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The Effects of Chitosan on Broiler White Striping

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The Effects of Chitosan on Broiler White Striping

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ABSTRACT

Chitosan is a compound that is shown to have numerous health benefit on chickens. It is shown to decrease heat stress, reduce pathogen load, and is also shown to have anti-inflammatory actions. More specific to the case of white striping, chitosan can also prevent sterol emulsification, which can help inhibit fat absorption, and is thought to be able to help with decreasing fat deposition. In this study, chitosan was used as a feed additive to mitigate the effects of white striping in broilers. We hypothesized that broilers fed chitosan would have improved meat quality and reduced incidence of white striping dose dependently. In this study, three groups of 42 broilers were fed different diets: conventional corn and soybean meal diet with no chitosan, 0.2% chitosan, and 0.4% chitosan added in the grower and finisher phases. There were 7 birds per cage and 6 cages per treatment, and were euthanized after 6 weeks of growth. To test our hypotheses, we examined weight progression, drip loss, cook loss, white striping scores, gross pathology, histopathology, and gene expression to discern the effects of chitosan on white striping. Overall, there were no significant difference in growth, showing how chitosan supplementation does not compromise growth. The 0.4% chitosan group showed the best results from chitosan supplementation with less cook loss and reduction in white striping incidence. There were unexpected results observed where the 0.2% chitosan group had a lower white striping incidence compared to the control group, but showed more expression of potential foam cell marker *CD36* and fat deposition marker *PPARG*. There were no statistical differences found with the other gene markers. Overall, chitosan supplementation improved meat quality and reduced white striping incidence, particularly at a 0.4% concentration. Chitosan supplementation has been adopted in poultry feed due to its broad benefits in broiler performance and health. Thus, further studies should be performed to further understand the mechanisms by which of chitosan mitigate the pathophysiology of white striping by regulating fat deposition in breast muscle.

TABLE OF CONTENTS

List of Figures/Tables

Chapter 1: Literature Review of Fast-Growing Broiler White Striping

1.1. Introduction

Annual broiler production has increased in the United States by approximately six-fold over the last 50 years. Approximately 60 billion pounds of live weight broilers were produced in 2021 [1]. The increased production is due to the increased demand and consumption of poultry products, as it increased from 15.5 kg per capita in 1960 to 51.4 kg per capita in 2021 in the United States [2]. In order to fulfill this increased need for poultry products, broilers were bred to grow faster with greater yields using genetic selection. In 1925, broilers reached a market weight of 1.13 kg in 112 days, while broilers reached a market weight of 2.93 kg in 47 days in 2021 [3]. Although this fast growth was meant to fulfill market expectations, it eventually led to unwanted pathological changes, such as white striping in meat. White striping is the deposition of fat along the muscle fibers during a bird's growth and development, mainly cranially to caudally in the direction of the myofibers of the pectoralis major muscle. Other broiler breast myopathies associated with fast-growing broilers include wooden breast, spaghetti meat, deep pectoral myopathy, and pale, soft, and exudative meat. White striping is characterized by the presence of increased intramuscular fat in the pectoralis major of broilers, whereas wooden breast is characterized by the paleness and hardness of the muscle tissues, and spaghetti meat is where the muscle tissues lost their integrity, so that the muscle fibers appear spaghetti-like. Kuttappan et al. [4] have shown that white striping has an incidence of 90% in all chicken breast products. White striping studies have included the investigation of phenotypes at the histological, pathological, and biochemical and biomolecular levels. Researchers identified cells, gene markers, and other molecular species that may contribute to the presence of white striping; however, no treatment has been discovered yet [5]. In this

literature review, we described (1) how chicken production has changed over the years to ultimately generate larger birds more quickly; (2) muscle growth physiology; (3) the suspected pathological process of white striping; (4) the causes of white striping; (5) how white striping is visualized, detected, and measured; (6) the effects of white striping on meat quality and production; and (7) strategies attempted to mitigate white striping and outcomes from their applications.

1.2. History of Chicken Production

Before the establishment of the industry in the twentieth century, poultry production was based upon individual households managing their own flocks of chickens. Moreover, production was limited due to a lack of knowledge about those factors that have the greatest impacts upon efficiency (e.g., housing, feed, sunlight and vitamin D, etc.). Thus, chickens were raised primarily for egg production as a source of income. Chicken meat was only a byproduct of egg production and was considered a delicacy that would only be consumed on special occasions [6]. Early efforts at broiler line development began in the 1920s in the United States. Confined housing and the caging system for birds were developed, which improved production and decreased mortality rates [7]. Then broiler production became more prevalent in areas such as New England, Arkansas, and Georgia. In the 1940s, broiler production was bolstered by an increased demand to feed troops in World War II. People were also encouraged to raise their own chickens, as they were easy to raise and had a high production value. At that point in time, hatcheries, farms, feed mills, and processors were all separate entities; however, as businesses began working together, an integrated industry grew. This made the production of chicken products more efficient and profitable. Moreover, chicken sales went from whole carcasses to offering specific cuts that were fabricated and packaged as ready-to-cook. Nevertheless, broilers were still not included as a separate statistic

for chicken data in the 1949 USDA reports [7]. In the 1950s, poultry meat was mainly fabricated from broilers—no longer as a delicacy. Moreover, the USDA recognized the importance of the broiler industry and introduced a program in which the quality of broiler products was monitored. At this time, the National Broiler Council was organized. Because of efficient production with minimal labor, 90% of chicken products came from the integrated operations.

In the 1970s, advancements in genetics, nutrition, medicine, and mechanization further advanced broiler production in the United States. Chicken surpassed beef and pork as the top selling meat in 1992. The United States also started exporting chicken products to other countries with billions of dollars of revenue, and the USDA initiated more programs and protocols to monitor quality control and reduce production hazards [6,8]. Advancements in poultry nutrition also contributed to improvements in broiler growth. Prior to the twentieth century, chickens were fed household scraps, such as vegetables, and were expected to find insects [9]. Books published in the early 1910s provided guidance into feeding chickens with the introduction of the concept of feeding grains [10]. Today, chickens eat a mix of starch-rich feedstuffs, such as corn grain and protein-rich feedstuffs, such as soybean meal, as well as vitamin and mineral supplements [11]. Starter, grower, and finisher diets have been adapted to the nutrient requirement of chickens at different stages of life. The feed ingredients for broilers and layers are similar; however, as meat birds, broilers have higher requirements of protein and energy to bolster their growth rates and yields. Feed efficiency is also increased as a result of these dietary changes. In 1985, 3.22 kg of feed was needed to produce a 1.40-kg broiler. In 2010, 3.66 kg of feed was needed to grow a 2.44-kg broiler [12]. In 2001, the breast muscle weights for Ross 308 were 8.4, 9.9, 10.3, and 9.8% higher than those in 1957 [13].

Genetic selection is another important reason that today's broilers grow larger at a faster rate. Producers selected broilers that grow faster, with better immunity and feed efficiency. The United States produces approximately 8.6 billion broilers every year, which has made it easy for the industry to select from a large pool of chickens for breeding. Advances in reproduction and DNA technology have also made the breeding process significantly faster [14]. Because of the short life of broilers, they grow expeditiously. Kuttappan et al. [15] observed that broilers who were fed a high-energy diet developed more severe degrees of white striping, suggesting a positive correlation between a high growth rate and the appearance of white striping. Furthermore, according to Alnahhas et al. [16], white striping is also highly heritable, with h^2 of 0.65 ± 0.08. Thus, although with advances in nutrition, selection for rapid growth has heightened the risk for white striping.

1.3. Muscle Growth in Broilers

Muscle growth is defined by both hyperplasia and hypertrophy. Hyperplasia happens *in ovo*, thus the number of muscle fibers that a chick can have is established before hatching. During development, muscular tissue is formed in myogenesis, in which, after cell proliferation and differentiation, the myoblasts fuse to become myofibers [17]. Post-hatching muscle growth occurs via increasing muscle fiber size that occur through the active satellite cells fusing to the muscle fibers [18]. After the somite specification and the determination of the sclerotome, different regions of the somite will then develop into their own respective derivatives. The ventral–medial portion of the somite gives rise to bones and cartilage, the dermatome gives rise to the skin and connective tissues, and the myotome gives rise to the skeletal muscle. Through different series of signaling and inhibition, myogenesis takes place [19].

Some somites diverge into myotome cells; then, myogenic bHLH (basic helix–loop–helix) proteins, such as transcription factors MyoD and MRF5, bind to muscle-specific genes to promote muscle differentiation. Thus, myotome cells start to produce the myogenic bHLH proteins and become committed muscle precursor myoblasts. The myogenic bHLH proteins also help with myoblast proliferation, specifically the fibroblast growth factors. After a few rounds of proliferation, the myoblasts undergo differentiation. The differentiation of some myoblasts can also signal other myoblasts to differentiate. Muscle cells begin to fuse to form fibers; fused cells must leave the cell cycle. During fusion, the cells align into chains by force generated by actin-based structures, eventually forming the multinucleated myotubules of the muscle [20]. The fusion of myoblasts activates myogenin - another bHLH protein - which then mediates the differentiation of other muscle cells. The myoblasts and muscle fibers are held together by fibroblasts, which can help arrange and orientate the muscle cells. Myoblasts then fuse through signaling pathways, such as the β1D-integrin–FAK pathway, the MEK5-ERK5-SP1-Klf2/4-Npnt pathway, and the NFATc2–IL-4pathway [21]. After fusing, the skeletal muscle fiber can then develop, mature, and form different isoforms, such as type I and type II muscle fibers [22,23]. Then, the formation of muscle fibers is completed in ovo.

Muscle fiber growth after hatching is caused by hypertrophy from satellite cell nuclei recruitment, which causes the enlargement of muscle fibers [24]. Muscles have vasculature and innervation. The vasculature maintains homeostasis for the muscles by providing the key nutrients and removing waste products, and it is also crucial in activating muscle regeneration [25]. In recent studies, it was shown that endothelial cells of the blood vessel can regulate myogenesis and myogenic cell expansion [26]. In addition, nerves are involved in muscle movement and are

closely associated with muscle growth. Thus, nerve damage can lead to muscle atrophy, which is also known as neurogenic atrophy [27]. Muscles that are no longer innervated have functional deficits, no longer contract anymore, and can degenerate [28].

