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Bone development of broiler chickens supplemented with chondroitin sulfate and manganese

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ABSTRACT

Musculoskeletal disorders in broiler chickens are often related to immature connective tissue. This study aimed to evaluate the effects of dietary supplementation with chondroitin sulfate (CS) and manganese (Mn) on performance, bone quality, and the optimal CS:Mn ratio for skeletal development in broilers. A total of 1152 male Cobb chicks were reared for 47 days in a completely randomized 4 × 3 factorial design comprising four CS levels (0.00, 0.06, 0.12, and 0.18 % w/w) and three Mn levels (0, 40, and 80 mg/kg), resulting in 12 treatments with eight replicates of 12 birds each. Supplementation with CS and Mn did not affect ($P > 0.10$) feed intake, body weight, weight gain, bone mineral content, bone mineral density, phosphorus and manganese levels, ash content, absolute bone weight, or diaphyseal perimeter of the tibiotarsus. A significant interaction between CS and Mn levels was observed for feed conversion (FC), which increased linearly with Mn inclusion in diets lacking CS ($P = 0.003$). In diets without Mn, CS levels exhibited a quadratic effect on FC ($P = 0.003$). Flock viability and productive efficiency index increased linearly with increasing CS inclusion. A significant CS × Mn interaction was also observed for maximum bone breaking strength, with a linear decrease with increasing Mn in diets containing 0.12 % CS ($P = 0.019$). CS had a quadratic effect on the Seedor index, bone area, and morphometric traits of the proximal and distal tibiotarsus, with 0.06–0.12 % CS yielding optimal outcomes. Mn supplementation showed quadratic effects on bone area ($P = 0.09$) and calcium content ($P = 0.005$), with peak values at 40 mg Mn/kg. The results suggest that supplementation with CS and the inclusion of 40 mg Mn/kg in broiler diets could be used as a nutritional strategy to improve tibiotarsal bone quality, particularly morphometric attributes, calcium content, and breaking strength. Furthermore, CS supplementation may contribute to reducing mortality and improving productivity metrics in broilers.

1. Introduction

The continuous advances in poultry production have been driven by scientific and technological progress in areas such as genetics, nutrition, health, and environmental management. Genetic selection accounted for approximately 80–85 % of performance gains in broilers, enabling birds to reach slaughter weight in half the time and to attain body weights nearly four times those recorded in the 1950s (Rutz et al., 2017). However, such rapid growth has had unintended consequences for skeletal development, as selection programs favored weight gain over

Abbreviations: CS, chondroitin sulfate; Mn, manganese; GAGs, glycosaminoglycans; USP, University of São Paulo; %w/w, percentage weight by weight (kg of CS/kg of feed); FC, feed conversion; FV, flock viability; PEI, productive efficiency index; Ca, calcium; P, phosphorus; GLM, generalized linear model; BMC, bone mineral content; BA, bone area; BMD, bone mineral density; BS, breaking strength; SI, Seedor index.

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proportional improvements in bone structure (Almeida-Paz et al., 2010; Araújo et al., 2012).

One of the main contributors to musculoskeletal disorders in broilers is the immaturity of connective tissue, which lacks sufficient mechanical strength to support the bird's rapid weight gain Almeida-Paz, 2008; Petracci & Cavani, 2012. Common skeletal abnormalities include tibial dyschondroplasia, chondrodystrophies, femoral degeneration, and bone deformities (Angel, 2007; Van Wyhe et al., 2012). These disorders are often associated with a disruption in the normal sequence of chondrocyte differentiation, leading to an accumulation of cells in the pre-hypertrophic stage and impaired cartilage maturation and calcification (Pizauro-Junior et al., 2017). Deficient ossification can alter the cartilage surface, resulting in proteoglycan loss and increasing susceptibility to fissures and mechanical damage (Castrogiovanni et al., 2016).

Although the clinical incidence of skeletal disorders in broilers is typically low (<3 %), subclinical cases are widespread and associated with locomotor impairments, reduced feed efficiency, and increased carcass condemnation rates (Oviedo-Rondón et al., 2009). These issues can significantly affect profitability, with estimated economic losses ranging from 10 % to 40 % of gross income (Almeida-Paz, 2008) and an increase in production costs of up to 1.19 % in conventional systems (Gocsik et al., 2014).

To address these challenges, nutritional strategies that support skeletal integrity have been explored. These strategies include the administration of glycosaminoglycans (GAGs), which are key components of the extracellular matrix and synovial fluid. GAGs have been applied as dietary supplements to maintain cartilage integrity in poultry (Sgavioli et al., 2017; Santos et al., 2018, 2019), cattle (De Mattei et al., 2002), horses (Hanson et al., 1997; Rodgers, 2006; Yamada et al., 2022), and dogs (Bwalya et al., 2017; Eleotério et al., 2012; Melo et al., 2008; Vieira et al., 2010). In addition to their anti-inflammatory properties, GAGs regulate anabolic and catabolic processes, stimulate collagen and proteoglycan synthesis, and support chondrocyte proliferation and bone matrix biosynthesis (Altman et al., 1989; Clark, 1991; Castrogiovanni et al., 2016).

One widely used GAG is chondroitin sulfate (CS). The presence of sulfate ions permits CS to bind to the carboxylate groups of uronic acid residues, enabling water retention in both proteoglycan and collagen matrices and thereby conferring the unique resilience to mechanical impact to articular cartilage (Nelson & Cox, 2014; Rodgers, 2006). Moreover, CS stimulates the synthesis of other GAGs and proteoglycans through extracellular and intracellular mechanisms and interacts with growth factors, cytokines, morphogenetic proteins, enzymes, inhibitors, and glycoproteins that stabilize the pericellular and extracellular matrix. These interactions influence cellular metabolism, cell proliferation, differentiation and migration, matrix synthesis/stabilization, and tissue remodeling during development, and are critical for cellular control of tissue homeostasis (Hayes et al., 2018).

Several studies have demonstrated the positive effects of CS and glucosamine sulfate supplementation in broilers. Testing CS at 0.00 %, 0.08 %, and 0.16 % and glucosamine sulfate at 0.12 % and 0.24 %, Sgavioli et al. (2017) observed improved feed conversion (FC), increased tibial chondrocyte count, and enhanced bone mineralization. Martins et al. (2023) used CS at 0.00 %, 0.05 %, and 0.10 % and glucosamine sulfate at 0.15 % and 0.30 % and reported greater articular cartilage thickness, enhanced tibial growth, increased type II collagen production, and gene expression modulation—downregulation of matrix metalloproteinase 9 (MMP9) and upregulation of tissue inhibitor of metalloproteinases 2 (TIMP-2) which promoted cartilage protection. Santos et al. (2018, 2019) fed 0.74 g/kg of an additive containing 30 % CS, 30 % glucosamine sulfate, and 5 % vitamin C and observed increases in tibial length and area, bone mineral content, and osteocyte count, indicating improved ossification.

Despite the promising outcomes associated with GAGs, their effect can be enhanced by the presence of manganese (Mn), an essential trace element present in all tissues and crucial for normal metabolism of amino acids, lipids, proteins, and carbohydrates (Aschner & Aschner, 2005; Zhu & Richards, 2017). As a nutrient, Mn is required for normal immune function, blood glucose regulation, cellular energy production, digestion, reproduction, connective tissue and bone formation, lipid and carbohydrate metabolism, and brain function (Aschner & Aschner, 2005; Bourre, 2006; Grandjean & Landrigan, 2014).

In bone matrix and connective tissues, Mn acts as a cofactor for galactosyltransferase and glycosyltransferase, enzymes that are essential for the formation of mucopolysaccharides. The latter are key components of proteoglycans, which are critical for growth plate development and cartilage formation in poultry (Favero et al., 2013; Reece, 2006; Tufarelli & Laudadio, 2017). Additionally, Mn is required for the function of arginase, a transaminase enzyme that converts arginine into ornithine and urea. Both substances are necessary for collagen synthesis, the main component of bone and cartilage (Reece, 2006; Tufarelli & Laudadio, 2017). Furthermore, dietary Mn supplementation has been shown to restore normal hexosamine levels in cartilage, thereby contributing to bone health. The inclusion of 25 mg Mn/kg in broiler diets was effective in maintaining normal hexosamine concentrations in epiphyseal cartilage (Stock & Latshaw, 1981).

