound that has also been found as a possible tool to reduce pulmonary hypertension.[115] Additional studies have also provided evidence that curcumin prevents the development of hypertension and vascular remodeling and protects against cadmium-induced vascular dysfunction and hypertension.[116–119]

1.4 Ascites

Ascites is defined as excessive accumulation of transudate fluid within the peritoneal spaces due to increased vascular permeability, increased tissue or decreased plasma oncotic pressure, obstructed lymph drainage, increased hydraulic pressure in the splanchnic venous system or a combination of these.[120] The most prevalent form of ascites in broilers is pulmonary hypertension induced ascites and is primarily associated with right ventricular failure secondary to pulmonary hypertension due to increased intravascular pressure in the portal system.[121] In addition to increased intravascular pressure in the portal system, ascites also increases the fluid pressure in the hepatic

capillary bed. Diagnosing ascites can be difficult as clinical signs are not specific and can include numerous symptoms that do not provide enough evidence the broiler is suffering from the syndrome.[120] Thus, death due to ascites usually occurs late in the production cycle and may even occur during transport to the slaughter house.[122] Post-mortem inspection of broilers who succumbed to ascites discovered accumulation of ascitic fluid in the peritoneal cavities, hydropericardium, right ventricular hypertrophy, and generalized venous congestion.[123] Physiological blood parameters indicative of ascites include a progressive increase in hematocrit and progressive hypercapnia and hypoxaemia.[124] An increase in the incidence of ascites induced by pulmonary hypertension can be attributed to improved rearing techniques and selection of fast growing and high meat-yielding broilers.[120] Due to a superior growth rate in modern broilers, physiological oxygen demand is higher to meet metabolic demands of the growing broiler.[121] In addition, relative heart and lung size is significantly reduced, diminishing cardiopulmonary capacity.[121] An increase in metabolic rate not only augments oxygen requirements at the tissue level, but also increases mitochondrial production or reactive oxygen species (ROS).[121] These increases in oxygen demand, metabolic rate, and reduced cardiopulmonary capacity is the central etiology of ascites. The use of preventative strategies is crucial due to the impact ascites syndrome has on the broiler industry. Since ascitic birds are rejected for consumption,[125] they are essentially fed for the entire rearing cycle but have no economic value for the producer.[126] Common preventative strategies used in the broiler industry are feed and lighting regimes that slow early growth of the broilers.[120,127–132] However, the method of feed restriction to reduce the incidence of ascites has been called into question due to studies suggesting inadequate

compensatory gain in later growth phases, and also extra costs in the form of non-nutritive fillers and higher transport costs per unit feed.[121] Reducing damage to the cardiopulmonary system caused by ROS is another approach to alleviate predisposition of fast growing broilers to ascites mortality.[133] Several studies have demonstrated that supplementing diets with antioxidants can reduce oxidative damage and improve pulmonary vascular performance.[134–136]

1.4.1 Quercetin and Ascites

"Quercetin is a dietary flavonoid which exerts vasodilator, antiplatelet and antiproliferative effects and reduces blood pressure, oxidative status and end-organ damage in humans and animal models of systemic hypertension." [40] The exact mechanism of action for quercetin has not yet been identified. In one study it was found that quercetin lowered pulmonary arterial pressure and vascular remodeling in male Wistar rats, which could have been a result of quercetin's positive vascular effects, such as vasodilation and smooth muscle antiproliferative and pro-apoptotic effects.[40] In another study, investigators discovered quercetin tended to reduce remodeling following the trend of blood pressure reduction in spontaneously hypertensive rats (SHR).[41] In addition, quercetin was found to attenuate cardiac hypertrophy by noting changes in the expression and localization of several proteins in the heart.[41] Quercetin has also been found to inhibit angiotensin 2-induced hypertrophy *in vitro* in cultured neonatal rat cardiomyocytes.[137] In another study, it was found that quercetin could potentially be used to treat hypoxia induced pulmonary arterial hypertension due to its effects on proteins and cells within the heart.[113] In this study, quercetin inhibited hypoxia-induced

pulmonary artery smooth muscle cells (PAMSC) proliferation, arrested cells in G1/G0 and inhibited cell migration in a dose-dependent manner.[113]

1.4.2 Curcumin and Ascites

Curcuma Longa (curcumin), another antioxidant used in this study as a feed supplement, has long been used in African traditional medicine to treat palpitation, hypertension and other related blood disorders.[138] The effects of curcumin have been investigated in rats and other models, but few studies have been conducted viewing its effects on the cardiopulmonary system of broilers. In one study, curcumin was found to be used in the regulation of hypertension.[115] With many studies suggesting the beneficial effects curcumin may have on hypertension, it was included in the chicken feed so the effects could be examined.