1.4. The Suspected Pathological Process of White Striping

White striping can be grossly seen as white lines extending down along with the muscle fibers. Samples with severe white striping demonstrated signs of tissue degeneration like necrosis, atrophy, fibrosis, and macrophage infiltration [29]. White striping manifests from the progressive deterioration of the muscle structure as dysfunction occurs in physiological processes of muscle metabolism, growth, and vascular homeostasis (**Figure 1.1**).

White striping is associated with vascular inflammation and macrophage infiltration as the fast growth of the pectoralis major muscle disrupts its metabolism and homeostasis [30]. Hypoxia is also involved in the formation of the white stripes. In hypoxic conditions, ATP production is reduced due to low cellular oxidative respiration, and the energy-dependent sodium and potassium pumps are disturbed that further affects the abilities of cells to control the influx and efflux of ions. Excess sodium can result in vacuole formation, which can be observed histopathologically [31]. Dysregulation in ion gradient homeostasis leads to cell swelling, cell rupture, and necrosis [32]. Muscle satellite cells play an important role in muscle regeneration and repair. As the tissues growing too fast, there is no room for satellite cells proliferation and vascularization, which leads to necrotic lesions [33,34]. In addition, collagen produced by satellite cells can further lead to the fibrosis in the affected tissues [35]. The inflammation of the tissues is also related to acetyl-CoA accumulation, where it can cause β-oxidation impairment, leading to increased fat accumulation

[36,37]. With inflammation, as well as lipid accumulation, the white blood cells will migrate to the tissue of interest from the basolateral membrane, and through the blood vessel walls after inside-out signaling and integrin activation [38]. The macrophages will phagocytose the dead cells [39] and can also ingest the lipids and become lipid-laden foam cells, which contribute to the visible white striping [40]. Foam cells can also release cytokines, which will recruit more immune cells, exacerbating the foam cell formation [41].

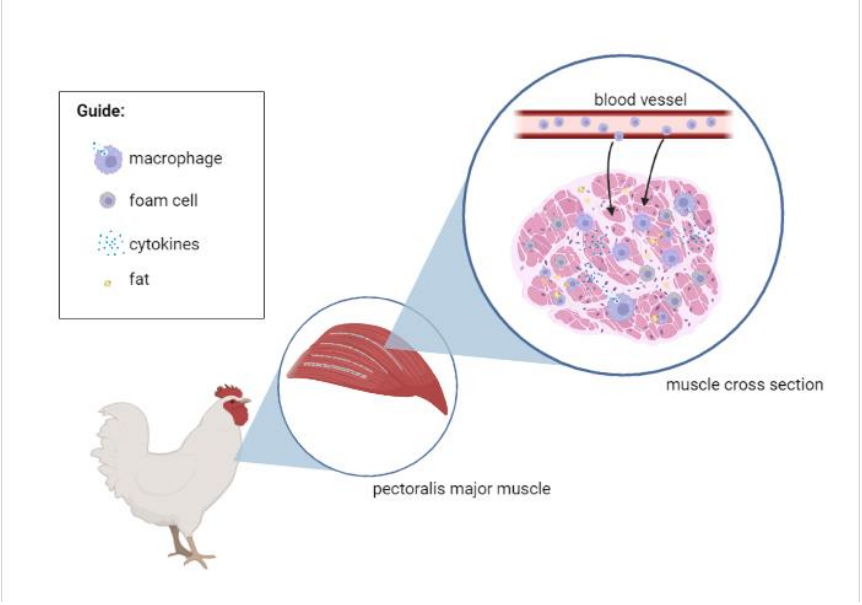

Figure 1.1. A schematic diagram of the suspected mechanism causing white striping. Created with BioRender.com. Accessed on 29 March 2023.

1.5. The Causes of White Striping

It is believed that hypoxia underlies the etiology of white striping, which is associated with metabolic disturbances, as well as heart and vascular diseases in rapid-growing broilers [42]. With an increased incidence of hypoxia, a broiler's metabolism can be described as becoming increasingly anaerobic. The pectoralis major muscles are mostly made up of type IIB fibers with low numbers of mitochondria; thus, they are more susceptible to hypoxic damage [43]. With less ATP produced from fewer mitochondria, reduced apoptosis and increased inflammatory responses

may occur, leading to inflammatory diseases in the body [44]. This was also reported in atherosclerosis, which has a similar disease mechanism as white striping in the muscles [45–47]. Several key metabolic pathways were altered in the broilers that had white striping, such as the TCA cycle, β-oxidation, and taurine metabolism [47]. In particular, there are increased taurine levels in pectoralis major muscle of birds that had white stripping [47]. Taurine plays a role in protecting the tissue from damage caused by hypoxia, and is also involved in regulating calcium homeostasis of the tissue. Increased taurine levels in the muscles led to increased calcium levels and osmosis in the muscle tissues, causing swelling. This swelling will further decrease the amount of oxygen reaching the tissues [48].

Boerboom's group suggested faulty β-oxidation might lead to white striping, as indicated with the increased levels of fatty acids with decreases in acylcarnitine esters. In a process characterized as lipotoxicity, several pathogenic processes are associated with fatty acid accumulation that substantially affect the tissue [49]. Fatty acid intermediates, such as ceramides, act as second messengers to initiate cell apoptosis. Free fatty acids are precursors to phospholipids that can affect membranes such as the inner mitochondrial membrane and result in reactive oxygen species (ROS) [48]. The subsequent accumulation of ROS results in damage to biomolecules that form cellular structures, organelles, proteins, nucleic acids, and lipids. If antioxidants in the cells are spent from the ROS, oxidative stress develops, which may further lead to pathogenesis. The lack of β-oxidation is also able to increase the intracellular calcium content, which can lead to cytotoxicity, eventually leading to cell death, which can also lead to the increase in fatty acids since they are not being broken down. Some intermediates of the TCA cycle increased, while others were reduced back to the previous intermediate. For example, where oxaloacetate was

originally converted to citrate, it was reduced back to malate in broilers with white striping. This backwards conversion is due to the electron transport chain not being able to oxidize the reducing equivalents under low oxygen circumstances [48].

Besides examining pathophysiology within the muscle, one could also consider etiologies of white striping from a more basic perspective. Recently developed diets provide more energy with greater amounts of starch and fat than diets from over sixty years ago [49]. Commercial growers today can capitalize on dietary advancements because of decades of genetic selection for growth and yield. Thus, it is important to note that selection and, thus, genetics have been a significant cause of white striping. In examining one line each of moderate- and high-yield broilers in the United Kingdom, Bailey et al. [50] noted greater levels of white striping in the high-yield broilers. They characterized the heritability of traits such body weight and body yield at h2= 0.271 – 0.418 with white striping at h2= 0.338. In Brazil, Panisson et al. [51] compared three lines, including high yield Cobb 500® and Ross 308® with moderate yield Embrapa021®, and their diets with varying nutrient densities. They reported that, regardless of diet, the higher yield lines had a greater incidence of white striping, with the higher yield lines being 24.4–28.0 and 11.0–25.1 times, respectively, more likely to have higher white striping scores by days 42 and 49. When describing the history of broiler production, comparisons were made between broilers from the 1950s and 1960s to today's broilers. A striking contrast was noted between the genetics of broilers from these two eras by Havenstein et al. [13,50]: in examining the growth and carcass composition of broiler lines from these two eras (2001 vs. 1957) when both were fed diets from the two respective eras, they observed that the 2001 broiler line had better food conversion, greater body weights, and greater carcass yields, but greater mortality rates, regardless of diet, relative to the

1957 line. Performance results like those of the 2001 line support the significant contribution that genetic selection has played on positive traits, such as yield, and negative traits, such as the incidence of white striping. One genome-wide analysis study has implicated three putative quantitative trait loci (QTL) regions on chromosomes1, 17, and 18 within the chicken genome to be associated with white striping when comparing one of each pHu+ and pHu- line [13]. More recently, 18 SNPs have been associated with white striping in Cobb 500® broilers with six QTLs on chromosome 5 with possible candidate genes involved in insulin secretion, cardiac electrical activity, and inflammation [54]. Genetic selection has significantly contributed to the incidence of white striping.

1.6. Visualization, Detection, and Measurement of White Striping

According to USDA poultry inspection regulations, disease check has to be performed on birds before slaughter to remove the unfit cuts. White striping is not often considered during trimming, as it is only considered an issue regarding meat quality but not necessarily meat safety [49,55]. However, Alnahhas et al. [16] revealed a positive correlation between the weight of the bird at the time of slaughter and the severity of white striping. In addition, Kuttappan's research reported that high-energy diets were positively correlated with white striping incidence. Thus, although antemortem detection for white striping has not been considered a priority, the prediction of white striping might be necessary. Many scoring methods have been adopted for white stripping antemortem detection. For example, Silva et al. [56] utilized real time ultrasound to measure the breast volume, area, and thickness in broiler chickens. Computer imaging and machine learning systems are also used to score white striping with models, which can be faster and more accurate

than human scoring [57]. Additionally, some researchers are considering the detection of white striping of a carcass with a spectrophotometry-based sensor when the skin is still attached [58].

Upon slaughtering broilers, white striping can be detected visually as white striations present along the muscle fibers. Striping scores have been created to describe the severity of white striping in the pectoralis major muscle [59]. White striping scores range from 0 to 3 depending on the amount of striping present on the breast muscle. A score of 0 denotes normal or no white striping. A score of 1 represents mild white striping, characterized as the stripes being under 1 mm thick; a score of 2 is for moderate white striping, where the white stripes are between 1 and 2 mm thick; and a score of 3 represents severe white striping, where the stripes are over 2 mm thick and cover most of the breast muscle (**Figure 1.2**) [59]. In a study performed by Trocino et al. [60], male broilers showed more severe white striping than females, whereas female broilers exhibited higher incidence of white striping. Diet restriction was reported to increase both incidence and severity of white striping cases [61].