Therefore, this study aimed to evaluate the effects of dietary CS and Mn supplementation—alone and in combination—on productive performance, cartilage structure, and bone development in broiler chickens. We hypothesized that combined supplementation would improve bone quality by enhancing the synthesis and stability of the extracellular matrix.

2. Material and methods

2.1. Ethics, animals and experimental design

The experiment was conducted at the Poultry Research Laboratory of the Department of Nutrition and Animal Production, School of Veterinary Medicine and Animal Science, University of São Paulo (USP), Brazil. The study was approved by the Animal Use Ethics Committee of the School of Animal Science and Food Engineering (CEUA/FZEA Protocol No 8401,180,618) during the meeting held on August 22, 2018.

For the experiment, 1152 one-day-old male Cobb broiler chicks were housed for 47 days in 96 boxes in a masonry barn equipped with nebulizers under positive pressure. The animals were allocated to the treatments in a completely randomized design arranged in a 4 × 3 factorial scheme: four levels of CS (0.00, 0.06, 0.12, and 0.18 % w/w) and three levels of Mn (0, 40, and 80 mg/kg), resulting in 12 treatments of eight replicates with 12 birds each, as described in Fig. 1. Each experimental unit was equipped with automatic nipple drinkers, tube feeders, and 250-W infrared heating lamps. The lighting program, management practices, and sanitation of the experimental project followed the recommendations of the Cobb manual (2014). The environmental conditions of the poultry house that would ensure the thermal comfort of the birds were monitored by calculating the enthalpy comfort index (ECI) of the Center for Studies in Agricultural Ambience and Animal Welfare (Queiroz et al., 2012) based on the average temperature and relative air humidity recorded with the aid of a digital thermo-hygrometer (Equitherm, model Th-439). Table 1 shows the weekly ECI calculated during the experimental period.

2.2. Experimental diets

The experimental diets were formulated with corn and soybean meal. Sodium CS ($[C_{14}H_{19}NO_{14}SNa_2]n$; 4-chondroitin sulfate derived from bovine cartilage, Extrasul, Ltd., Brazil, production batch number 180,319,023,945 had a purity of 98.5 %) was included in the diets as a substitute of the inert material (sand) at the concentrations defined for each treatment.

Regarding Mn levels in the experimental diets, the mineral requirements were based on the nutritional recommendations for broiler

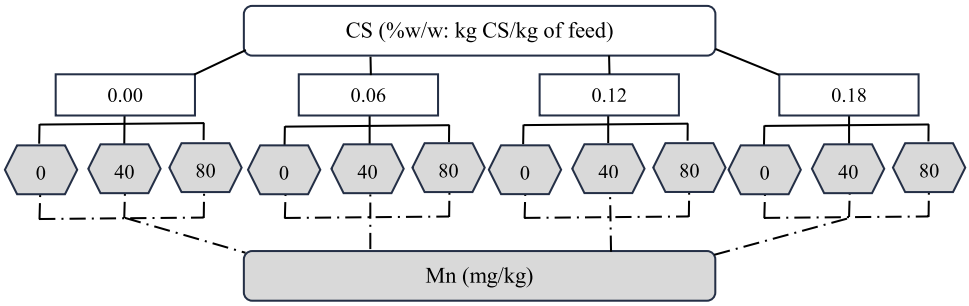

Fig. 1. Distribution of treatments according to the 4 × 3 factorial scheme. Four levels of CS (0.00, 0.06, 0.12 and 0.18 %: kg CS/kg of feed) and three levels of Mn (0, 40, and 80 mg/kg), totaling 12 treatments of eight replicates with 12 birds each.

Table 1
Environmental conditions during the experiment.

Period /day	* Environmental parameters					
	¥07:00 a.m.			¥02:00 p.m.		
	TºC	RAH (%)	ECI (kJ/kg)	T ºC	RAH %	ECI (kJ/kg)
7	28.40	58.21	79.93	29.96	57.21	80.08
21	23.65	71.50	68.51	27.49	65.71	82.73
35	23.89	69.57	67.38	26.24	69.79	77.99
47	22.70	80.75	66.99	26.64	78.75	86.86

Comfort **Alert** **Critical** **Lethal**

*Temperature (TºC), relative air humidity (RAH) and enthalpy comfort index (ECI).
¥Schedule for monitoring the environmental conditions of the poultry house.

*Temperature (T °C), relative air humidity (RAH) and enthalpy comfort index (ECI).
¥Schedule for monitoring the environmental conditions of the poultry house.

chickens and supplied via Premix (Table 2). Excess Mn methionine hydroxyl analog chelate (Mn-MHAC) (MINTREX® Mn, Novus International, Inc., containing a minimum of 13 % Mn) was added according to the experimental levels (0, 40, and 80 mg/kg) to facilitate the availability of the mineral for processes related to the prevention of musculoskeletal diseases.

The feeding program consisted of four phases: pre-starter (1–7 days of age), starter (8–21 days), grower 1 (22–35 days), and finisher (36–47 days), following the recommendations of the Broiler Performance and Nutrition Supplement (Cobb 500TM, 2015). The 47-day experimental period was established to provide additional time for bone matrix development. According to Almeida-Paz (2008), broiler chickens up to 42 days of age (the typical slaughter age) are still in the growth phase and exhibit relatively immature ligaments, tendons, and bones, with limited development of compact bone tissue.

The diets were formulated using the Super Crac 5.7 Master® program for Windows (copyright 1983–2010). Table 2 shows the percent composition and nutrient calculation of the starter and finisher diets.

2.3. Performance

For performance monitoring, the animals and diet leftovers were weighed. Next, the body weight was recorded and weight gain, feed intake, FC, flock viability (FV), and the productive efficiency index (PEI) were calculated for the period from 1 to 47 days of age. Mortality was recorded daily for correction of the performance variables and FV according to Sakomura and Rostagno (2016).

2.4. Slaughter and sample collection

At 47 days of age, the birds were slaughtered in accordance with the regulations on the Industrial and Sanitary Inspection of Products of Animal Origin (RIISPOA - MAPA, 2017). One animal per experimental unit was selected based on its body weight, considering that the incidence of bone disorders is higher among heavier birds. The animals were then weighed individually, identified with a leg ring, and submitted to fasting for 10 h. After this period, the birds were electrically stunned, bled through a transverse cut of the jugular vein, scalded, plucked, cleaned, and eviscerated (without removing the head, feet, and neck). The carcasses were then stored in cooling chambers at 0 °C for 24 h.

During deboning, the carcasses were weighed and the thighs and drumsticks were removed. Next, the tibiotarsus was removed from the left thighs and approximately 1 cm of articular cartilage was collected from the proximal epiphysis for histological analysis. The right thighs and drumsticks were packed in polyethylene bags and stored in a freezer (−18 to −20 °C) for subsequent evaluation of tibiotarsal bone quality.

2.5. Morphometric analysis of tibiotarsus

Morphological analysis was performed according to the method proposed by Santos et al. (2019). The tibiotarsus of the thighs was cleaned, removing residues (meat and cartilage), and weighed to obtain the absolute and relative weights (in relation to the carcass). Next, tibiotarsal length and perimeters of the proximal epiphysis, diaphysis and distal epiphysis were measured with a ruler and tape measure. The measurements per treatment were used to identify possible differences

Table 2
Percent composition and nutrient calculation of diets.