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Chapter 2

Study 1: Effect Of The Flavonoid Antioxidant Rutin On Gastrointestinal Permeability And Susceptibility To Hypoxia-Induced Pulmonary Hypertension In Holstein Calves

2.1 Abstract

The purposes of this study were to determine if the naturally occurring flavonoid quercetin, as its glucorhamnoside rutin, has favorable effects on reducing gastrointestinal permeability and reducing susceptibility to hypoxia-induced pulmonary hypertension in neonatal Holstein calves. A 2 x 2 between-subjects factorial design was conducted using Holstein steers (n = 16). Factors included oxygen-level (simulated altitude of 4,572 m versus 975 m) and quercetin supplementation as its glucorhamnoside rutin (4g of quercetin per day versus 0 g per day). Two-days post-arrival (Day 0 of study) the calves were blocked by body mass into treatment groups and both treatments initiated. Pulmonary arterial pressures were measured on Day 12. On Day 14, calves were orally administered lactulose (0.45 g/kg) and mannitol (0.15 g/kg) and serum concentrations of these substances measured by high performance liquid chromatography mass spectrometry four-hours post feeding. Calves were euthanized on Day 15 and tissue sections collected from the lung, liver, jejunum, and ileum for histological evaluation and semi-quantitative scoring of lesions. Hypoxia, but not rutin ($P \geq 0.31$), was found to be associated with intestinal permeability. The lactulose-mannitol ratio was 0.54 ± 0.13 in hypoxic calves and 0.02 ± 0.13 in normoxic controls ($P = 0.02$). Hypoxia ($P < 0.001$) and, possibly, rutin ($P = 0.07$) were associated with mean pulmonary arterial pressure. There was a tendency toward a hypoxia-rutin interaction ($P = 0.13$). Calves fed rutin under hypoxic conditions (mPAP = 59 ± 7 mm Hg) had a lower mPAP than calves that

were not fed rutin (mPAP = 80 ± 7 mm Hg) (P = 0.13). Under normoxic conditions calves fed rutin (mPAP = 30 ± 7 mm Hg) had similar mPAP to control calves (mPAP = 28 ± 7 mm Hg). Paradoxically, however, a greater proportion of calves fed rutin had histological evidence of pulmonary arteriolar hypertrophy (P = 0.045) and adventitial hyperplasia (P = 0.01) than negative controls. In conclusion, the findings of this study indicate that hypoxia deleteriously increases intestinal permeability in neonatal calves. The flavonoid quercetin, as its glucorhamnoside rutin, had no protective effect on intestinal permeability and, although it tended to reduce the severity of hypoxia-induced pulmonary hypertension, a greater proportion of calves fed rutin had histological lesions consistent with pulmonary arteriolar remodeling.

2.2 Introduction

Increased gastrointestinal permeability is deleterious to health. In cattle, this is most commonly problematic during the neonatal period and when fed diets high in starch, as is typical in beef cattle finishing systems. In the US, 10-20% of feedlot cattle[1] are reported to have liver abscesses at slaughter; consequently, 71.2 ± 4.4% of all feedlots feed the broad-spectrum, macrolide antibiotic Tylosin as a prophylactic measure.[2] Antimicrobial-free interventions to improve gut barrier function could, therefore, have broad benefits across the cattle industry. Evidence from rodent and animal studies indicates that the naturally occurring dietary flavonoid quercetin has two major physiological effects that would also be of great benefit to health: reducing gastrointestinal permeability[3,4] and reducing susceptibility to pulmonary hypertension[5–8], a potentially lethal physiological states that cattle are particularly susceptible to.

We hypothesized that dietary supplementation with the naturally occurring flavonoid quercetin, provided as its glucorhamnoside rutin to improve bioavailability, will decrease mucosal permeability to the nondigestible, nonmetabolizable sugars mannitol and lactulose and reduce the susceptibility of calves to hypoxia-induced pulmonary arterial hypertension.

2.3 Materials and Methods

2.3.1 Study site

One-day old Holstein bull calves were obtained from a large commercial dairy farm within 40-miles of Texas Tech University Research farm, New Deal, Texas. Two blocks of eight-calves per block were studied: the first block was collected on February 19, 2018, and the second block was collected on March 20, 2018. Calves were fed four liters of colostrum within six hours of birth according to calf management protocol. Serum total proteins were measured to assess adequacy of passive immunoglobulin transfer.

Calves were group-housed (n = 4; 1.5 m^2 per calf) in pens (Agri-Plastics, Grassie, ON, Canada) on raised perforated flooring within one of two chambers (41 m^3). Temperature (15 ± 2°C), light (12 h light, 12 h dark), and oxygen levels were controlled within the chambers. Each chamber was equipped with a dehumidifier (HomeLabs 4 Gallon Dehumidifier, model HME020030N) fitted with a continuous drain hose. Temperature and humidity ranges were checked daily using an indoor digital monitor (ThermoPro TP50 Digital Hygrometer).

The calves were weighed one-day post-arrival and the heaviest four calves assigned to one chamber and the four lightest calves assigned to the other chamber. Calves were stratified into dietary treatment groups according to failure of passive transfer (FPT) status (< 55 g/L). Within each stratum, calves were randomly assigned to dietary treatment groups so that within each chamber two calves were fed the dietary additive rutin and two calves served as negative controls. The first block of calves was housed under normoxic conditions (altitude 975 m) and the second block under hypoxic conditions (simulated altitude 4,572 m).

2.3.2 Feed and husbandry

Starting on the morning post-arrival, calves were individually fed 2.5 liters of milk replacer (Purina® High Energy Nurse Chow®), 22% protein and 12% fat, twice per day using standard, commercially available plastic calf bottles and nipples. Milk replacer was prepared in a large bucket using warm water (approximately 43 ℃) and a balloon whisk. Texturized calf starter (Purina® Ampli-Calf® Starter 22), 22% protein and 2.5% fat, and water was available ad libitum throughout the study. Feed was checked daily for spoilage and replaced every other day.

Treatment exposure began two-days post-arrival (Day 0 of the study). Calves received 4 g of rutin trihydrate (97%, Alfa Aesar, Heysham, Lancashire, United Kingdom) per day divided into 2 g per meal.