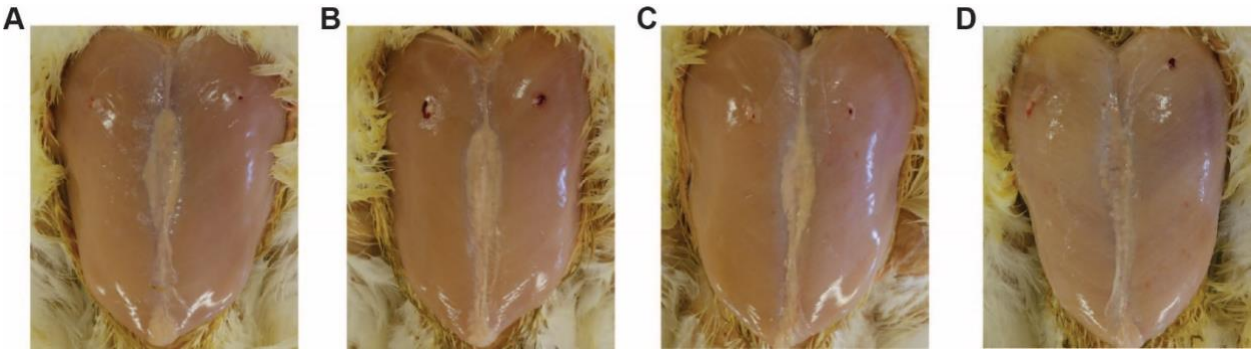

Figure 1.2. White striping scores for pectoralis major muscles. White striping in broiler breast can range from almost no striping in a normal breast to severe levels of striping. These photographs of the pectoralis major muscles of 42-day old Cobb 500 broilers at market weight demonstrate the range in the scoring system from Kuttappan et al. [58]. Breasts could be: (A) score 0, normal with little to no striping, or could have (B) score 1, mild striping, (C) score 2, moderate striping, or (D) score 3, severe striping.

Sections of grossly necropsied samples can be evaluated histopathologically. There is apparent fat infiltration around the muscle fibers affected by white striping, accompanied by different levels of necrosis, atrophy, vacuolar degeneration, fibrosis, and macrophage infiltration [60]. Necrosis can be visualized microscopically around the muscle fibers if there is a presence of a bundle of dead cells due to the increased β-oxidation of long-chain fatty acids [48] with macrophage infiltration present to clear away the dead cells [62]. Vacuolar degeneration is the swelling of cells observed due to cell injuries, which might also derive from the increased β-oxidation, where the cell is trying to repair itself [63] **(Figure 1.2)**. Atrophy, a reduction in the size of the muscle fibers, is also present in tissues with white striping. When examining the tissues, fibroblasts can be seen initiating scar-like fibrous connective tissue [64]. Further analyses can be performed with immunohistochemistry by staining biomarkers such as MYH15 and NCAM, as studies have shown increases in their levels were proportional to the severity of white striping [5].

1.7. The Effects of White Striping on Meat Quality and Production

Chicken breast products with white striping do not appeal to customers because of the decreased meat quality, and some scientists point to white striping as a symptom of other metabolic issues [65]. In the studies of Petracci et al. [66], normal chicken breast muscles contained 0.78±0.09% fat, while breast muscles with severe white striping had 2.53±0.30% fat. This increase in intramuscular fat might affect the palatability negatively, as cooked breast muscle with white striping tasted tougher [67]. The increase in fat content is also related to a decrease in protein content. Compared to normal broilers, broilers with severe white striping had 2% lower protein content [66]. Broilers with severe white striping also had 0.13% greater collagen content [66], representing a decrease in the protein quality. The calorie values are also higher (13.56 kcal/100

g) in severe white stripping than normal broilers [15]. In summary, white striping reduces the overall nutritional values of the breast muscles of broilers. White striping also led to a 12% of loss in production [67], therefore, it is important for researchers to solve this problem.

White striped broiler breast meat features are listed in **Table 1.1**. Decreased meat quality were largely reported in the chicken breast muscles exhibiting white striping. For example, Mudalal et al. [30] observed that breast muscle with white striping had a higher pH level and lower drip loss, as high pH levels can allow the muscles to retain liquid better, thus having greater cooking loss values because the increased liquid retained are loss after the meat is being cooked. Breast muscles with white striping had higher compression values, suggesting that the meat is less tender than the breasts without white striping. The breasts with white striping are also less juicy [68] and chewier [69] than the normal chicken breasts, as they have higher toughness values, which may be due to the increase in the collagen content. Kong et al. [70] reported that breast fillets with white striping contained less phospholipids, which might lead to a lower sensory aroma profile, since phospholipids have shown to increase the aroma in beef. In addition, histidine that contributes to the taste activity value was also decreased in white striped breasts, which might also relate to the decreased palatability of breast fillets with white stripes [71]. It should be noted that while there do not seem to be animal health concerns specifically related to white striping, there are health and welfare issues associated with the management of fast-growing broilers.

Table 1.1. Literature describing the effects of white striping on meat composition and quality.

White Striping on Meat Composition and Quality	Study
Decreased palatability	Kuttappan et al. 2012 [15]
Increased calorie	Kuttappan et al. 2012 [15]
Increased pH level	Mudalal et al. 2015 [30]
Decreased drip loss	Mudalal et al. 2015 [30]
Indicator of hypoxia	Boerboom et al. 2018 [48]
Indicator of decreased β oxidation activity	Boerboom et al. 2018 [48]
Increased fat content	Petracci et al. 2014 [66]
Decreased protein content	Petracci et al. 2014 [66]
Increased collagen content	Petracci et al. 2014 [66]
Decreased juiciness	Lee et al. 2021 [68]
Increased chewiness	Brambila et al. 2016 [69]

1.8. Strategies Attempted to Reduce White Striping

Nutritional interventions have been adopted to mitigate white striping in broilers while avoiding negative effects on growth performance (Table 1.2.). For example, Bodle et al. [72] evaluated increasing digestible arginine to 111-125%, supplementing vitamin C at 94.4mg/kg, increasing the vitamin premix supplementation 2-fold, reducing the dietary amino acid density in the grower phase by 15%, and combinations of these four strategies. Hypoxia is associated with white striping. Arginine supplementation could help with increasing vasodilation [73], thus supplying the muscles with better oxygen resources. It was also theorized that vitamin C has strong antioxidant effects, thus supplementing vitamin C could against free radicals to prevent cell damage and reduce white striping [74]. Moreover, the reduction in amino acid supplementation, such as lysine, could slow muscle growth, thus allowing more blood flow into the muscle and possibly reduce white striping incidence. Lysine bolsters proteins synthesis, thus reductions might slow the growth [75]. Broilers fed an 85% dietary lysine diet had a lower white striping incidence and less tissue damage [76]. Similar results were seen in another study as well, where the reduction

of dietary lysine (75% and 85%) at different growth time points (18 to 26d and 28 to 40d) also decreased the severity of white striping at 48 and 61 days of age [77]. However, since lysine is the first limiting amino acid for chickens, it has been observed that there was a decrease in body weight compared to the control group [68], and Bodle et al. concluded that the overall treatment effects for each strategy were not significant. In addition, the supplementation of methionine from natural sources of roasted cowpeas and sunflower seed meal also reduced white striping incidence by a decrease in *PPARG*, *PTGS2*, and *CD36* expression in the pectoralis major muscles of the treatment groups (0.50 and 0.49% for the starter phase, 0.47 and 0.44% for the grower phase, and 0.46 and 0.47% for the finisher phase) [78]. Overall, there is no a single solution to the resolution of white striping due to complicated underlying factors leading to the disease. Nevertheless, the ultimate goal is for producers to raise their chickens with less white striping, reduced oxidative stress, and with normal muscle growth.

Table 1.2. Successful dietary intervention to reduce white striping.

Strategy	Mechanisms	Outcomes	Reference
Decrease lysine supplementation	Lessens proteins synthesis; reduces growth, vasoconstriction, and hypoxia	Lower white striping incidence and less tissue damage	Ahsan et al. 2022 [16] Meloche et al. 2018 [77]
Feed restriction	Less energy for muscle growth	White Striping incidence increased	Trocino et al. 2015 [60]
Decrease feed intake	Less energy for muscle growth	White Striping incidence decreased	Meloche et al. 2018 [77]
Alternative methionine source	Legume antioxidant properties	Lower white striping incidence	Sachs et al. 2019 [78]
Vitamin E supplementation	Antioxidants; cytoprotection.	White Striping incidence decreased	Kuttappan et al. 2012 [79]

Feeding the birds with antioxidants, such as vitamin E, was thought to be helpful in mitigating the cell damage that leads to adipose tissue replacing muscle tissue growth. However, there were

not any significant effects associated with it [79]. Feed restriction studies have also been performed to discern their effects on white striping. Meloche et al. [80] found that a decrease in feed intake was associated with a decreased white striping severity. However, a second study is conflicting: Trocino et al. [60] found that feed restrictions led to the increase of white striping in breasts. Researchers have also attempted to use genetic selection to reduce the incidence and severity of white striping. It was reported that CTSD (codes for peptidases), LSP1 (codes for F-actin binding protein, which participates in muscle filament formation), TNNI2 (codes for skeletal muscle proteins), SYT8 (codes for membrane proteins important in exocytosis), and MOB2 (regulates protein phosphorylation) might be responsible for the appearance and inheritance of white striping, as they are related to pancreatic β cell and insulin secretion [53]. We speculate that restoration of beneficial genetics variants to these genes specifically and selectively breeding the birds may help with decreasing white striping incidence. Variability exists between different mitigation techniques. Further studies need to be conducted to better understand how to mitigate white striping while preserving favorable traits.