Ingredient (%)	Diets	
	Starter Manganese[3] 0–40–80 mg/kg	Finisher Manganese[3] 0–40–80 mg/kg
Corn (7.88 %)	55.57	67.86
Soybean meal (45 %)	37.83	24.96
Dicalcium phosphate	1.86	1.50
Soybean oil	2.54	3.58
Limestone	0.78	0.73
Salt	0.37	0.34
L-lysine HCl	0.20	0.16
DL-methionine	0.33	0.20
L-threonine	0.10	0.05
Premix[1,2]	0.20	0.40
Choline chloride 70 %	0.03	0.03
Chondroitin sulfate[3]	0.00–0.06–0.12–0.18	0.00–0.06–0.12–0.18
Sand[4]	0.18 a 0.00	0.18 a 0.00
Total	**100.00**	**100.00**
Metabolizable energy and calculated nutrient composition (%)		
ME (kcal/kg)	2.98	3.19
Crude protein	22.00	18.00
Calcium	0.90	0.76
Available phosphorus	0.45	0.38
Sodium	0.16	0.16
Chlorine	0.29	0.25
Dig. Lysine	1.22	0.90
Dig. Met+cys	0.91	0.70
Dig. Methionine	0.61	0.44
Dig. Threonine	0.83	0.61

[1] Vitamin and mineral premix per kilogram of feed (used during the period from 1 to 21 days): Folic Acid (min) 250.00 mg; Pantothenic Acid (min) 3750.00 mg; Cu (min) 25.00 g/; Choline (min) 86.56 g; Fe (min) 12.50 g; I (min) 300.00 mg; Mn (min) 17.50 g; niacin (Niacin) 10.00 g; Se (min) 50.00 mg; A Vitamin (min) 2000,000.00 IU; B1 Vitamin (min) 600.00 mg; B12 Vitamin (min) 3500.00 mg; B2 Vitamin (min) 1500.00 mg; B6 Vitamin (min) 1000.00 mg; D3 Vitamin (min) 600,000.00 IU; E Vitamin (min) 3000.00 IU; K3 Vitamin (min) 500.00 mg; Zinc (min) 12.50 g; Virginiamycin 3750.00 mg; Nicarbazin 31.25 g.

[2] Vitamin and mineral premix per kilogram of feed (used during the period from 22 to 47 days): Folic Acid (min) 200.00 mg; Pantothenic Acid (min) 2866.50 mg; Copper Sulfate (min) 3000.00 mg; Choline (min) 60.00 g; Iron Sulfate (min) 9525.00 mg; Calcium iodate (min) 254.40 mg; Manganese monoxide Mn (min) 13.50 g; niacin (Niacin) 6996.00 mg; Sodium selenite (min) 75.65 mg; A Vitamin (min) 2044,800.00 UI; B1 Vitamin (min) 494.80 mg; B12 Vitamin (min) 3380.00 mg; B2 Vitamin (min) 1260.00 mg; B6 Vitamin (min) 551.25 mg; D3 Vitamin (min) 508,360.00 UI; E Vitamin (min) 3825.00 UI; K3 Vitamin (min) 688.40 mg; Zinc oxide (min) 12.54 g; Biotin (min) 17.00 mg; Halquinol 7500.00 mg/kg; Salinomycin 16.50 g.

[3] Amount of inclusion of sodium CS ($[C_{14}H_{19}NO_{14}SNa_2]n$; 4-chondroitin sulfate derived from bovine cartilage, Extrasul, Ltd., Brazil, production batch number: 180,319,023,945 had a purity of 98.5 %)), and excess Mn methionine hydroxyl analog chelate (Mn-MHAC) (MINTREX® Mn, Novus International, Inc) according to the experimental treatments: **T1:** 0.0 % CS and 0 mg/kg Mn; **T2:** 0.0 % CS and 40 mg/kg Mn; **T3:** 0.0 % CS and 80 mg/kg Mn; **T4:** 0.06 % CS and 0 mg/kg Mn; **T5:** 0.06 % CS and 40 mg/kg Mn; **T6:** 0.06 % CS and 80 mg/kg Mn; **T7:** 0.12 % CS and 0 mg/kg Mn; **T8:** 0.12 % CS and 40 mg/kg Mn; **T9:** 0.12 % CS and 80 mg/kg Mn; **T10:** 0.18 % CS and 0 mg/kg Mn; **T11:** 0.18 % CS and 40 mg/kg Mn; **T12:** 0.18 % CS and 80 mg/kg Mn. [4]Dietary sand: Concentrations decrease in the same proportion as CS (kg CS/ kg of feed).

in bone development between birds.

2.6. Seedor index

The weights and lengths of the tibiotarsus obtained in the morphometric analysis were used for calculation of the Seedor index (SI). The SI is obtained by dividing bone weight (mg) by bone length (mm) and is an indicator of bone density (Seedor et al., 1991).

2.7. Surface area, bone mineral density and bone mineral content

The cleaned right tibiotarsus (free of connective tissue and meat residues) was radiographed in the proximal, distal, and diaphyseal regions by dual-photon X-ray absorptiometry (Horizon Discovery DXA Hologic®, Massachusetts, USA), following the specifications of Sgavioli et al. (2017) and Santos et al. (2019). Dual-photon X-ray absorptiometry is widely recognized as a non-destructive, accurate, and reproducible technique for measuring parameters that are highly correlated with the biomechanical strength of bone tissue. The resulting images were analyzed using the device's software for small animals, which provides values for bone mineral content (BMC, g), bone area (BA, cm^2), and bone mineral density (BMD, g/cm^2). Prior to scanning, the device was calibrated according to the manufacturer's instructions (Hologic®, Massachusetts, USA).

2.8. Breaking strength

For the analysis of breaking strength (BS), the tibiotarsus was predried for 24 h at room temperature. After this period, the samples were submitted to the three-point bending test (axial compression) using a universal testing machine (EMIC 23–5S, INSTRON/EMIC) equipped with the BlueHill® LE 3.72 program, as described by Sgavioli et al. (2017) and Santos et al. (2019). In the machine, the bones were placed in the same position to permit calibration according to diaphyseal length. The length was set at 6 cm for all bone samples since this distance was sufficient to ensure that the load was applied to the geometric midpoint of the bones. The tests used a preload of 5 N and an adaptation time of 5 s. The speed of load application was 5 mm/min using a 5-kN load cell to determine the maximum BS (N).

2.9. Mineral matter of tibiotarsus

The procedures of Silva and Queiroz (2009) were used to determine mineral matter or bone ashes. Two grams of the right tibiotarsus were collected, pre-dried for 24 h at room temperature, transferred to crucibles, and incinerated in a muffle for 8 h at a temperature of 600 °C. After this period, the crucibles were cooled in a desiccator until room temperature was reached and weighed. The ash content of the samples was calculated as the difference between initial and final weight (expressed as percentage).

2.10. Mineral profile

The calcium (Ca), phosphorus (P), and Mn content of the tibiotarsus was determined by plasma optical emission spectroscopy according to the analytical methods of Caputi (2013). For this procedure, 2 g of bone was defatted by immersion in petroleum ether for 8 h. Next, the samples were removed from the thimble of a Soxhlet extractor and transferred to crucibles for burning in a muffle for 8 h at a temperature of 600 °C. After this period, the bones were triturated in the crucible and 20 ml HCl (1:1) was added immediately to dehydrate the silica and solubilize the minerals (the sample was left to stand for 20 min). The content of the crucible was filtered through a 100-ml balloon. The crucible was washed several times to avoid sample loss. The resulting solution was stirred and analyzed in a spectrometer (ICPE-9000, Shimadzu) according to the dilutions and calibration curve pre-established for each element. The mineral content (Ca, P, and Mn) of the bones was calculated. Ca and P content is reported as percentage. Since Mn is a micromineral, it is reported as mg/kg.

2.11. Histological evaluation of proximal epiphyseal cartilage

For histological evaluation, approximately 1 cm of proximal epiphyseal cartilage was collected from the left tibiotarsus to quantify chondrocyte density according to the method of Lillie (1954). The articular

cartilage was extracted with a scalpel, fixed in a 10 % buffered formalin solution for 24 h at room temperature, and immersed in 70 % alcohol.

For dehydration, the samples were immersed in an increasing alcohol series (90 %, 95 %, and absolute alcohol [4x]). Next, the samples were cleared in ethanol-xylene solution (1:1), followed by xylene (100 % [2x]), and transferred to a mixture of xylene-paraffin (1:1) previously heated in an oven to 60 °C. The samples were then embedded in histological paraffin and 6-μm-thick semi-serial sections were cut with a microtome. The material was mounted on a slide, stained with hematoxylin-eosin (Tolosa et al., 2003), and protected with a coverslip to quantify the number of chondrocytes by light microscopy.

2.12. Quantification of chondrocytes

Histological cartilage sections were photographed using microscope software (Leica QWin, Leica Imaging Systems, Wetzlar, Germany) under a 40 × objective. Twenty-five photomicrographs were obtained per histological slide, totaling 200 images per treatment. Chondrocytes visible in the superficial, middle, and deep regions of the cartilage were counted using the ImageJ software. The analysis area measured with ImageJ was standardized to 17,063.27 μm^2. Chondrocyte counts per treatment were expressed as cell density (number of cells per unit area; see Fig. 2).