Rutin was weighed in disposable plastic boats to a precision of 0.01 g (Ohaus SPX1202, Ohaus Corporation, Parsippany, NJ). The rutin was then poured into the bottle containing milk replacer, which was then capped and inverted several times. Calves were

hand fed using designated and clearly labelled bottles to minimize the risk of ingestion of the incorrect diet. Bottles were hand washed and dried after each feeding.

2.3.3 Health monitoring

Calves were assessed daily for signs of respiratory distress, tachypnea, and scours throughout the study and rectal temperatures measured on alternate days. Electrolytes (Re-sorb®, Zoetis, Kalamazoo, NJ) were given to calves with scours in place of the morning feed.

2.3.4 Intestinal permeability evaluation

Calves were weighed on Day 14 immediately prior to the oral administration of mannitol (0.15 g/kg) and lactulose (0.45 g/kg). Both substances were weighed using disposable plastic boats to a precision of 0.01 g (Ohaus SPX1202, Ohaus Corporation, Parsippany, NJ) before mixing with 0.5 L of water (approximately 43 ℃). The mannitol and lactulose solution was then fed using an esophageal feeder to minimize the amount of solution lost during the feeding process. Calves were fed their regular morning milk replacer (2.5 L) two-hours after the mannitol and lactulose challenge.

2.3.5 Pulmonary arterial pressure measurement

Calves were manually restrained using a lamb trimming stand for pulmonary arterial pressure measurement on Day 12. The neck was clipped and cleaned with chlorhexidine solution. A scalpel blade was used to create a full-thickness stab incision through an intradermal lidocaine bleb placed in the jugular grove. A 7-French peel-away introducer (IS-07AS, Vascor Medical Corporation, Tarpon Springs, FL, USA) was placed in the jugular vein prior to inserted of a 110 cm, 7 French, polyurethane, modified J-tip

wedge pressure catheter (172-110P, Vascor Medical Corporation, Tarpon Springs, FL, USA). A pressure transducer (TranStar DPT, Smiths Medical ASD, Inc., Dublin, OH, USA) was interposed between the catheter to the data acquisition system (IX-TA-220, iWorx Systems, Inc., Dover, NH, USA). The pressure waveforms were recorded and analyzed offline (LabScribe3, iWorx Systems, Inc., Dover, NH, USA). Pressures were analyzed at the end of expiration. The position of the catheter tip was determined by monitoring the change in the pressure waveform as the catheter tip was advanced through the right atrium, right ventricle, and finally into the pulmonary artery.

2.3.6 Echocardiography

Right parasternal short-axis views of the left ventricle, mid-way between the papillary muscles and mitral valve, were obtained on Day 13 using a portable ultrasound machine (GE Vivid i) and 1.5 – 4.0 MHz sector probe (3Sc-RS, General Electric, Boston, MA). End-systolic left ventricular eccentricity index values were calculated from the anterior-inferior and septal-posterolateral cavity dimensions.

2.3.7 Histology

Calves were euthanized on Day 15 (pentobarbital sodium 85 mg/kg iv) and exsanguinated immediately post-mortem. A gross examination of all major organs was performed, and tissue sections approximately 1 cm thick obtained from the dorsal aspect of the left diaphragmatic lung lobe, quadrate liver lobe, mid-jejunum and mid-ileum collected and preserved in neutral buffered formalin (10%; 10:1 formalin to tissue volume). Tissues were trimmed and paraffinized approximately one week later. Slides containing 4 µm thick sections were stained with hematoxylin and eosin.

2.3.8 Lung weight

The right lung of all calves was collected, weighed, and placed on a metal wire drying rack located in a temperature-controlled chamber set at 100°F. The lungs were kept in the chamber until there was less than a one gram change in the lung weight over consecutive days.

2.3.9 Statistical analyses

Statistical analyses were performed using commercially available software (STATA 15.1, College Station, TX). Data were descriptively analyzed graphically and numerically to assess data distribution and check for the presence of outliers. Backwards step-wise linear regression was performed with the full model containing both factor variables and their interaction. Results are presented as marginal means ± SEM unless otherwise stated. Likelihood ratio tests were used to test the overall statistical significance of a variable where an interaction was statistically significant ($\alpha = 0.05$) or where an interaction was hypothesized. Repeated rectal temperature measurements were assessed using generalized estimating equations and an exchangeable correlation structure. Two-sample proportions tests were undertaken to evaluate whether the proportion of calves with at least mild histological lesions differed between treatments.

2.4 Results

2.4.1 Descriptive

Calves weighed 40.8 ± 4.5 kg (mean ± SD) on arrival at the university farm and 42.1 ± 3.3 kg on the day of euthanasia. Four calves had FPT: two calves per block. These calves were equally distributed among treatments. Two calves were fed milk replacer

once by esophageal feeder because of feed refusal. Four calves were administered electrolytes for the treatment of scours.

2.4.2 Gastrointestinal permeability to lactulose and mannitol

Hypoxia, but not the feeding of the rutin, was associated with intestinal permeability (Table 2.1). There was no interaction between the dietary additive rutin and hypoxia on blood serum concentrations of lactulose ($P = 0.09$), mannitol ($P = 0.75$), and the ratio of lactulose to mannitol ($P = 0.15$).