1.9. Conclusions

Improvements in genetics, nutrition, and management have led to a significant increase in broiler production. With advances in production came an increased demand for poultry meat. However, these advances have led to some trade-offs in meat quality, as is the case with myopathies that have arisen in broiler muscles. White striping is one example of myopathies that causes financial losses due to meat quality and consumer satisfaction concerns. Multiple factors may contribute the development of white striping in broilers. Hypoxia, inflamed muscle tissues, accumulation of lipids, and the over-recruitment of macrophages into the tissue likely leads to

foam cells, much like an atherosclerotic process with the accumulation of fat and fibrosis, leading to the white stripes. More investigation is ongoing to better understand the pathophysiology of white striping. Other researchers are examining myopathies such as wooden breast and spaghetti meat - two other serious broiler conditions - with some theorizing that they all share similar metabolic dysfunctions. All of these conditions are likely the product of the efficacy of fast-growing broilers.

There is much still to attempt in further examining mitigation strategies to decrease the incidence of these myopathies in broiler chickens. Nutritional strategies are mainly focusing on including nutrients and substances that aid in decreasing inflammation, reducing fat accumulation in the breast, or providing antioxidant properties. In addition, slowing down the bird growth was proved to be effective controlling white striping. However, the trade-off is reduced efficiency, which could lead to decreases in overall production and increased management costs per bird. Additionally, genetic selection for broilers without myopathies or introduction of new genetic stock could lead to reductions in white striping. Ultimately, a reexamination of the nutrition and genetics of fast-growing broilers could lead to reductions in myopathies, such as white striping.

Chapter 2: The Effect of Supplemental Dietary Chitosan on Broiler Performance and Breast Myopathy

2.1 Introduction

White striping has become one of the most commonly seen commercial broiler conditions. This myopathy is characterized by the appearance of white fat striations throughout the pectoralis major muscle of fast-growing commercial broilers. The breast meat of broilers affected by white striping (WS) has reduced palatability and thus meat quality is lessened [69, 81]. Kuttappan et al. also reported that chicken breast fillets with white striping have lower consumer acceptance due to unappealing aesthetics as they look fattier [82]. WS is highly heritable, likely an outcome of genetic selection for fast growth [51]. Causes of white striping have been recently reviewed by Lee & Mienaltowski [83]. Briefly, WS is associated with myovascular inflammation within broiler breast muscle that is growing faster than the vasculature's ability to maintain metabolic demands. Lipid accumulation in the muscle and vascular walls leads to macrophage migration to the region; macrophages intake lipids and become fat-laden foam cells. Mitigations for this process have already been pursued, either through dietary interventions or genetic selection, but only a few have successfully decreased white striping without compromising growth [83]. In this study, we investigated a supplementary diet approach with chitosan in an attempt to reduce white striping via a nutritional approach.

Chitosan is a polysaccharide derived from the deacetylation of chitin, which is one of the most abundant carbohydrates found in the biosphere. Chitosan is a component of fungal cell walls as well as the exoskeleton of insect and arthropod shells, such as shrimps and crabs, with an annual

estimated production of one billion tons [84]. Chitin extraction and chitosan synthesis techniques were recently review by Pellis et al. [85]. Given the positively charged nature of chitosan in neutral conditions, it possesses a lot of beneficial life properties, and has become one of the most promising substances in life sciences and agricultural research. It has antimicrobial, antifungal, antiviral, anti-inflammatory, and antioxidant properties, and can act as a prebiotic and inhibit fat absorption [86].

Chitosan has been added to chicken diets in previous studies for several reasons. Menconi et al. found that dietary chitosan supplementation decreased the overall colony-forming units of *Salmonella enterica* serovar Typhimurium maintained and transmitted in broiler chickens [87]. Moreover, Lee et al. reported the bifidogenic effect of chitosan as it stimulated the growth of *Lacticaseibacillus casei* and *Lactobacillus brevis* [86]. Chitosan was suggested to have similar characteristics to the known prebiotic substances like β-glucan and inulin [88]. Furthermore, Chang et al observed that chitosan alleviated heat stress and its negative effects by decreasing the production of reactive oxygen species (ROS), activating the Nrf2-antioxidant signaling pathway, as well as stimulating glutathione peroxidase activity [89]. Thus, chitosan has been previously safely fed to chickens.

Studies have also detailed how chitosan interacts with lipids. Chitosan has been shown to reduce serum cholesterol in humans [90], as well as improved cases of fatty liver and hyperlipidaemia induced in high-fat fed mice by inhibiting intestinal fat absorption [91]. It is believed that positively charged chitosan interacts with the anionic carboxyl group of fatty acids and bile acids and can prevent sterols from emulsifying [90]. According to Deuchi et al., chitosan

supplementation increased fecal lipid excretion and reduced the apparent fat digestibility in rats [92]. Thus, in this study, we hypothesized that the fat-absorption-inhibiting and antioxidant properties of dietary chitosan could play important roles in the reduction of white striping. Its antioxidant property could reduce the tissue impairment caused by ROS [93], therefore reducing inflammation and decreasing the numbers of immune cells being recruited to the breast muscles. Additionally, with less fat being absorbed, chitosan supplementation may further decrease the amount of ROS damage to the tissues. We also hypothesized that there could be less fat for the macrophages to intake, thus reducing the number of foam cells formed that lead to the white fat striations on the pectoralis major muscles.

2.2. Methods

Experimental Design, Diets, and Animal Housing

The current study was approved by the Institutional Animal Care and Use Committee at University of California, Davis (UC Davis). A total of 126 one-day-old Cobb-500 broilers were received from Foster Farms, weighed, and sorted randomly into 18 pens (4ft × 4ft), with 7 birds per pen. The broilers were housed in the animal rooms at the Hopkins Avian Facility on the UC Davis campus. The animal room temperature was set at 30°C for the first few days, then decreased by 2°C–3°C weekly until it eventually reached 20°C, and maintained until the end of the study. Birds were randomly allotted into 3 treatment groups with 6 pens per treatment group. Feed and water were provided *ad libitum* throughout the experiment. All groups started with the same conventional corn/soybean meal starter diet **(Table 2.1)** for 10 days. Grower diets were fed days 11-21, and finisher diets were fed days 22-42. Group 1 broilers were fed the conventional grower and finisher diets. Group 2 birds were fed the conventional grower and finisher with the addition

of 0.2% food grade chitosan oligosaccharide (3000 Da, Matexcel) replacing 0.2% corn on an as-fed basis. Broilers in Group 3 were fed the conventional grower and finisher with the addition of 0.4% food grade chitosan oligosaccharide (3000 Da, Matexcel) replacing 0.4% corn on an as-fed basis. Birds were weighed weekly and sexed before culling. After six weeks, a total of 114 birds were culled via CO_2 inhalation.

Necropsy

After each bird was euthanized, it was laid dorsally. 70% ethanol was sprayed on their breast to wet the feathers and disinfect. Tweezers were used to lift up the skin, and a scalpel was used to make an opening in the skin. Scissors were then used to cut the skin vertically along the midline, exposing the full pectoralis major muscle for inspection, white striping scoring, and photographing gross pathology of breast muscles. Then samples were collected.

White Striping Analysis

The breast muscles of all of the broilers were exposed in necropsy, and white striping analysis of the pectoralis major muscles was performed. WS scores of 1, 2, and 3 were given, corresponding to normal (absent), moderate (<1mm thick), or severe (>1mm thick), based on the extent of WS [94].

Gross Pathology Analysis

During necropsy, photographs of the pectoralis major muscles for all broilers were also taken for gross pathology analysis. The images were blinded and examined for muscle pathology scores of

Table 2.1. Control Diet Ingredients.

Ingredients As Fed (kg) or percent composition	STARTER (DAYS 0-10) 298 g/bird	GROWER (DAYS 11-22) 1011 g/bird	FINISHER (DAYS 23-45) 3477 g/bird
Organic corn, yellow (kg)	48.13	55.11	58.29
Organic soybean meal (kg)	46.40	39.34	35.86
Organic soybean oil (kg	1.8	2.11	2.72
Dicalcium phosphate (kg)	1.68	1.49	1.38
Limestone, ground (kg)	1.03	1.07	0.91
Salt (kg)	0.45	0.4	0.38
DL-methionine 99% (kg)	0.26	0.23	0.19
NRC Vitamins/Minerals (kg)	0.25	0.25	0.25
Calculated nutrients			
% Dry Matter	90.8	90.6	90.3
% Acid Detergent Fiber	5.1	4.8	4.4
% Total Nitrogen	4.7	3.8	3.5
% Protein	29.2	23.9	21.6
% Total Digestible Nutrients	70.7	70.9	71.2
% Crude fat	6.8	7.1	7.6
% Ash	7.9	7	6.7
% Cellulose	4.6	4.3	4
% Hemicellulose	7.4	7.1	7.2
% Asx	2.27	3.13	1.98
% Thr	0.78	1.06	0.7
% Ser	0.97	1.28	0.84
% Glx	3.8	5.06	3.34
% Pro	1.11	1.38	0.98
% Gly	0.89	1.05	0.73
% Ala	0.86	1.17	0.84
% Val	1.66	1.23	0.83
% Ile	0.71	1.21	0.78
% Leu	1.66	2.15	1.48
% Tyr	0.71	0.96	0.58
% Phe	1.06	1.46	0.94
% His	0.56	0.75	0.51
% Lys	1.21	1.69	1.09
% Arg	1.5	2.11	1.31
% Cys	0.36	0.44	0.31
% Met	0.5	0.47	0.46
% SAA	0.86	0.91	0.77

Asx, asparagine or aspartate; Glx, glutamate or glutamine; SAA, sulfur amino acids methionine and cysteine. 0.2% chitosan and 0.4% chitosan were added at grower and finisher phases for the 0.2% and 0.4% chitosan groups.