2.13. Statistical analysis

The effects of supplementation with CS (CS_i) and Mn (Mn_j), as well as of their interaction $(CS.Mn)_{ij}$, on bone quality were analyzed using the following model: $Yijk = \mu + CS_i + Mn_j + (CS.Mn)_{ij} + eijk$. All experimental variables were evaluated by the Shapiro-Wilk test at $P < 0.05$ to assess the normality of errors. Any variable that did not follow a normal distribution was transformed using the RANK procedure of SAS Institute (2013). The data were submitted to analysis of variance using the Generalized Linear Model (GLM) procedure of SAS Institute (2013) at a level of significance of 10 % ($P < 0.10$). When a significant difference was detected between treatment means, the levels of CS and Mn and their interactions were analyzed as orthogonal contrasts of linear and quadratic effects considering a probability of 5 % ($P < 0.05$).

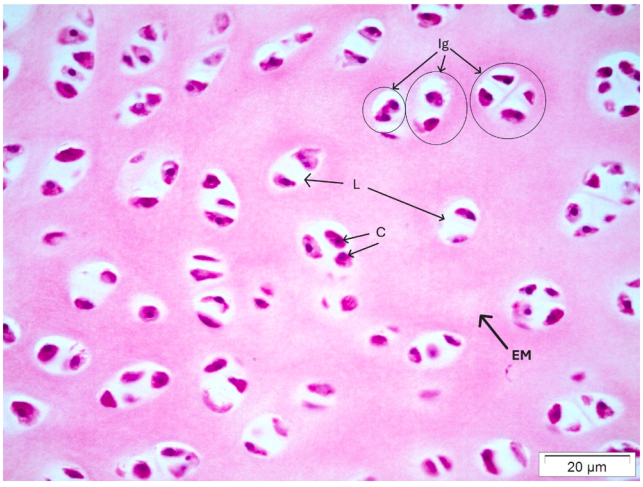

Fig. 2. Photomicrograph of articular cartilage stained with hematoxylin and eosin, highlighting the main histological components. C, chondrocytes, rounded cells with basophilic nuclei located within lacunae; L, lacunae, clear spaces surrounding the chondrocytes, resulting from cell shrinkage during histological processing; Ig, isogenous groups, clusters of two or more chondrocytes derived from a single progenitor cell, indicating recent mitotic activity; EM, extracellular matrix, eosinophilic region (pink-stained) rich in type II collagen and proteoglycans.

3. Results

3.1. Zootechnical performance

Table 3 shows the results of analysis of variance of performance traits and bone physical quality in broiler chickens at 47 days of age. Feed intake, body weight, or weight gain was not affected by the different levels of CS and Mn supplementation in broiler diets ($P > 0.10$). However, a significant interaction between CS and Mn levels was observed for FC ($P = 0.004$). Regression analysis revealed a quadratic effect of dietary Mn levels without CS supplementation (0 % CS) on FC ($P = 0.003$), as illustrated in Fig. 3 and described by Equation 1. FC increased progressively with increasing Mn levels, reaching a critical point at 64.3 mg/kg, at which the estimated FC was 1.73. Beyond this point, FC tended to stabilize or slightly decrease with further increases in dietary Mn.

Similarly, a quadratic effect of CS supplementation levels was observed for the diet containing 0.0 mg Mn/kg (Fig. 4 and Equation 2). FC increased with increasing CS levels up to the estimated critical value of 0.10 %, at which the FC was 1.72 ($P = 0.003$). Beyond this point, FC tended to decrease with further increases in dietary CS.

As shown in Table 3, FV was influenced by CS levels ($P < 0.10$), regardless of Mn inclusion. The viability rate improved (lower mortality) with each increase in dietary CS according to the linear effect shown in Fig. 5 and Equation 3 ($P = 0.005$). PEI increased linearly with each increment of CS (Fig. 6 and Equation 4; $P = 0.01$), indicating that the zootechnical performance of the broiler batch increased with each inclusion level of CS.

The addition of CS and Mn to the broiler diets did not influence BMC or BMD ($P > 0.10$). However, for BA, analysis of variance revealed an effect of CS and Mn levels ($P = 0.008$ and $P = 0.037$, respectively). Regression analysis showed a quadratic effect of CS level on BA ($P = 0.002$; Fig. 7 and Equation 5). An increase in BA was observed with increasing CS levels, reaching a maximum at the estimated critical point of 0.096 %, at which the predicted BA was 8.07 cm^2. Above this critical level, BA decreased progressively with further increases in dietary CS.

Diets containing Mn also exerted a quadratic effect on tibiotarsal BA, as illustrated in Fig. 8 and described by Equation 6 ($P = 0.089$). An increase in BA was observed with increasing Mn levels, reaching a maximum at the estimated critical point of 40.5 mg Mn/kg, at which the predicted BA was 8.06 cm^2. Above this level, a sharp reduction in BA was observed with further increases in Mn supplementation.

Table 3 shows an effect of the interaction between CS and Mn levels on BS ($P < 0.10$). Further analyses indicated that increasing CS levels did not significantly affect BS within each Mn level ($P > 0.10$). However, a significant interaction for BS was observed when Mn was added to diets containing 0.12 % CS, as shown in Fig. 9 ($P = 0.019$). The inclusion of increasing levels of Mn in this diet resulted in a linear reduction in BS. According to Equation 7, for each 1 mg/kg increase in Mn, BS decreased by approximately 1.26 N. These results highlight the importance of controlling dietary Mn concentrations for optimizing the mechanical properties of broiler bones.

3.2. Mineral profile of tibiotarsus and number of chondrocytes

Table 4 shows the mineral profile (ashes, Ca, P, and Mn) of the tibiotarsus of broiler chickens. CS levels did not influence bone ash content or the content of the minerals analyzed ($P > 0.10$). Likewise, Mn supplementation levels did not affect P, Mn, or ash content. However, Mn levels influenced tibiotarsal Ca content ($P = 0.041$). An increase in Ca levels was observed with increasing Mn supplementation, reaching a maximum at the estimated critical point of 39.5 mg Mn/kg, at which the predicted Ca concentration was 10.74 % ($P = 0.005$; Fig. 10 and Equation 8). Further increases in Mn supplementation resulted in a progressive reduction in bone Ca content.

Regarding the number of chondrocytes in articular cartilage, analysis

Table 3
Analysis of variance of performance parameters and physical quality of the tibiotarsus in broiler chickens from 1 to 47 days of age.

Factor	*FI	BW	WG	FC	FV	PEI	BA	BMC	BMD	BS
¥Chondroitin sulfate (CS)										
0.00 %	5441.43	3252.02	3207.92	1.71	89.43b	362.92	7.59	1.42	0.18	366.19
0.06 %	5509.87	3280.39	3236.10	1.71	89.98ab	372.68	8.01	1.45	0.18	357.26
0.12 %	5592.57	3288.53	3244.47	1.73	96.13a	395.22	8.04	1.49	0.19	397.97
0.18 %	5588.55	3322.16	3278.13	1.71	94.25ab	397.26	7.70	1.35	0.17	359.83
Manganese (Mn)										
0 mg/kg	5585.19	3338.43	3294.13	1.70	94.53	397.56	7.90	1.44	0.18	375.29
40 mg/kg	5495.39	3251.02	3206.94	1.72	91.07	371.90	8.00	1.44	0.18	376.96
80 mg/kg	5518.74	3267.88	3223.89	1.72	91.74	376.58	7.65	1.41	0.18	357.59
ANOVA	**Probability**									
CS	0.386	0.791	0.791	0.043	0.012	0.089	0.008	0.378	0.371	0.224
Mn	0.567	0.308	0.311	0.001	0.197	0.146	0.037	0.986	0.953	0.435
CS x Mn	0.617	0.759	0.761	0.004	0.982	0.955	0.124	0.381	0.373	0.094
CV** (%)	6.30	7.31	7.41	1.92	8.43	13.57	6.76	19.69	14.80	18.71
Regression	**Probability**									
CS	ns	ns	ns	ns	[3]L (0.005)	[4]L (0.010)	[5]Q (0.002)	ns	ns	ns
Mn	ns	ns	ns	ns	ns	ns	[6]Q (0.089)	ns	ns	ns
Interaction CS x Mn	**Probability**									
CS x 0.0 mg Mn/kg	ns	ns	ns	[1]Q (0.003)	ns	ns	ns	ns	ns	ns
Mn x 0.00 % CS	ns	ns	ns	[2]Q (0.003)	ns	ns	ns	ns	ns	ns
Mn x 0.12 % CS	ns	ns	ns	ns	ns	ns	ns	ns	ns	[7]L (0.019)

* FI (g) - feed intake; BW (g) - body weight; WG (g) - weight gain; FC - feed conversion; FV (%) - flock viability; PEI - productive efficiency index; BMC (g) - bone mineral content; BMD (g/cm²) - bone mineral density; BS (N) - breaking strength. ¥Chondroitin sulfate (%CS: kg CS/ kg of feed); ns: not significant.