Table 2.1. Influence of dietary rutin and environmental hypoxia on blood serum lactulose and mannitol concentrations four hours after feeding by esophageal intubation

	Rutin treatment			Hypoxia treatment			
Item	Control	Rutin	*P*-value	Control	Hypoxic	*P*-value	SEM
Lactulose, mg/L	0.33	0.40	0.75	0.13	0.60	0.03	0.13
Mannitol, mg/L	0.68	0.93	0.50	0.45	1.16	0.07	0.25
Lactulose:Mannitol ratio	0.18	0.38	0.31	0.02	0.54	0.02	0.13

2.4.3 Effect of rutin and hypoxia on cardiopulmonary pressure

Hypoxia ($P < 0.001$) and the dietary additive rutin ($P = 0.07$) were associated with mPAP. There was a tendency toward interaction between hypoxia and rutin ($P = 0.13$). Under hypoxic conditions, calves fed rutin (mPAP = 59 ± 7 mm Hg) had a lower mPAP than calves that did not consume rutin (mPAP = 80 ± 7 mm Hg) ($P = 0.13$). Under

normoxic conditions calves fed rutin (mPAP = 30 ± 7 mm Hg) had similar mPAP to control calves (mPAP = 28 ± 7 mm Hg).

2.4.4 Echocardiography

No statistically significant effect of hypoxia (P = 0.81) or rutin (P = 0.83) on left ventricular end-diastolic eccentricity index (1.21 ± 0.05; mean ± SE). Nor was there a statistically significant effect of hypoxia (P = 0.82) or rutin (P = 0.93) on left ventricular end-systolic eccentricity index (1.26 ± 0.09; mean ± SE). No pairwise correlation between mean PAP and left ventricular end-diastolic eccentricity index (P = 0.57) and between mean PAP and left ventricular end-systolic eccentricity index (P = 0.66).

2.4.5 Rectal temperature changes

Hypoxia (*P* = 0.42) and the dietary additive rutin (*P* = 0.91) were not associated with calf rectal temperature. On average, rectal temperature increased by 0.019 ± 0.009°C per day of the study (*P* = 0.033).

2.4.6 Lung weight

It was found that rutin (*P* = 0.67) had no effect on lung weight. However, the proportion of lung mass attributable to water was marginally lower in calves exposed to hypoxia (80.3 +/- 0.3%) than normoxic controls (81.6 +/- 0.3%) (P = 0.017).

2.4.7 Histology

Five hypoxic calves and three normoxic calves had at least mild lesions of pulmonary arteriolar hypertrophy (*P* = 0.31) (Table 2.2). Six calves fed rutin and two control calves had at least mild lesions of pulmonary arteriolar hypertrophy (*P* = 0.045).

Five hypoxic calves and four normoxic calves had at least mild pulmonary arteriolar adventitial hyperplasia ($P = 0.61$). Seven calves fed rutin and two control calves had at least mild pulmonary arteriolar adventitial hyperplasia ($P = 0.01$). All normoxic calves and five of the hypoxic calves had mild or moderate hepatic lipidosis (Table 2.2).

Table 2.2. Influence of dietary rutin and environmental hypoxia on liver lesion, pulmonary arteriolar hypertrophy, pulmonary arteriolar adventitial hyperplasia, and hepatic lipidosis.

		Normoxic (n = 8)		Hypoxic (n = 8)	
Lesion	Severity	No rutin	Rutin	No rutin	Rutin
Pulmonary arteriolar hypertrophy	None	3	2	3	0
	Mild	0	2	0	3
	Moderate	1	0	1	1
Pulmonary arteriolar adventitial hyperplasia	None	3	1	3	0
	Mild	0	3	1	4
	Moderate	1	0	0	0
Hepatic lipidosis	None	0	0	1	2
	Mild	1	1	1	1
	Moderate	3	3	2	1

2.5 Discussion

The findings of this study suggest that the naturally occurring flavonoid quercetin, as its glucorhamnoside rutin, has no effect on intestinal permeability in neonatal Holstein calves. Hypoxia, however, was found to significantly increase intestinal permeability, a finding that points to hypoxemia in calves with respiratory compromise as a contributing cause of neonatal sepsis. Although, rutin tended to reduce mean pulmonary arterial pressure in calves housed under hypoxic conditions, pulmonary histological evaluation indicated that rutin was deleteriously associated with pulmonary arteriolar remodeling.

It is well known that liver abscesses in cattle are a result of the entry, growth, and establishment of pyogenic bacteria.[9] In feedlot cattle, these bacteria most commonly enter the liver via the portal vein due to the large blood volume of blood flow and proximity to the gastrointestinal tract, a major source of bacteria.[9,10] Due to cattle's high oxygen demand and relatively small lung size, cattle are prone to hypoxemia.[11,12] As we have shown in our study, hypoxia increases gastrointestinal permeability. This increase in permeability may offer these pyogenic bacteria mentioned above more access to the circulatory system, allowing more access to the liver, leading to an increase in liver abscessation.

Exogenous antioxidants play a key role in keeping the delicate equilibrium between oxidation and antioxidation reactions in living systems.[13,14] However, at high doses or in the presence of metal ions, these antioxidants can demonstrate pro-oxidative activities. Quercetin, for example has been shown to exhibit prooxidative activity at high concentrations (>50µM) *in vitro*.[13,15] Studies using low doses of quercetin (0.1-20µM) observed the antioxidant activity of this compound while higher doses (>50µM)

decreased cell survival and viability *in vitro*.[16] *In vivo* studies using rat models have also been conducted with quercetin and found that concentrations of 0.33 mg/kg exhibited displayed protective activities.[17] A different study investigating the bioavailability of quercetin and rutin in neonatal calves used an amount of nine milligrams and eighteen milligrams, respectively.[18] This study did not investigate the activity of the antioxidants, but found that quercetin was much more bioavailable than its glucorhamnoside rutin.[18] In our study we used a concentration of four grams of rutin per day. Using other studies as reference, a concentration of this magnitude could have caused rutin to exhibit prooxidative activities in the calves, which might explain why rutin had no effect on intestinal permeability.