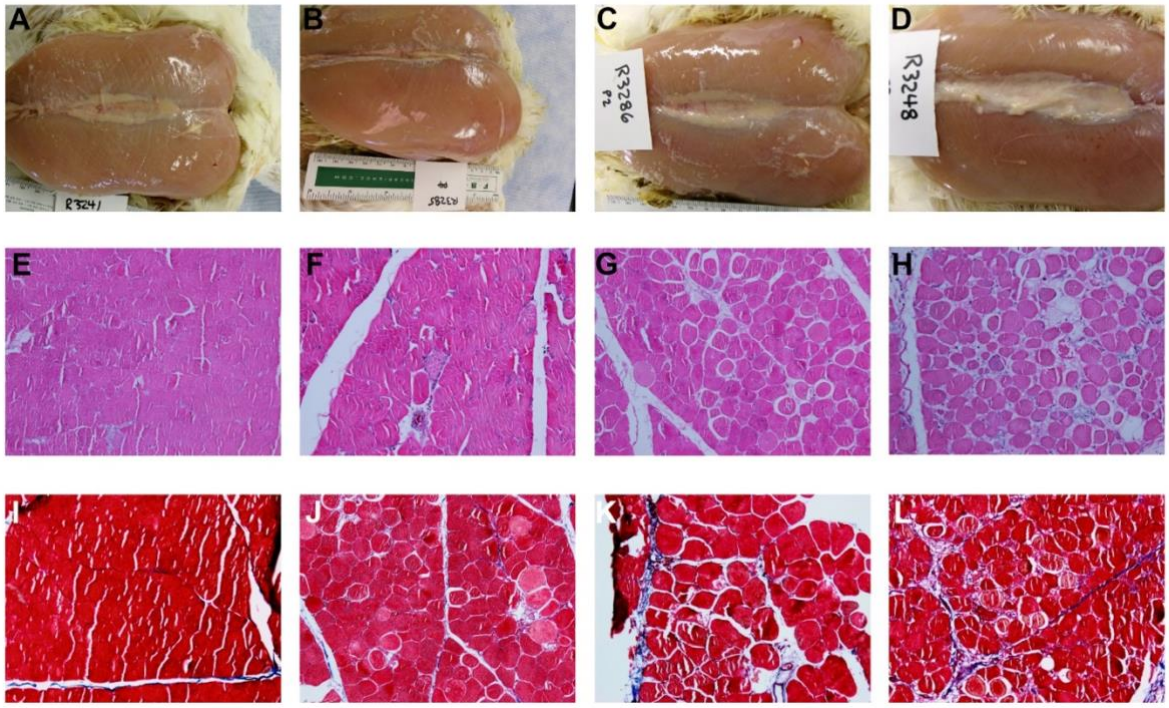

Figure 2.1. Representative images used for gross pathology and histopathology analyses. Gross pathology of broiler breasts was scored for each bird. Scores ranged from 0-4; from this study, scores found included: 0, normal; 1, no presence of WS only **(A)**; 2, presence of surface hemorrhaging near the sternal apex **(B)**; 3, presence of intramuscular hemorrhaging near the sternal apex **(C)**; and 4, ischemia **(D)**. Histopathology of pectoralis major muscle was examined for each broiler culled. Scores were: 0, normal **(E,I)**; 1, mild **(F,J)**; 2, moderate **(G,K)**; 3, severe **(H,L)**. H&E staining was performed for tissues in panels **E, F, G,** and **H**; Masson's trichrome staining was performed for tissues in panels **I, J, K,** and **L**. Representative broilers displayed were: R3241 **(A)**, R3285 **(B)**, R3286 **(C)**, and R3248 **(D)**, and R3221 **(E,I)** from the 0.2% Chitosan Group; R3231 **(F,J)** and R3260 **(G,K)** from the 0.4% Chitosan Group; and R3258 **(H,L)** from the Control Group.

0 to 4, which were given based on the extent of muscle damage: 0, no presence of WS; 1, presence of WS only; 2, presence of surface hemorrhaging near the sternal apex; 3, presence of intra-muscular hemorrhaging near the sternal apex; and 4, ischemia **(Figure 2.1A-D)** [29, 95].

Sample Collection

Following white striping scoring and photography for gross pathology analysis, two 1 cm × 2 cm × 0.5 cm portions were taken from the left anterior pectoralis major muscles for all birds. For each

broiler, one such sample was snap frozen using liquid nitrogen and stored at -80°C for RNA isolation, and the other sample was stored in 10% neutral buffered formalin at 22°C to be processed for histological analyses. The right anterior pectoralis major muscles for selected broilers were dissected out, divided into 2 equal sized pieces, weighed, vacuum sealed, and placed on ice for subsequent drip-loss and cook-loss analyses.

Drip Loss and Cook Loss

Two approximately equal sized pieces were isolated from the right anterior pectoralis major muscles for selected broilers using a 5 cm diameter circular mold and then weighed, vacuum sealed in a polyethylene food storage bag, placed on ice and transported to the laboratory. Samples from one sample set were placed in cotton meat netting in an inflated plastic bag at 4°C for 7 days. Drip-loss was calculated based on the following equation: drip-loss (%) = (raw weight - stored weight)/raw weight × 100 [96]. Samples from the second sample set were used for cook loss, where the samples were kept at -20°C for 7 days. On Day 6 they were thawed overnight; On Day 7, samples were cooked at 80°C for 20 minutes, cooled on slurry ice for 20 minutes, blotted dry, and weighed. Cook loss values were calculated based on the following equation: cooking loss (%) = (raw weight - cooked weight)/raw weight × 100 [96].

Histopathology

Tissues were taken out of the 10% neutral buffered formalin, trimmed, processed through Sakura Tissue-Tek VIP 5, and embedded in paraffin. The paraffin embedded tissues were sectioned into 4-5 μm on a microtome (Leica RM2255). The sections were stained with hematoxylin and eosin (H&E) and Masson's Trichrome stains using an adapted chicken breast muscle specific protocol

[29]. Images of stained sections were captured using a BX43F microscope fitted with a DP80 digital and cellSens Dimension software v.1.12 (Olympus). Images were examined and scored looking for macrophage infiltration, tissue damage, adipose cell and collagen presence, etc. Scores were given based on the status of the tissues: Mild (some macrophage infiltration), Moderate (few macrophage phagocytosis and appearance of fibrotic tissue), and Severe (high levels of macrophage infiltration with complete muscle destruction) **(Figure 2.1E-L)** [94].

Gene Expression

Snap-frozen pectoralis major tissue were powdered using liquid nitrogen. Portions of the powdered tissue were used to isolate total RNA using an adapted protocol using TRIzol tri-reagent and a QIAGEN Micro RNeasy Kit [78]. A NanoDrop microvolume UV spectrophotometer (ThermoFisher Scientific) was used to determine the concentrations and purities of the total RNA samples. DNA-free total RNA was reverse-transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). Amplified cDNA template was added to a reaction with Fast Advanced TaqMan Master Mix (Life Technologies) for RT-qPCR analysis in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Chicken specific Taqman primer probe sets were used for *RER1* (internal control gene), *CCR7* (chemokine receptor leukoycte migration), *LECT2* (leukocyte-derived chemotaxin), *PPARG* (fat marker), *PTGS2* (inflammation), and *CD36* (foam cell marker) [78, 97]. Gene specific efficiencies were calculated using LinRegPCR v7.5 software for each qPCR plate with relative expression found for each replicate [78, 98, 99]. GraphPad Prism software was used to analyze gene expression.

Statistical Analyses

Weights of culled broilers, drip-loss, and cook-loss were each analyzed using two-way ANOVA analyses, with Dunnett's multiple-comparison test for weight, and Tukey's multiple-comparison tests for drip-loss and cook-loss, by sex and chitosan group. White striping scores, pathology ranks, and histopathology scores of the culled birds were analyzed using Goodman and Kruskal's gamma nonparametric measure of correlation for all broilers in each chitosan group, as well as two-way ANOVA analyses with Tukey's multiple-comparison tests for striping scores, pathology ranks, and histopathology scores by sex and chitosan group. GraphPad Prism (GraphPad Software, La Jolla, CA) was used to perform these statistical analyses and non-parametric analyses were done manually.

2.3 Results

Growth Performance

There was no statistical difference in weight amongst treatment groups throughout the course of the study **(Figure 2.2A).** Additionally, after 6 weeks on experimental diets, differences were detected between sex within each group **(Figure 2.2B)**. Pen and sex differences can be delineated when examining differences all broiler weights individually by sex and pen at 6 weeks post-hatching **(Figure 2.2C)**.

Drip Loss and Cook Loss

No differences in drip loss were detected amongst treatment groups or sex **(Figure 2.3A)**. However, differences in cook loss were detected with significance overall for differences by treatment ($p=0.0301$), sex ($p=0.0499$), and the treatment-sex interaction. A significant interaction was seen