** Coefficient of variation.

Equation 1 FC (Mn x 0.00 % CS) = −0.000015 × 2 + 0.00193x + 1.669; $R^2 = 1.00$

Equation 2 FC (CS x 0.0 mg/kg Mn) = $-4.5833\times^2 + 0.9183x + 1.6731$; $R^2 = 0.79$

Equation 3 FV (CS) = 34.35x + 89.356; $R^2 = 0.66$

Equation 4 IEP (CS) = 209.27x + 363.19; $R^2 = 0.92$

Equation 5 BA (CS) = $-52.778\times^2 + 10.1x + 7.591$; $R^2 = 0.99$

Equation 6 BA (Mn) = $-0.0001\times^2 + 0.0081x + 7.9$; $R^2 = 1.00$;Equation 7 BS (Mn x 0.12 % CS) = −1.2631x + 446.75; $R^2 = 0.85$.

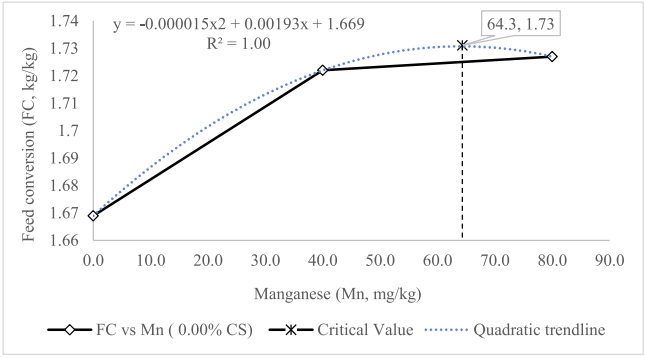

Fig. 3. Regression analysis of feed conversion (FC) in broiler chickens as a function of increasing dietary manganese (Mn) levels in diets without chondroitin sulfate (0.00 % CS). A quadratic model was fitted to the data (R^2 = 1.00), with the highest FC being observed at an estimated Mn level of 64.3 mg/kg.

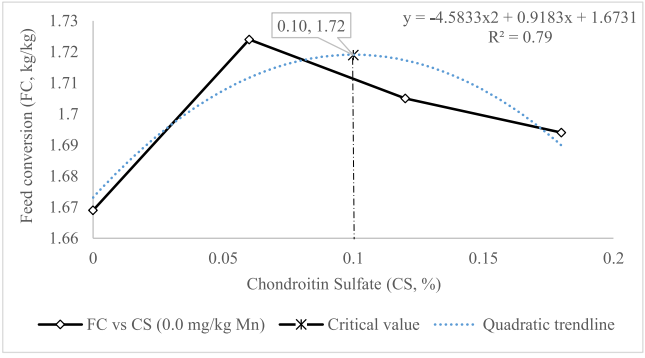

Fig. 4. Regression analysis of feed conversion ratio (FC) in broiler chickens as a function of increasing dietary chondroitin sulfate (CS) levels in diets without manganese supplementation (0.0 mg Mn/kg). A quadratic regression model was fitted to the data (R^2 = 0.79), with the highest FC being observed at 0.10 % CS.

of variance revealed an effect of Mn addition to the broiler diets, regardless of CS level ($P < 0.10$; Table 4). Regression analysis showed a quadratic effect of dietary Mn levels on chondrocyte density in cartilage, as illustrated in Fig. 11 and described by Equation 9 ($P = 0.023$). A progressive reduction in chondrocyte density was observed as Mn levels increased, reaching a critical point at 33.79 mg Mn/kg, corresponding to 6486.43 chondrocytes/mm². Above this concentration, the values increased significantly, suggesting that higher Mn levels may stimulate chondrocyte proliferation.

3.3. Morphometry of tibiotarsus

Table 5 shows the effects of CS supplementation on broiler bone development at 47 days old ($P < 0.10$). Dietary CS supplementation exhibited a quadratic effect on tibial length, SI as a measure of bone density, relative weight, proximal epiphyseal perimeter, and distal epiphyseal perimeter, as shown in Figs. 12–15 ($P = 0.012$, $P = 0.021$, $P = 0.016$, $P = 0.021$, and $P = 0.007$, respectively). The SI reached a maximum value of 172.50 mg/mm at 0.08 % CS, while the relative weight peaked at 0.648 % at the same supplementation level (Equations 12 and 13). Proximal epiphyseal and distal epiphyseal perimeter also showed a quadratic response, with maximum values of 8.08 and 6.68 cm at 0.07 % and 0.08 % CS, respectively (Equations 14 and 15). Tibial length increased with CS supplementation, reaching a peak of 11.02 cm at the estimated critical point of 0.14 % CS (Equations 16). Beyond the critical point, there was a decreasing trend in the morphometric parameters of the tibiotarsus, indicating that higher CS levels may not provide additional benefits for bone development and could potentially offset the improvements observed at moderate supplementation levels.

Likewise, the addition of different Mn levels to the broiler diets had no effect on tibiotarsal morphometry ($P > 0.10$), with similar lengths,

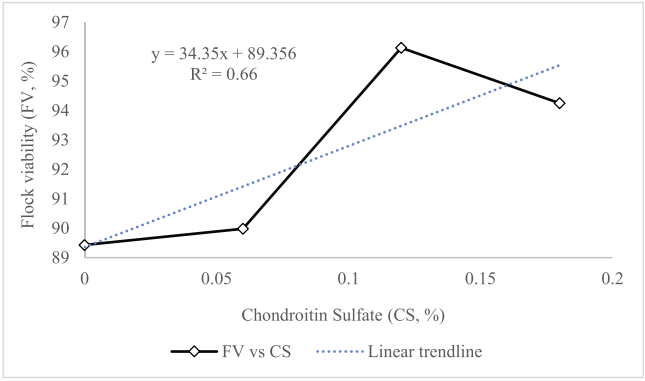

Fig. 5. Regression analysis of flock viability (FV) in broilers fed increasing levels of chondroitin sulfate (CS), regardless of dietary manganese (Mn) inclusion. A linear model was fitted to the data ($R^2 = 0.66$), indicating a positive association between CS level and flock viability.

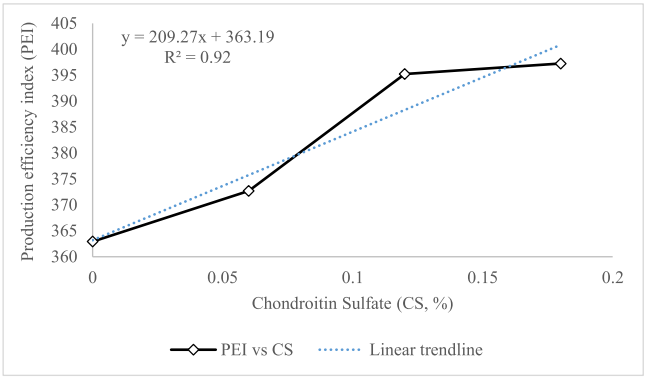

Fig. 6. Regression analysis of productive efficiency index (PEI) in broilers fed increasing levels of chondroitin sulfate (CS), regardless of dietary manganese inclusion. A linear model was fitted to the data ($R^2 = 0.92$), indicating a positive association between CS level and productive efficiency.