To our knowledge, the association between hypoxia and increased intestinal permeability specifically has not been previously reported in cattle. There is, however, a study using vasa vasorum endothelial cells (VVEC) isolated from pulmonary artery adventitia of control and chronically hypoxic neonatal calves that demonstrated hypoxia induced enhanced paracellular permeability and disordered purinergic control of vascular barrier function.[19] Although increased intestinal permeability has not yet specifically been reported in cattle, there are numerous studies using *in vivo* mouse or rat models that suggest hypoxia does in fact increase intestinal permeability,[19–26]. By using these models these researchers have been able to discover some of the mechanisms that allow or inhibit permeability of epithelial cells. It is now clear that responses to hypoxia include transcriptionally regulated gene expression.[27–29] One of the most well-known and characterized hypoxia signaling mechanisms is hypoxia inducible factor 1 (HIF-1).[30,31] This signaling mechanism responds to low oxygen levels where it binds to and induces

the expression of several genes whose products are involved in initiating the cellular adaptation to hypoxic stress.[31–33] Nuclear factor kappa B (NF-κB), is another central regulator of innate immunity and inflammatory processes. It too is activated by a hypoxic, both in vitro and in vivo.[34]

According to numerous studies, quercetin and its rhamnoglucoside rutin, have a wide range of biological activities and pharmacological effects, such as antihypertensive, vasoprotective, and cardioprotective activities.[6,8,35–39] In one study it was found that quercetin lowered pulmonary arterial pressure and vascular remodeling in male Wistar rats, which could have been a result of quercetin reducing the number of muscular arteries.[8] In another study, investigators discovered quercetin tended to reduce remodeling following the trend of blood pressure reduction in spontaneously hypertensive rats (SHR).[7] Therefore, using these studies as evidence, it does not seem likely for quercetin, as its rhamnoglucoside rutin, to decrease mPAP while also promoting arteriolar remodeling.

The bioavailability of quercetin and rutin has been intensively investigated in monogastric animals, however there is limited knowledge on their bioavailability in ruminants,[40]especially neonatal ruminants. In monogastric species, the bioavailability of quercetin depends on the glycoside moiety of the quercetin glycosides present in the diet.[40,41] Consequently, the oral bioavailability of rutin compared to quercetin applied as aglycone is lower in monogastric species.[40] In contrast to these findings, one study found the bioavailability of quercetin after intraruminal application of rutin is higher compared to quercetin aglycone.[40] Although neonatal calves are technically ruminants they do not have fully developed rumens until approximately 4 months old.[42,43] Therefore, rutin may

not have been as bioavailable as found in fully mature cattle and thus not be able to be absorbed and cause an effect on intestinal permeability. The use of quercetin applied as aglycone may have had better bioavailability in the neonatal calves used in our study.

Although we had a small sample size, reducing the power of the study, we were still able to find that hypoxia was deleterious to gastrointestinal permeability. Furthermore, given the experimental design using neonatal cattle we cannot make inferences about feedlot cattle, but the findings of our study do suggest that hypoxia in cattle[11,12] could contribute towards an increase in gastrointestinal permeability, due to considerable supporting evidence for an inter-relationship between the pulmonary and gastrointestinal systems.

The purposes of this study were to determine if the naturally occurring flavonoid quercetin, as its glucorhamnoside rutin, has favorable effects on reducing gastrointestinal permeability and reducing susceptibility to hypoxia-induced pulmonary hypertension in neonatal Holstein calves. Although the study found that the naturally occurring flavonoid quercetin, as its glucorhamnoside rutin, had no effect on intestinal permeability in neonatal Holstein calves, it was found that hypoxia significantly increases permeability.

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Chapter 3

Study 2: Effect Of The Natural Antioxidant Quercetin On Broiler Growth Performance And Arterial Oxyhemoglobin Saturation

3.1 Abstract

The purposes of this study were to determine if the natural antioxidants quercetin and curcumin have favorable effects on growth performance and physiology of growing broiler chickens. A completely randomized design was conducted using Cornish x White Rock cross commercial broilers (n = 72). Three weeks after arrival (Day 0 of the study), the birds were randomly divided into four treatment groups: control, quercetin, quercetin plus curcumin (low), and quercetin plus curcumin (high). Oxyhemoglobin saturations and weight of the birds were measured once per week. At six weeks of age the broilers were euthanized, and the hearts of three representative birds from each pen were dissected and weighed for calculation of the right: total ventricular weight ratio (RV:TV ratio). The spleen and liver were also dissected and weighed to compare organ weights across all treatment groups. In addition, feed consumption was measured daily by weighing feed containers each time new feed was added. There were no significant differences in final weights among treatments ($P = 0.15$). The mean +/- SD of the birds at the end of the study (wk. 6) was 2666 +/- 316 g. The feeding of antioxidants had no effect on oxyhemoglobin saturation ($P = 0.46$). Paradoxically, however, oxyhemoglobin saturation increased by two percent per week over the course of the study ($P < 0.001$). Liver weight ($P = 0.31$), spleen weight ($P = 0.23$), and total ventricular weight ratio ($P = 0.34$) were all not effected by treatment. In addition, feed conversion ratio by week was not found to be different among treatment groups ($P = 0.97$). The feeding of antioxidants did effect blood antioxidant concentration among treatment groups ($P = 0.0001$), with controls having the

lowest blood antioxidant concentration, followed by low dose quercetin and curcumin, quercetin only group, and finally high dose quercetin and curcumin. In conclusion, the findings of this study suggest that supplementation of the diet fed with quercetin, curcumin, or combination of both did not have a positive effect on growth performance or improve broiler arterial oxyhemoglobin saturation.