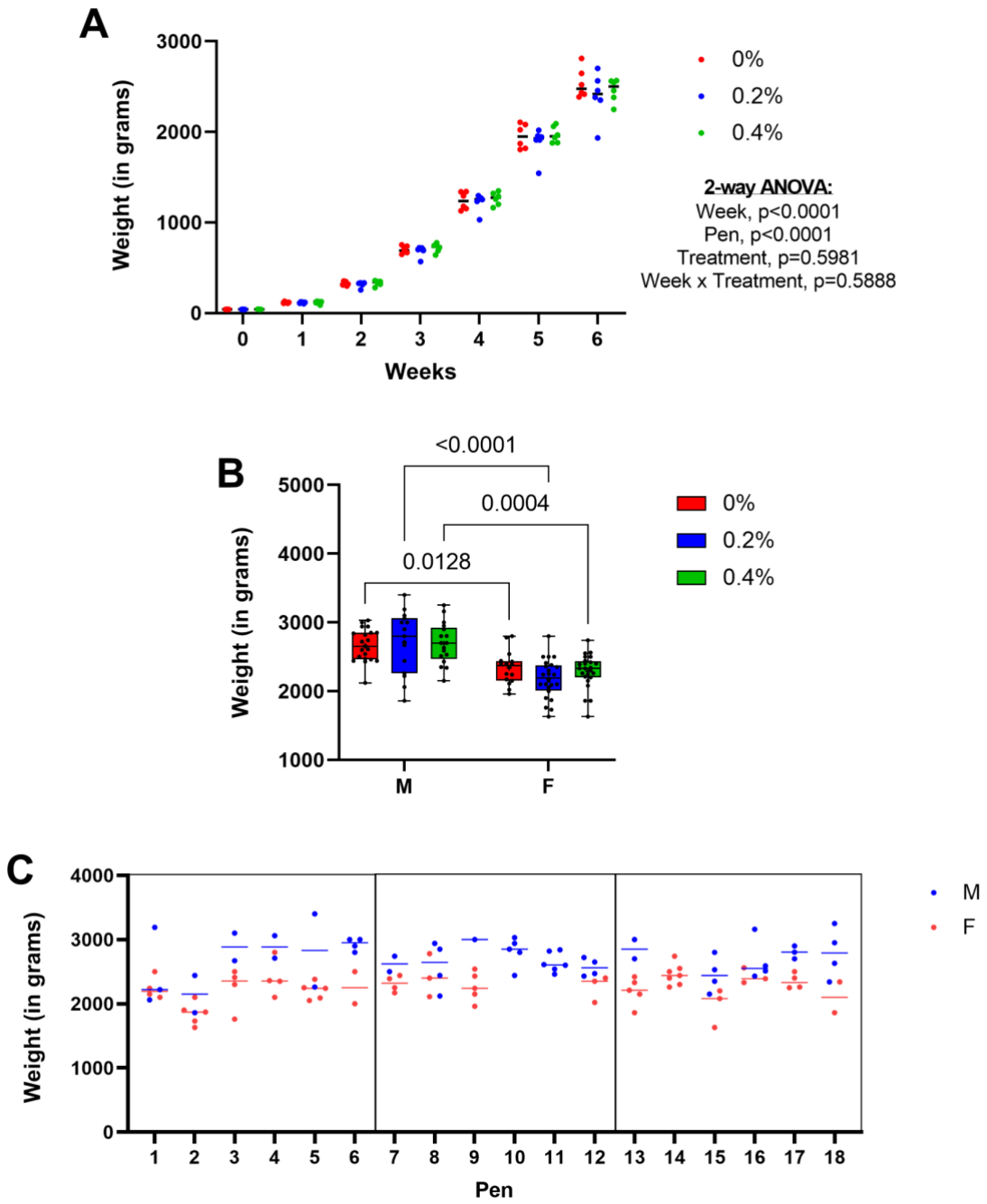

Figure 2.2. Broiler weights. Weights for each broiler were tracked each week; a dot plot provides mean bird weights by pen **(A)**. A box plot gives the weights of broilers by supplementation group and sex, providing the median, first and third quartiles, with whiskers representing range **(B)**. A dot plot providing Week 6 weights of each bird individually by pen is provided to show the differences between sex in each pen **(C)**. Individual birds were represented as dots **(B, C)**. No significant differences were found between treatments overtime. A two- way ANOVA with Tukey-multiple comparison test revealed significant differences between male and female 0%, 0.2%, and 0.4% chitosan supplemented birds at p=0.0128, p=0.0004, and p<0.0001, respectively.

in cook loss between sex and treatment (p=0.0171), where the most significant difference was observed between males and females fed 0.4% chitosan (p=0.0135), showing the overall lower cook loss in males compared to females **(Figure 2.3B)**.

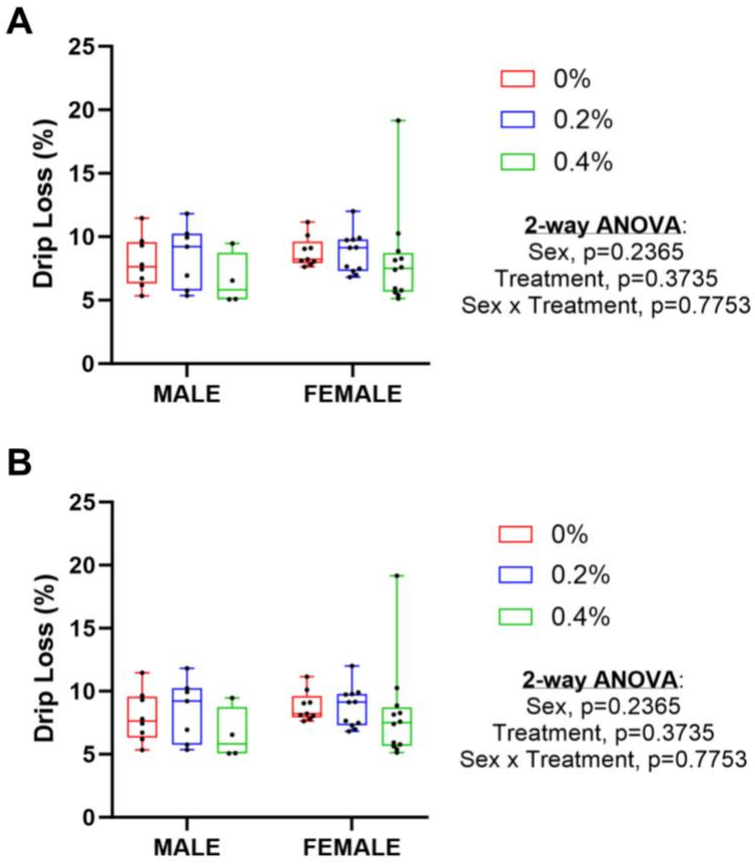

Figure 2.3. Effect of chitosan supplementation on broiler breast drip loss and cook loss. Box plots display drip loss **(A)** and cook loss **(B)** for broiler breast meat. Box plots provide the median, first and third quartiles, with whiskers representing range. Data are divided by sex and treatment groups (0%, 0.2%, 0.4% supplemented chitosan). Individual birds were represented as dots (n=4-13 per group due to distribution of sex). For cook loss, comparisons between treatment, sex, and treatment x sex were all significant by two-way ANOVA; multiple comparison test revealed significant cook loss differences (p=0.0135) between male and female 0.4% chitosan supplemented birds ($p_{tukey} < 0.05$, two-way ANOVA with Tukey multiple-comparison test). No significant differences were found for drip loss.

White Striping Scores

Broilers fed 0.4% chitosan had an overall lower white striping score compared to 0% and 0.2% chitosan; a greater percentage of the 0.4% chitosan supplemented broilers had a white striping score of 0 than with the other two diets **(Figure 2.4A)**. When comparing mean white striping scores, the 0.4% chitosan supplemented broilers had the lowest mean striping score of 0.87, while the 0.2% chitosan supplemented broilers had a mean score of 1.14 and broilers fed the control diet had a mean score of 1.17 **(Figure 2.4B)**. However, the difference in mean white striping scores overall was not significant. There were differences in white striping based upon sex with females having lower white striping scores overall ($p=0.0159$) than male birds. Multiple-test comparisons also revealed statistical differences between the female 0.4% chitosan supplemented broilers versus the male 0%, 0.2%, 0.4% chitosan supplemented birds at $p=0.0020$, $p=0.0080$, $p=0.0318$, respectively.

Gross Pathology Ranks

The mean gross pathology scores for 0%, 0.2%, 0.4% chitosan supplemented birds were 1.95, 1.87, and 1.70, respectively. There were statistical differences by diet ($p=0.0164$) and by sex ($p=0.0159$), with the male 0.4% chitosan supplemented broilers having an overall lower pathology score **(Figure 2.4D)**. The male 0.4% chitosan supplemented broilers had the lowest pathology scores, while the 0% chitosan supplemented females had the highest pathology scores **(Figure 2.4C)**. The most significant difference was observed between the 0% chitosan supplemented females and the 0.4% chitosan supplemented males ($p=0.0083$). However, there were no significant statistical contribution of sex towards the treatment groups ($p>0.05$).