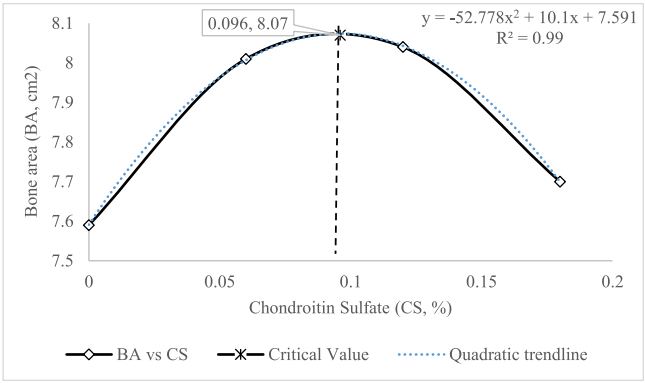

Fig. 7. Regression analysis of bone area (BA) in broilers fed increasing levels of chondroitin sulfate (CS), regardless of dietary manganese inclusion. A quadratic model was fitted to the data ($R^2 = 0.99$), indicating a peak bone area at 0.096 % CS.

weights, epiphyseal perimeters (proximal and distal), diaphyseal perimeter, and SI at all Mn levels tested in this study.

4. Discussion

Bone disorders have become increasingly frequent in broiler chickens, particularly as a consequence of genetic selection for rapid growth. Monitoring males of commercial lines revealed a 12 % increase in the incidence of severe skeletal disorders (Shim et al., 2012). Within this context, dietary supplementation with CS and Mn has been evaluated for its potential to interact with the bone matrix, to mitigate skeletal development issues, and to enhance productive performance in poultry. This strategy is supported by previously reported promising results regarding FC, bone development, type II collagen synthesis, chondrocyte proliferation, and bone mineralization (Martins et al., 2023; Sgavioli et al., 2017).

In the present study, FC was the trait most affected by CS and Mn supplementation, with an increase in dietary Mn concentration up to approximately 64 mg/kg worsening the animals' feed efficiency (Table 3 and Fig. 3). According to Macari et al. (2002) and Gajula et al. (2011), high Mn concentrations can interfere with the absorption of iron, copper, Ca, and P by competing for the same uptake sites. This interference can cause deficiencies through reduced plasma transport and increased intracellular retention. In addition, excessive Mn intake can lead to its accumulation in the liver, the main storage organ for this mineral in broiler chickens. Roy et al. (2015) demonstrated that Mn toxicity in domestic birds induces histopathological liver changes such as hepatocyte necrosis and apoptosis, along with the formation of oxygen-derived free radicals, indicating significant oxidative stress in liver tissue. These hepatic alterations can compromise the liver's capacity to metabolize nutrients and eliminate toxins, with a negative impact on feed efficiency. Therefore, high dietary levels of Mn may negatively affect broiler performance since liver accumulation can

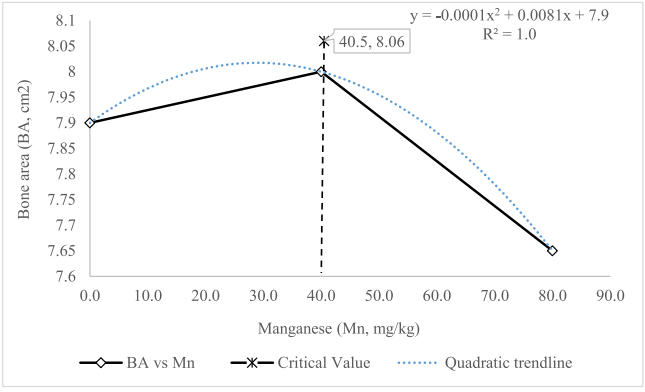

Fig. 8. Regression analysis of bone area (BA) in broilers fed increasing levels of dietary manganese (Mn), regardless of dietary chondroitin sulfate inclusion. A quadratic model was fitted to the data ($R^2 = 1.0$), indicating a peak bone area at 40.5 mg Mn/kg.

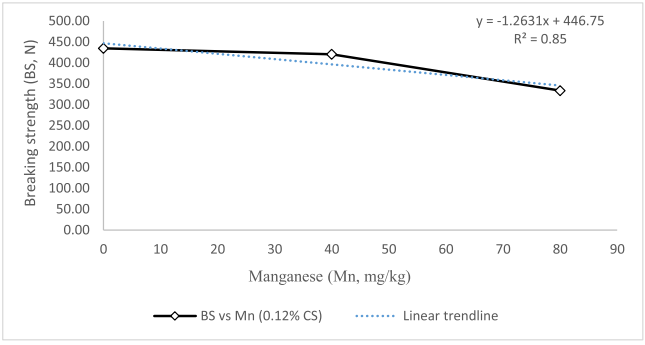

Fig. 9. Regression analysis of bone breaking strength (BS) in broilers fed increasing levels of dietary manganese (Mn), regardless of chondroitin sulfate (CS) inclusion. A linear model was fitted to the data ($R^2 = 0.85$), indicating a negative association between Mn level and bone breaking strength.

Table 4
Analysis of variance of mineral profile and number of chondrocytes in the tibiotarsus of broiler chickens.

Factor	Ashes (%)	Ca (%)	P (%)	Mn (mg/kg)	Chondrocytes
Chondroitin sulfate (CS)					
0.00 %	50.70	10.52	5.79	4.40	6780.73
0.06 %	50.27	10.31	5.67	4.82	6910.45
0.12 %	50.43	10.21	5.64	4.60	6738.10
0.18 %	50.94	10.16	5.73	4.55	6696.69
Manganese (Mn)					
0 mg/kg	50.34	9.96	5.64	4.31	6786.48
40 mg/kg	50.96	10.79	5.77	4.76	6493.24
80 mg/kg	50.44	10.12	5.72	4.71	7064.75
ANOVA	Probability				
CS	0.824	0.769	0.528	0.758	0.893
Mn	0.609	0.041	0.349	0.309	0.024
Interaction CS x Mn	0.454	0.842	0.790	0.896	0.670
CV** (%)	5.22	11.87	6.43	33.67	13.21
Regression	Probability				
CS	ns	ns	ns	ns	ns
Mn	ns	[8]Q (0.005)	ns	ns	[9]Q (0.023)
Interaction CS x Mn	ns	ns	ns	ns	ns

Ca (%) - calcium; P (%) – phosphorus.
Chondroitin sulfate (%CS: kg CS/kg of feed).
ns: not significant.
** Coefficient of variation.
[8] Calcium (Mn) $= -0.0005\times^2 + 0.0395x + 9.96$; $R^2 = 1.0$.
[9] Chondrocyte (cell/mm^2) $y = 0.2669\times^2$ - $18.035x + 6791.1$; $R^2 = 1.0$.

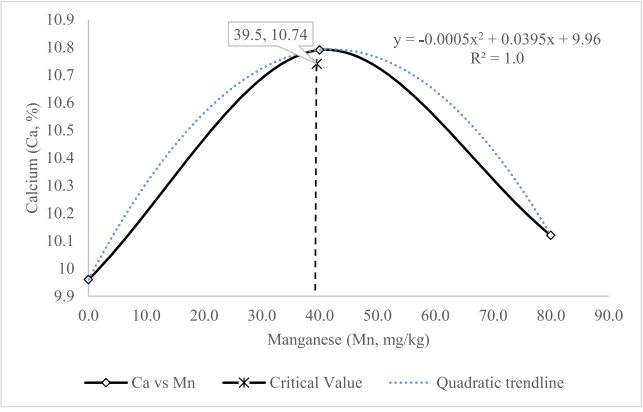

Fig. 10. Regression analysis of bone calcium content (Ca) in broilers fed increasing levels of dietary manganese (Mn), regardless of chondroitin sulfate (CS) inclusion. A quadratic model was fitted to the data ($R^2 = 1.0$), indicating a peak calcium content at 39.5 mg Mn/kg.

disrupt mineral homeostasis and lead to metabolic stress, factors that together compromise FC.