3.2 Introduction

The control of ascites in broilers is of major concern to the broiler industry. A multifactorial syndrome, ascites was most commonly found at high altitudes but is now seen to effect broiler production at lower altitudes.[1] Because of its relationship to oxygen demand, ascites is affected and/or precipitated by factors such as growth rate, altitude (hypobaric hypoxia) and environmental temperature.[2] In addition, the increased selection for fast growing, early maturing broilers has led to the increased occurrence of metabolic disorders, such as ascites and sudden death syndrome, that cause mortality in broilers.[3] Many methods have been implemented to control ascites. The ideal permanent solution to the ascites problem would be genetic selection for ascites resistant broiler lines,[4] but this may take time. Therefore, a few preventative strategies to combat ascites currently utilized in the poultry industry are feed restriction and lighting regimes that slow early growth of broilers.[5,6] Another preventative strategy using antioxidants may be gaining popularity due to mounting evidence from rodent and animal studies.[7–12] These studies indicate that the supplementation of antioxidants, such as quercetin and curcumin, in the diet may reduce the susceptibility to ascites while maintaining the growth rate seen in today's broilers.

We hypothesized that the supplementation with the dietary antioxidants quercetin and curcumin would have favorable effects on the arterial oxygenation and growth performance and, consequently, reduce susceptibility to ascites.

3.3 Materials and Methods

This study was approved by the Texas Tech University Institutional Animal Care and Use Committee (Protocol 18018-02).

3.3.1 Study site

Eighty Cornish Rock broiler chickens were purchased for this study (Stromberg's Chicks and Game Birds LLC, Pine River, MN). Birds arrived on March 8th, 2018, two-days post-hatching (Day 0 of study). Chicks were group-housed on pine shavings (7 to 10 cm deep) in one of three pens (2.4 m^2 per pen) so that there were approximately equal numbers of birds per pen. Shavings were replaced weekly. The brooding temperature was approximately 95 °F (33 °C) at floor level. The temperature was reduced to 85 °F (29 °C) at 5 days of age, to 80 °F (27 °C) at 10 days of age, and to 75 °F (24 °C) after 14 days of age. Temperatures remained within 3 °C of the target temperature throughout the study. The photoperiod was 24 hours of light throughout the study. At one-week of age, birds were individually identified with zip wing bands.

3.3.2 Feed

Birds had *ad libitum* access to fresh drinking water and feed meeting or exceeding all NRC requirements throughout the study. Feed and water were suspended above ground. The birds were fed prestarter, starter, and grower-finisher diets that were medicated (amprolium, 0.0125%) for the prevention of coccidiosis (Honor Show

Chow®, Purina®, Gray Summit, MO) (Table 1). The diets were mixed to provide a gradual reduction in crude protein over six weeks: 30%, 28%, 26.5%, 25%, 23.5%, and 22% for weeks one through six, respectively. All feed was reground using the grind feature on a commercial blender (BESTEK CB-606D 1500W) so that supplemental dietary antioxidants, if added, would be more uniformly distributed throughout the feed than the original crumbled or pelleted feed. A maximum of one kilogram of feed was ground per cycle.

Table 3.1. Nutrient composition of the diets

Nutrient	Prestarter, %	Starter, %	Grower-Finisher, %
Crude Protein (Min)	30	26	22
Lysine (Min)	1.8	1.5	1.15
Crude Fat (Min)	5	6.5	6
Crude Fiber (Max)	3.5	3.5	3.5
Calcium (Min – Max)	1.05 to 1.55	1.05 to 1.55	1.0 to 1.2
Phosphorous (Min)	0.8	0.8	0.7
Methionine (Min)	0.6	0.55	0.4
Salt (Min – Max)	0.25 to 0.75	0.25 to 0.75	0.25 to 0.75

3.3.3 Treatments

At three weeks of age, birds were randomly allocated among 12 pens. Random numbers between 1 and 12 were generated using a random number function in Excel (Microsoft Corporation, Redmond, WA). Birds were group housed for the random

allocation process and assigned to the appropriate pen in the order in which they were caught. There were three pens or replicates per treatment and four treatments in the study: negative control, quercetin only (0.75 g/kg feed), low dose quercetin and curcumin (0.25 g/kg feed), and high dose quercetin and curcumin (0.50 g/kg feed).

Each pen had one designated and labeled feeder and one waterer to prevent cross-contamination between pens. Feeders were weighed before and after the addition of new feed. Up to 1 kg of new feed was first weighed (Ohaus SPX1202 Scout Analytical Balance, 1200 g x 0.01 g) in a plastic container and the appropriate dose of antioxidants to be added calculated. Antioxidants were then weighed (Ohaus SPX1202 Scout Analytical Balance, 1200 g x 0.01 g) in disposable plastic weigh boats before being added to the feed. The weigh boat containing the antioxidant was "washed" in the feed to maximize the transfer of the antioxidant to the feed. The feed was then poured into a blender, as previously described, and the resultant mash transferred to the appropriate designated feeder. The feeder, with a pre-recorded empty weight, was then weighed to determine the total amount of feed available. The control diet was prepared first, and the low and high-dose quercetin and curcumin diets prepared last so that there was no cross-contamination among treatments. The blender was hand-washed in warm soapy water and dried between feedings.