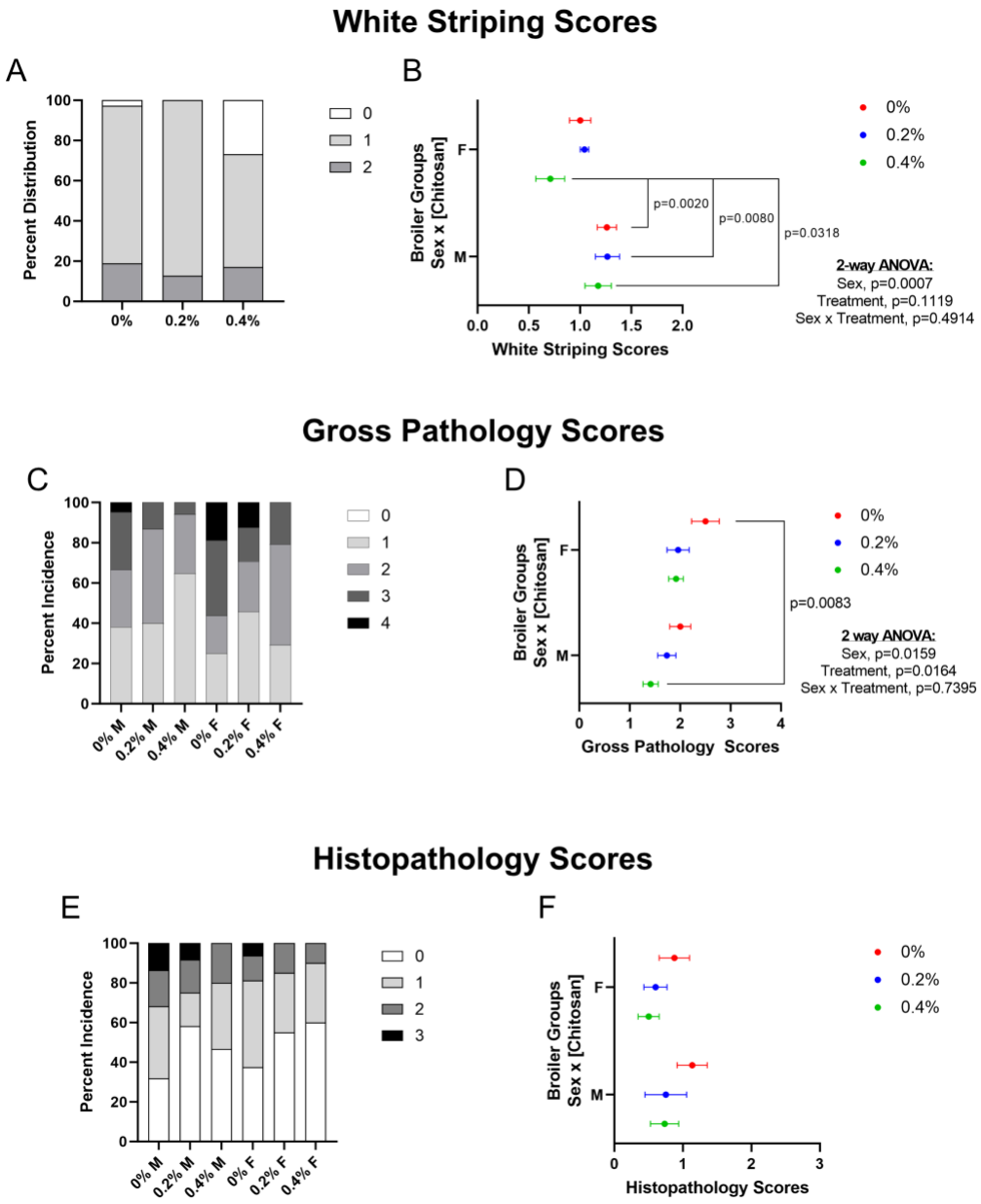

Figure 2.4. Striping scores, gross pathology, and histopathology of all birds. White striping scores **(A)**, gross muscle pathology ranks **(C)**, and histopathology ranks **(E)** are displayed as stacked bars of percentage incidence. Scores analyzed using Goodman and Kruskal's gamma test demonstrated no significant difference. Additionally, incidence of white striping **(B)**, gross pathology ranks **(D)** and histopathology ranks **(F)** relative to sex and treatment groups were showed in dot plots and analyzed using two-way ANOVA with Tukey multiple-comparison test. For white striping, a significant difference for sex was seen; individual comparisons accounting for this are displayed. The effects of sex and treatment were significant in gross pathology ranks. No significant differences were seen at the histopathology level.

Histopathology

The 0.4% chitosan supplemented broilers had the lowest histopathology score of 0.60, followed by the 0.2% chitosan supplemented broilers at 0.66, with the 0% chitosan supplemented broilers having the highest score of 1.03. However, the results by treatment were not significant ($p>0.05$). More of the 0.4% chitosan supplemented females had a score of 0, and more of the 0% chitosan supplemented males had a score of 3 **(Figure 2.4E)**, but there was also no statistical contributions nor difference between sex ($p>0.05$) **(Figure 2.4F)**.

Gene Expression

We examined the gene expression of *CCR7* (chemokine receptor leukoycte migration), *CD36* (foam cell marker), *LECT2* (leukocyte-derived chemotaxin), *PPARG* (fat marker), and *PTGS2* (inflammation marker). When considering all broilers, there were no significant differences in expression amongst treatment groups for *CCR7*, *LECT2*, *PPARG*, and *PTGS2* **(Figure 2.5A, C-E)**. However, the 0.2% chitosan supplemented broilers had higher levels of *CD36* ($p=0.0445$) and tended to have increased *PPARG* in muscle compared with the 0% and 0.4% chitosan supplemented birds ($p=0.0276$) **(Figure 2.5B, D)**. Because some differences in growth, white striping, and gross pathology were observed between male and female birds, gene expression was further evaluated by considering the sex of the birds. Expression patterns for *CCR7* and *CD36* were both equivalent between males and females when considering each feeding group **(Figure 2.6A, B)**. Transcript abundance of *LECT2* was lower in female broilers fed chitosan compared to male birds fed chitosan ($p=0.0446$) **(Figure 2.6C)**. Moreover, levels of *PPARG* and *PTGS2* trended be lower in female broilers fed chitosan, relative to male broilers fed chitosan at either level, 0.2% or 0.4%, though statistical significance was not met **(Figure 2.6D, E)**. An overall diet

effect was seen in PPARG (p=0.0276), but this could not be attributed to a specific comparison between diet group or sex.

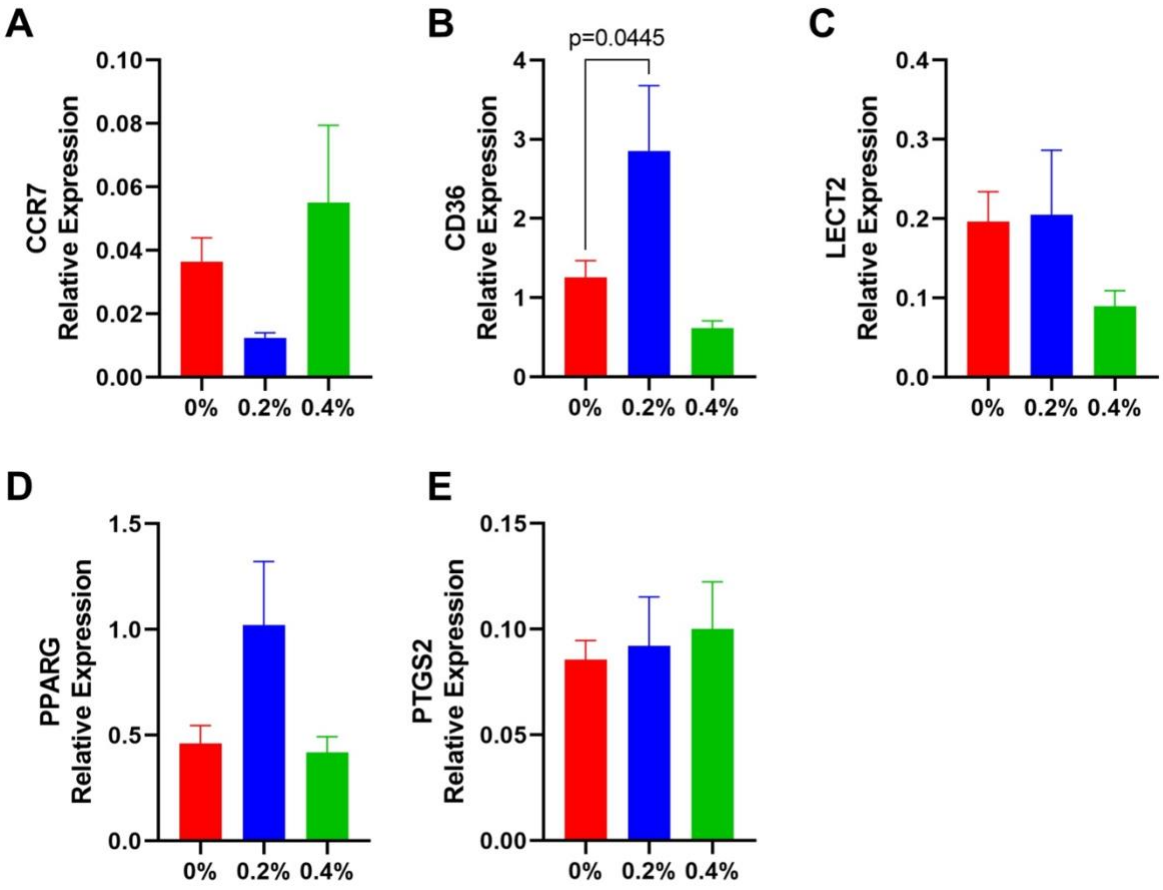

Figure 2.5. Analysis of differentiation markers in pectoralis major muscles. RT-qPCR results for **(A)** *CCR7*, **(B)** *CD36*, **(C)** **LECT2**, **(D)** *PPARG*, and **(F)** ***PTGS2*** genes, relative to housekeeping gene *RER1*, in pectoralis major muscles for birds fed 0%, 0.2%, and 0.4% chitosan diet groups. Values given as mean ± SEM; comparisons were analyzed by one-way ANOVA with Dunnett multiple comparison tests of 0.2% or 0.4% chitosan to 0% control. Comparison between 0% and 0.2% chitosan groups demonstrated that *CD36* expression was significant (p=0.0445).