Similarly, CS exerted a quadratic effect on FC, especially when the animals were fed the diet containing 0.0 mg Mn/kg (Fig. 4, Table 3). Poor FC ratios were observed when the supply of CS reached the critical value of 0.10 %. The negative effects of CS on FC using this diet are probably related to an imbalance in the homeostasis of cations such as sodium and Ca. The large amount of negative charges in CS can attract water, a condition that increases the viscosity of the extracellular matrix, facilitating interactions between matrix molecules and the cell surface (Souza & Pinhal, 2011). However, large amounts of CS can lead to an unnecessary increase in ionic reactions, enhancing cellular stress and consequently compromising the development of the animals as demonstrated by the decrease in FC when this experimental diet was offered.

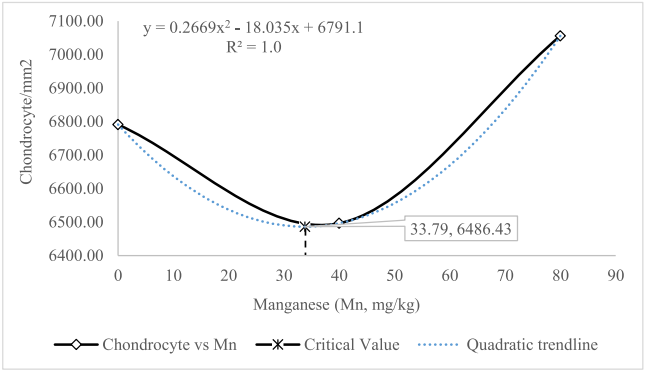

Fig. 11. Regression analysis of chondrocyte density (chondrocytes/mm^2) in response to increasing dietary manganese (Mn) levels. A quadratic model was fitted to the data ($R^2 = 1.00$), indicating the lowest chondrocyte density at 33.79 mg Mn/kg (6486.43 chondrocytes/mm^2).

According to the Broiler Performance and Nutrition Supplement (Cobb 500TM, 2018, 2022), the FC ratio of male batches at 47 days of age ranges from 1.68 to 1.62, values higher than those shown in Table 3. However, the higher FC of the experimental diets did not compromise animal development or the efficiency of converting ingested nutrients into body mass since neither CS nor Mn levels affected performance traits such as feed intake, body weight, and weight gain. In addition, FV and PEI increased with increasing CS level in the broiler diet.

The PEI is an indicator that considers weight gain, FC, and FV to assess the zootechnical performance of the broiler batch. Thus, CS improved productivity since it did not affect the overall FC of the batches or the weight gain of the birds. In addition, a 6.7 % reduction in mortality was observed compared to the diet without CS (Table 3), with FV increasing with each increment of CS (Fig. 5 and Equation 3).

The reduction in mortality associated with CS inclusion can be explained by the ability of this GAG to form covalent bonds with the core protein of proteoglycans, enabling its participation in processes that are essential for controlling metabolic stress in poultry, such as extracellular matrix repair, cell proliferation, immune response, regulation of cell trafficking, and basement membrane selectivity (Souza & Pinhal, 2011). This role is particularly relevant in modern commercial lines, which face intense metabolic stress due to the high oxygen demand required to sustain their rapid growth. Hartcher and Lum (2020) found that broilers with proportionally smaller hearts and lungs exhibit reduced cardiac output, compromising blood oxygenation and increasing mortality.

This hypothesis gains further support when considering that the environmental conditions in the present study exceeded the birds' thermoneutral zone (Table 1). Heat stress induces a series of physiological alterations, including acute hyperthermia, respiratory alkalosis, electrolyte imbalance, reduced feed intake, decreased growth rate, and increased mortality (Brossi et al., 2009). Thus, the presence of CS in the diet may help attenuate the effects of metabolic stress, reducing the physiological changes that contribute to increased mortality in broiler chickens.

Conversely, the addition of CS and Mn to the broiler diets did not affect BMC or BMD (Table 3). These parameters are believed to be the best tool to screen individuals at risk for osteoporosis and are the main determinants of fracture risk (Van Der Schouw, 2009). Bone mineral density is the ratio of BMC to bone cross-sectional area. Within this context, densitometry is a highly accurate technique for detecting variations in bone mass gain or loss and is useful for monitoring diseases that affect bone mineralization (Barreiro et al., 2009). The results obtained for these two variables indicate that there was no loss of bone mass and that the tibiotarsus did not contain weak or very porous areas that would increase the risk of fractures or bone deformities.

As can be seen in Table 3, the largest BA (cm^2) was obtained with the inclusion of 0.096 % CS, calculated based on the quadratic effect of

Table 5
Analysis of variance of morphometric attributes of the tibiotarsus in broiler chickens.

Factor	AW	TL	SI	RW	PEP	DEP	DP
Chondroitin sulfate (CS)							
0.00 %	18.13	10.79	167.17	0.63	7.98	6.57	3.66
0.06 %	18.49	10.90	169.49	0.65	8.08	6.63	3.71
0.12 %	18.99	11.06	173.46	0.64	8.03	6.70	3.76
0.18 %	18.28	10.98	161.20	0.62	7.85	6.51	3.73
Manganese (Mn)							
0 mg/kg	18.54	10.92	170.45	0.63	8.06	6.65	3.72
40 mg/kg	18.50	10.98	165.91	0.64	7.97	6.58	3.71
80 mg/kg	18.38	10.90	167.14	0.63	7.93	6.57	3.72
ANOVA	Probability						
CS	0.536	0.032	0.063	0.090	0.044	0.019	0.335
Mn	0.890	0.631	0.434	0.766	0.140	0.223	0.900
Interaction CS x Mn	0.928	0.610	0.912	0.654	0.884	0.722	0.414
CV** (%)	11.14	2.86	7.76	8.37	3.44	3.14	5.86
Regression	Probability						
CS	ns	^{9}Q (0.012)	^{10}Q (0.021)	^{11}Q (0.016)	^{12}Q (0.021)	^{13}Q (0.007)	ns
Mn	ns	ns	ns	ns	ns	ns	ns
Interaction CS x Mn	ns	ns	ns	ns	ns	ns	ns

*AW (g) - absolute weight; TL (cm) - tibial length; SI (mg/mm) - Seedor index; RW (%) - relative weight; PEP (cm) - proximal epiphyseal perimeter; DEP (cm) - distal epiphyseal perimeter; DP (cm) - diaphysis perimeter.
Chondroitin sulfate (%CS: kg CS/kg of feed).
ns: not significant.
** Coefficient of variation.
Equation 10 $TL = -13.194\times^2 + 3.5917x + 10.776$; $R^2 = 0.89$.
Equation 11 $SI = -1010.4\times^2 + 158.59x + 166.28$; $R^2 = 0.80$.
Equation 12 $RW = -2.7778\times^2 + 0.4333x + 0.631$; $R^2 = 0.96$.
Equation 13 $PEP = -19.444\times^2 + 2.7667x + 7.981$; $R^2 = 0.99$.
Equation 14 $DEP = -17.361\times^2 + 2.9417x + 6.5565$; $R^2 = 0.82$.

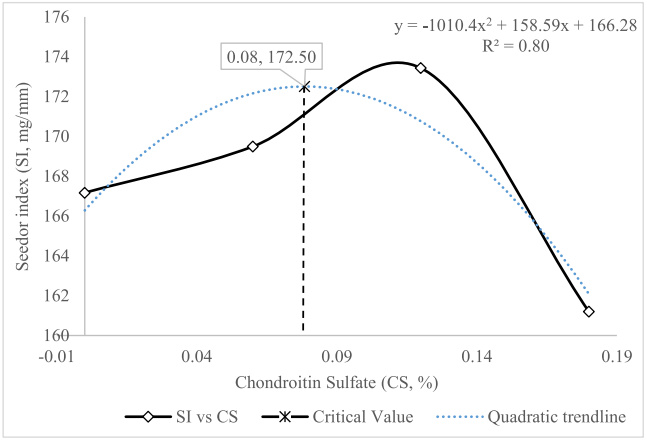

Fig. 12. Regression analysis of Seedor index (SI) in broilers fed increasing levels of dietary chondroitin sulfate (CS), regardless of manganese (Mn) inclusion. A quadratic model was fitted to the data ($R^2 = 0.80$), indicating a peak SI at 0.08 % CS (172.50 mg/mm).