3.3.4 Health monitoring

Broilers were assessed daily for signs of ascites or other miscellaneous health problems, such as lameness. Broilers demonstrating signs of lameness or ascites were euthanized by CO_2 inhalation followed by cervical dislocation.

3.3.5 Body mass

The birds were weighed once every seven days starting on Day 14. To do this, birds were placed in a clear plastic tray on the scale (Ohaus Valor 2000 V22XWE6T, 6000 g x 1 g). The mass was recorded to the nearest gram after the reading stabilized for several seconds.

3.3.6 Oxyhemoglobin and heart rate measurement

A pulse oximeter clamp (Criticare Systems, Inc., Milwaukee, WI 53226) was placed between the radius and ulna. The probe was repositioned as necessary to maintain a pulse reading of ≥ 250 beats per minute to maximize the likelihood of measuring the greatest possible oxy-hemoglobin saturation in blood supplied by the arteria radialis profunda.

3.3.7 Ventricular hypertrophy and organ weights

The right ventricle and left ventricle and septum, and the liver, and spleen were weighed to the nearest 0.1 g (Ohaus SPX1202 Scout Analytical Balance). The ratios of right ventricular mass to total ventricular mass (RV:TV) were calculated.

3.3.8 Antioxidant capacity of serum

Using a Biotech Synergy HT microplate reader, plasma total antioxidant capacity was measured with a colorimetric antioxidant assay kit (Cayman Chemical Company, Ann Arbor, MI). Blood from the broilers was collected using the anticoagulant heparin, then centrifuged in order to collect the plasma layer. Next, the samples were stored in a -80°C freezer until ready to use. The plasma was diluted to 1:20 with Assay Buffer before assaying.

3.3.9 Statistical analyses

Statistical analyses were performed using commercially available software (STATA 15.1, College Station, TX). Likelihood ratio tests were used to test the overall statistical significance of a variable where an interaction was statistically significant ($\alpha = 0.05$) or where an interaction was hypothesized. Data were descriptively analyzed graphically and numerically to assess data distribution and check for the presence of outliers. Backwards step-wise linear regression was performed with the full model containing both factor variables and their interaction. Results are presented as marginal means ± SEM unless otherwise stated.

3.4 Results

3.4.1 Descriptive

There were six male and twelve female birds in the control group, ten male and eight females in the quercetin only group, four males and fourteen females in the low dose quercetin and curcumin group, and nine males and nine females in the high dose quercetin and curcumin group. In general, the individual weights of birds among all treatment classes increased over the course of the study, with no treatment outperforming any other.

3.4.2 Treatment effect on oxyhemoglobin

There was no significant effect of treatment on oxyhemoglobin ($P = 0.46$). However, it was found that oxyhemoglobin saturation increased over time by two percent per week ($P < 0.001$).

Table 3.2. Treatment effect on average liver weight, average spleen weight, and total ventricular weight ratio (RV/TV) (n=72).

Treatment	Margin	Std Error	t	P>|t|	95% Conf. Interval	
Control	0.246	0.0223	10.81	0.000	0.120	0.292
Low dose quercetin and Curcumin	0.299	0.0223	13.17	0.000	0.253	0.346
Quercetin only	0.309	0.0223	13.61	0.000	0.263	0.356
High dose quercetin and curcumin	0.323	0.0223	14.20	0.000	0.277	0.369

3.4.3 Treatment effect on organ weight

It was found that treatment had no effect on liver weight (P = 0.31), spleen weight (P = 0.23), or total ventricular weight ratio (P = 0.34).

3.4.4 Treatment effect on feed to gain ratio

Feed conversion ratio was not affected by treatment (P = 0.97). However, there was a significant change in feed conversion ratio from week 3 to 6 ($P < 0.001$) but no difference among treatments ($P = 0.99$).

3.4.5 Treatment effect on blood antioxidant concentration

Blood antioxidant concentration was found to be different among treatment groups ($P = 0.0001$). Controls had the lowest concentration of antioxidants (1.79mmol ± 0.08), followed by low dose quercetin and curcumin (2.26 ± 0.07), quercetin only (2.02 ± 0.07), and finally high dose quercetin and curcumin (2.00mmol ± 0.07).

3.5 Discussion

The findings of this study suggest that the naturally occurring antioxidants quercetin and curcumin do not have favorable effects on growth performance and physiology of growing broiler chickens. Although the antioxidant concentration of blood was found to be significantly different among treatment groups, there was no antioxidant effect on organ weight, feed to gain ratio, or oxyhemoglobin saturation.

Despite oxyhemoglobin saturation not being significantly affected by the different treatments, statistically speaking, it was surprising to find that saturation levels increased by two percent each week instead of decreased. This is counterintuitive because studies have shown that heavier chickens have lower oxygen saturation levels than lighter chickens.[13] This increase could be explained by the presence or absence of ascites. In the presence of ascites, oxyhemoglobin saturation would likely decrease over time due to pulmonary hypertension. In the absence of ascites, oxyhemoglobin saturation would likely remain the same or even increase, as it did in this study. The presence of ascites is usually indicated by an increase in the right ventricle to total ventricle weight (RV/TV) ratio.[13] A RV/TV ratio greater than 0.30 is indicative of right ventricular hypertrophy, pulmonary hypertension, and ultimately ascites syndrome.[13–15] In our study, no treatment

had an average RV/TV ratio greater than 0.30 (table 2). This observation was not due to an abnormal growth rate, or insufficient feed to gain ratio since the average feed conversion efficiency (FCE) observed in this study (1.94) was slightly better than the industry standard (1.82)[16]. This may suggest the birds enrolled in this study may have been genetically less susceptible to ascites than other commercial varieties.