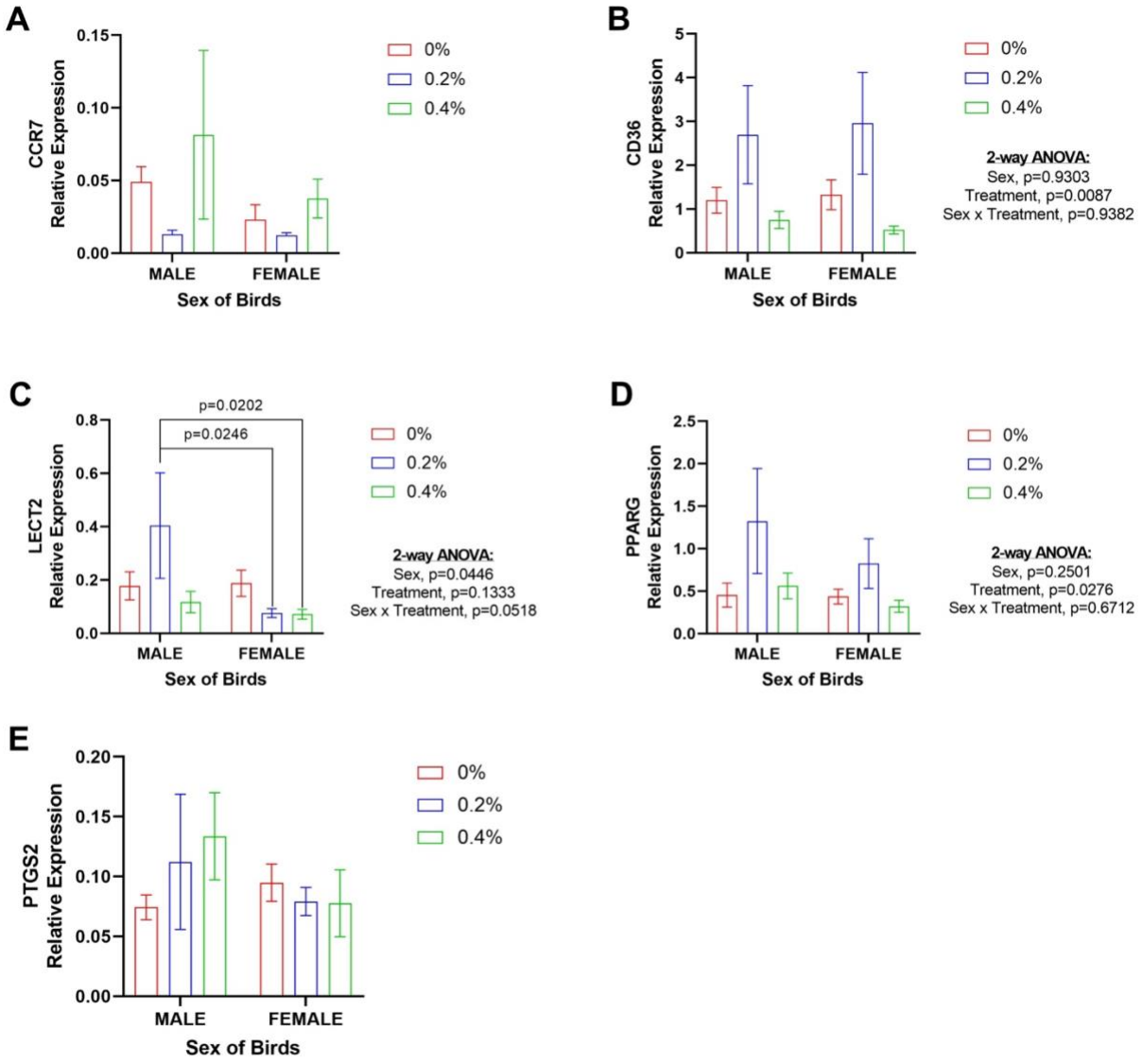

Figure 2.6. Analysis of differentiation markers in pectoralis major muscles by sex. RT-qPCR results for **(A)** *CCR7*, **(B)** *CD36*, **(C) LECT2**, **(D)** *PPARG*, and **(F)** ***PTGS2*** genes, relative to housekeeping gene *RER1*, in pectoralis major muscles for the control (0%) and chitosan diet groups (0.2% and 0.4%). Values given as mean ± SEM; comparisons were analyzed by two-way ANOVA with Tukey multiple comparison tests between diet groups and sex. Comparisons between groups were significant for *LECT2*, *PPARG*, and *CD36* expressions ($p_{tukey} < 0.05$, one-way ANOVA with Tukey multiple-comparison test). Significant differences are depicted in panels.

2.4 Discussion

Due to increasing demands for poultry products, broilers have been selected to grow faster though there are risks to compromising meat quality. In this study, we focused on a disease of the pectoralis major in broilers called white striping. We investigated how the addition of a feed additive chitosan could affect the broiler performance, meat quality, and white striping. There were 3 groups of 42 chickens, which were either fed no chitosan, 0.2% chitosan in their grower and finisher diets, or 0.4% chitosan in their grower and finisher diets. Broiler weights were measured throughout the lives of the birds. Upon euthanasia of the birds, we assessed drip loss, cook loss, white striping score, gross pathology, histopathology, and gene expression of the pectoralis major muscles. Not only did we compare between treatment groups, we also compared between sex. Although some statistical differences were observed between treatment groups, more were observed between sex.

There were no significant differences observed in final weights amongst treatment groups, suggesting that growth performance of the broilers was not compromised with the addition of chitosan. At six weeks just before culling, there were statistically significant weight differences observed between sexes within each diet group. The male broilers were relatively heavier than the female broilers, likely because male broilers typically have a higher feed intake as well as efficiency than female broilers [100, 101].

When drip loss is low, less water is lost in the freezing preservation process of the muscle, signifying better meat quality. Drip loss is related to the water holding capacity of the muscle. Drip loss also contributes to a loss of iron and proteins from the meat [102]. Using indigenous yellow-

feathered chickens, the most common meat bird in China, Wang's group noted that drip loss decreased when broilers were fed approximately 0.6% chitosan for 56 days [103]. Contrary to a study by Wang et al. [103], no difference in drip loss was observed among diet groups in the present study. The differences between this study and Wang et al. [103] were likely due to the relatively low chitosan supplementing levels in the current study. There were significant differences observed between the breasts of the 0.4% chitosan supplemented males and females. Breasts from the 0.4% chitosan supplemented males exhibited the lowest cook loss; therefore, the 0.4% chitosan supplemented broilers' breasts lost the least amount of water from the meat in the cooking process and thus might be the juiciest. The decrease in cook loss signifies less shrinkage of the collagen and muscle fibers, making the muscle more tender, overall showing better meat quality [104]. This might be due to the positively charged nature of chitosan giving it its gelation properties [105]. It can potentially hold muscle fibers together better, decreasing water loss and muscle shrinkage during cooking. The sex difference demonstrated in our study favoring the 0.4% chitosan supplemented males could be due to the higher feed intake [103] and better feed efficiency generally seen in males [101], where they can potentially better utilize the chitosan consumed, though this is speculation because feed intake and feed efficiency were not specifically examined in this study.

The 0.4% chitosan supplemented group had the lowest white striping score, followed by the 0.2%, then the 0% chitosan supplemented broilers. Likewise, the 0.4% chitosan supplement group had the lowest gross pathology and histopathology scores, although the results were not statistically significant due to the low replication numbers. In previous studies, chitosan was shown to decrease fat absorption, lowering the total plasma cholesterol in broilers [106]. This is achieved

through the suppression of lipase activity in the small intestines [107]. Chitosan can disrupt the access of lipase to fat by forming a Gibbs monolayer between them since chitosan is soluble [108]. With less fat absorption, it can be inferred that less fat is deposited in the body, in this case decreasing white striping. Such results were also reported in Wang et al.'s study, where the size of the liver and abdominal adipose deposit decreased after the supplementation of chitosan [103]. Razdan et al. [106] also concluded that different concentrations of chitosan would have different effects on the broilers, where birds fed higher chitosan concentration showed better results of reducing dietary fat absorption, which were consistent with our results in the current study. More research is suggested to investigate the inclusion rate of chitosan higher than 0.4% on white striping incidence.

White striping is caused by the inflammation of the breast muscles due to hypoxia, therefore recruiting macrophages which eventually uptake accumulated lipids and transition into foam cells, leading to a unique form of fat deposition in the pectoralis muscles of broilers. Therefore, to gain insight into a muscle's white striping status, it was important to examine expression profiles of genes related to inflammation and fat deposition. In the case of white striping, fatty acid accumulation in the muscle and its associated vasculature will stimulate the recruitment of macrophages to the area [65]. Therefore, increased expression of certain inflammation, chemotaxis, and chemokine markers were expected. However, in the present study, chitosan supplementation had no significant effects on inflammation marker *PTGS2*, the chemokine causing leukocyte migration *CCR7* [109], and leukocyte chemokine marker *LECT2* [110]. Birds in 0.2% chitosan supplemented group, however, expressed more *CD36* (a foam cell marker), and trended having more fat deposition marker *PPARG* than birds in the 0% chitosan supplemented

group. The combination of *CD36* and *PPARG* expression could signal greater fat accumulation, triggering the recruitment of more macrophages and more active transition of macrophages into foam cells within the breast muscles [110]. However, the 0.2% chitosan supplemented birds in the present study had a lower white striping incidence than the 0% chitosan supplemented birds, which was unexpected.

Research findings in the present study also suggest that supplementing 0.4% chitosan could deliver promising outcomes of reducing incidence of myopathies in broilers. For example, the 0.4% chitosan supplemented birds expressed less *CD36*, *LECT2*, and *PPARG* in muscle. The 0.4% chitosan supplemented males had better cook loss values, the 0.4% chitosan supplemented females had lower white striping scores and histopathology scores, and the 0.4% chitosan supplemented birds had the lowest white striping scores and gross pathology scores among all treatments. Taken all together, it is likely that dietary supplementation of at least 0.4% chitosan for Cobb500 broilers in their grower and finisher phases could be beneficial to controlling white stripping. Follow-up studies would help advance our understanding of dietary supplementing chitosan on reducing myopathies like white striping in broilers.

Daily feed intake data were not recorded in the present study, thus, the impacts of chitosan supplementation on performance of broilers would be further evaluated in the future study by including feed intake and digestibility of nutrients with more replication cages. Moreover, in this study we evaluated the breast muscle using scoring systems for white striping, gross pathology, histopathology, and through gene expression profiling of the breast muscles in a similar strategy to previous studies [18, 20]. Measuring lipid content in muscle and liver and plasma metabolites

should be considered in the future study to better delineate fat deposition. Furthermore, immunohistochemistry, spatial transcriptomics or proteomics could be used to better define expression of muscle, fat deposition, inflammation, and foam cell markers within the muscle and its supporting vasculature throughout grades of myopathic severity and across dietary treatment groups. In this study, measuring mRNA abundance globally within muscle tissue was not totally sufficient to understand the pathophysiological mechanism of a myopathy like white striping. Transcripts represent one snapshot in expression of a gene, but there could be accurate or detectable differences in protein products, post-translational modifications, or even in localization of targets within tissues. For example, one strength to the addition of such an analysis would be discernment of the origin of *CD36* expression, whether it be the macrophages transitioning to foam cells, smooth muscles in the vasculature, or for use to translocate fatty acids into skeletal muscle for energy.

2.5 Conclusions

In conclusion, the present study indicates that dietary chitosan supplementation in the grower and finisher phases helped reduce the incidence of white striping without compromising growth. Moreover, adding at least 0.4% low molecule weight chitosan seemed to reduce the incidence of myopathies and improve cook loss. More experiments could be done in the future to better discern broiler metabolic and muscle physiologic mechanisms that contribute to broiler performance when such dietary chitosan is supplemented.

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