In spite of using two different levels of antioxidants in this study, there is a possibility that the amount of antioxidant supplemented in the diet could have caused pro-oxidative effects. Exogenous antioxidants play a key role in keeping the delicate equilibrium between oxidation and antioxidation reactions in living systems.[17,18] However, at high doses or in the presence of metal ions, these antioxidants can demonstrate pro-oxidative activities. Quercetin, for example has been shown to exhibit pro-oxidative activity at high concentrations (>50µM) *in vitro*.[17,19] Studies using low doses of quercetin (0.1-20µM) observed the antioxidant activity of this compound while higher doses (>50µM) decreased cell survival and viability *in vitro*.[20] In a study examining quercetins effect on expression levels of Mdr1 mRNA in different tissues of broilers by real-time RT-PCR, they found quercetin has positive effects when administered at two different concentrations (15 and 60 mg/kg).[9] Our study used a much larger concentration of quercetin in all treatment groups, with the lowest concentration of quercetin administered being 125 mg/kg. Another study was conducted on mice using curcumin at a concentration of 100 mg/kg and found treatment with curcumin caused a significant drop of the increased blood pressure caused by N-nitro-L-arginine-methylester (L-NAME).[21] Even with this finding, the concentration of curcumin used to elicit this positive effect was much less than the concentration used in this study. Thus, the

concentration used in our study could have elicited pro-oxidative effects in the broilers. This might explain why the low-dose quercetin and curcumin had greater antioxidant levels than high-dose groups.

Though this study measured oxyhemoglobin saturation levels, a limitation of the study was not measuring pulmonary arterial pressures. In addition, this study was only conducted at one site, limiting the application of the results obtained from this study to the climate and altitude of Lubbock, Texas. Housing of the birds may also be considered a limitation of the study, as the environment of our study did not completely replicate that of the industry. Despite these limitations, the results of this study may be used in future studies investigating the effects of quercetin and curcumin on broiler chickens.

The purposes of this study were to determine if the natural antioxidants quercetin and curcumin have favorable effects on growth performance and physiology of growing broiler chickens. Although this study found that quercetin and curcumin do not have favorable effects on growth performance and physiology of growing broiler chickens, it was found that oxyhemoglobin saturation increased over time instead of decreased, contrary to the hypothesis of the researchers.

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Chapter 4

Discussion

With increasing pressure from consumers to decrease antibiotic use in food-animal production, antibiotic-free methods to control disease are becoming more necessary to incorporate into production systems. Antioxidants could potentially be one of these antibiotic-free methods used to control or prevent disease in food-animal production. This belief stems from the numerous studies that have shown the positive effects antioxidants can have on an animals immune system, cardio-pulmonary system, and also their gastrointestinal system.[1–9] The addition of antioxidants into animal feed could potentially reduce the impact stressful periods have on an animal throughout the production period such as weaning, branding, transport, or heat stress, as these stressful events have been shown to increase oxidative stress biomarkers in multiple species.[10–13]

Historically, antioxidants have been added to livestock feed to prevent lipid peroxidation and increase shelf life of the feed.[14] In the near future, antioxidants may be incorporated into livestock feed not only to increase shelf life, but also for their positive health effects in livestock. However, before the addition of antioxidants into livestock feed becomes standard in the industry, more research should be conducted investigating concentration and administration of these compounds. This is because it has been shown high doses of isolated compounds (antioxidants) may be toxic, owing to pro-oxidative effects at high concentrations or their potential to react with beneficial concentrations of ROS normally present at physiological conditions that are required for optimal cellular functioning.[15] For example, in our studies we used concentrations much larger than other studies used and it resulted in no interaction between treatments, meaning you cannot add

any random antioxidant at any concentration and expect positive results. Therefore, future research needs to be conducted on specific antioxidants in specific species in order to determine the bioavailability of the antioxidant in the species in question and to also develop correct dosages and administration techniques, like what is common when using antibiotics.

In today's world, we have much greater growth rates, faster growing broilers, and higher producing dairy and beef cattle than ever before. By achieving an animal's maximum growth potential, it has become evident that these animals can develop chronic oxidative stress that could lead to tissue destruction and possibly mortality. In a recent study, it was found that 89.3% of cattle tested on the kill floor presented with chronic oxidative stress.[16] In broilers, a mismatch between oxygen demanding organs (i.e. muscles) and oxygen-supplying organs (i.e. heart and lungs) has emerged and resulted in increased blood pressure within the pulmonary arteries, which subsequently can lead to the progressive development of pulmonary arterial hypertension syndrome (ascites).[17] By supplementing livestock with exogenous antioxidants, the prevalence of chronic oxidative stress and ascites could be reduced significantly, increasing production and decreasing the monetary loss lost by producers when animals succumb to the diseases caused in part by oxidative stress.

As consumer pressure to decrease antibiotic use in livestock production keeps mounting, alternative methods to control and prevent disease will eventually become a reality for livestock producers. Based on antioxidants relative high availability, low cost, and positive health effects at the correct dosage, researchers should continue researching antioxidants as an alternative to antibiotics.

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