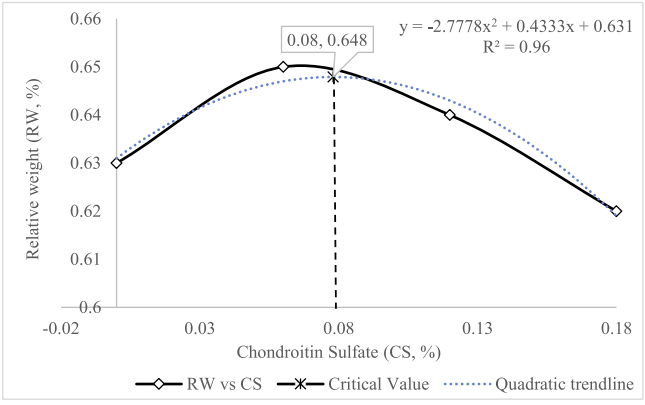

Fig. 13. Regression analysis of relative weight (RW) of the tibia in broilers fed increasing levels of dietary chondroitin sulfate (CS), regardless of manganese (Mn) inclusion. A quadratic model was fitted to the data ($R^2 = 0.96$), indicating a peak RW at 0.08 % CS (0.648 %).

Fig. 7 and Equation 5. In addition, the largest BA was obtained with the addition of 40.5 mg Mn/kg (quadratic effect, Fig. 8 and Equation 6). These results may be due to the greater body growth of the birds since bone development is intrinsically associated with an increase in the growth rate of body components and bone is involved in the mechanical protection of tissue and organs and in muscle support (Junqueira & Carneiro, 2017; Pizauro-Junior et al., 2017). It is noteworthy that Mn has affinity for the same absorption sites as other minerals such as Ca and P (Macari et al., 2002). Thus, the smaller BA found at the supplementation level of 80 mg Mn/kg may be the result of the antagonism observed at higher levels of this element, increasing competition with Ca and P.

Analysis of the effects of the interaction between CS and Mn levels on BS (Table 3) showed that, although this parameter was not affected by CS increases within each Mn level, a decreasing linear effect was observed with each addition of the mineral to the diet containing 0.12 % CS (Fig. 9 and Equation 7). In this case, the assumption was that Mn, a cofactor required for various enzymes such as galactotransferase and glycosyltransferase that participate in the production of cartilage, GAGs and glycoproteins (Reece, 2006), would provide stronger bones when combined with 0.12 % CS. However, as reported earlier, the increase in Mn led to the loss of tibiotarsal strength. This result can be explained by the antagonism of Mn. According to Bassi et al. (2021), the concentration of Ca in bones decreases with increasing dietary Mn supplementation. Thus, the lower amount of Ca due to the competition with Mn causes deficiencies in bone mineralization, reducing bone strength. According to Shim et al. (2012), in the presence of deficient bone mineralization processes, the bone loses its ability to resist gravity and the bird's weight leads to locomotor problems.

Bone rigidity results from two matrices: the inorganic and the

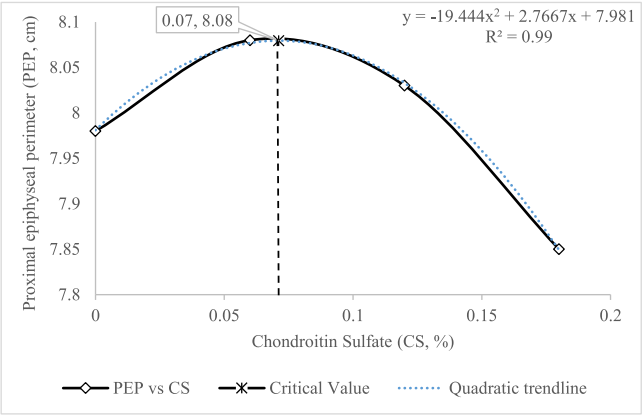

Fig. 14. Regression analysis of proximal epiphyseal perimeter (PEP) in broilers fed increasing levels of dietary chondroitin sulfate (CS), regardless of manganese (Mn) inclusion. A quadratic model was fitted to the data (R^2 = 0.99), indicating a peak PEP at 0.07 % CS (8.08 cm).

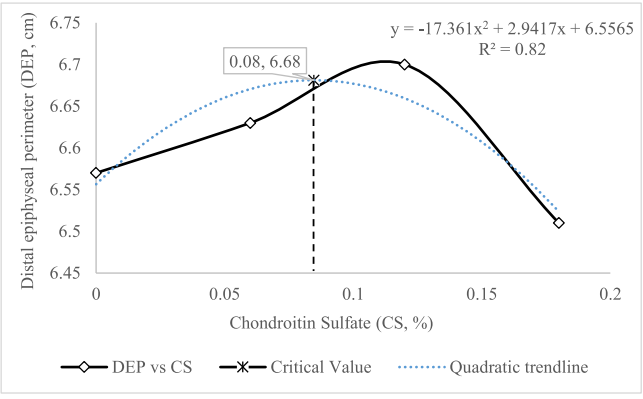

Fig. 15. Regression analysis of distal epiphyseal perimeter (DEP) in broilers fed increasing levels of dietary chondroitin sulfate (CS), regardless of manganese (Mn) inclusion. A quadratic model was fitted to the data (R^2 = 0.82), indicating a peak DEP at 0.08 % CS (6.68 cm).

organic matrix. The inorganic matrix is rich in Ca and P (phosphate) ions that occur in the form of hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$] during the mineralization process (Johnsson et al., 2015; Junqueira & Carneiro, 2017). The organic matrix mainly consists of collagen, lipids, proteoglycans, and other structural proteins that confer elasticity to bones (Johnsson et al., 2015). Within this context, the level of 0.12 % CS without Mn in the broiler diet promoted stability of the organic bone matrix, directly influencing mineral concentrations, preventing ionic imbalances, and protecting the inorganic matrix. It also reduced excessive bone resorption, favoring the balance between bone formation and degradation, thereby preventing bone fragility (thus explaining the higher BS).

According to Favero et al. (2013), Reece (2006) and Tufarelli and Laudadio (2017), the capacity of bone to withstand compression is also related to the action of glycosyltransferase, an enzyme that plays a key role in the formation and elongation of GAG chains during the biosynthesis of proteoglycans in connective tissue. These proteins are essential for growth of the bone plate and for cartilage formation in birds. According to Anderson et al. (2005), GAGs play an important role in endochondral ossification, a process that is responsible for the longitudinal growth of long bones through calcification of the epiphyseal disc.

With regard to the ash, Ca, P, and Mn content and the number of chondrocytes in the tibiotarsus, CS apparently was not sufficient to influence the mineral composition of the tibiotarsus in a way that would improve bone quality (Table 4). However, the absence of an effect of CS on mineral concentrations resulted in a state of equilibrium between the bone's inorganic components (mainly Ca, P, and micromineral ions) and the organic matrix (collagen, lipids, proteoglycans—GAGs, and other structural proteins) (Junqueira & Carneiro, 2017), promoting the proper formation of bone tissue through endochondral ossification. Moreover, Müller et al. (2012) reported that the mineral composition of bones is not fixed and that minerals can be mobilized in severe pathological conditions, altering their concentrations. This was not observed in the present study at the CS levels evaluated.

Another finding that supports the effect of CS on the mineral profile and the stability of the tibiotarsal organic matrix was the absence of an effect of CS on the number of chondrocytes in articular cartilage (Table 4). This result suggests that the composition of the extracellular matrix in the proximal tibial epiphyseal cartilage was preserved, with no signs of degenerative changes that could compromise bone formation processes. According to Martins et al. (2023), supplementing the diet of birds with 0.05 % CS increases chondrocyte density and the concentration of type II collagen and proteoglycans, which are key components for the structural and functional integrity of cartilage. Additionally, Wollenweber et al. (2006) demonstrated that maintaining the number of chondrocytes is associated with preserving their metabolic activity. These cells are responsible for producing and maintaining the cartilage matrix and biomechanical properties of the tissue. Thus, the absence of an effect on chondrocyte density observed in this study indicates that chondrogenic activity was preserved, reflecting a structural and functional balance in articular cartilage, even with increasing levels of CS supplementation.

In addition to the effects observed with CS, the Mn supplementation results also revealed important aspects of bone metabolism. Mn levels did not significantly affect P, Mn, or bone ash content (Table 4), supporting the hypothesis of ionic balance between the organic and inorganic fractions of the tibiotarsus. This stability may be associated with the absence of excessive Mn accumulation in the bones of broilers fed the experimental diets. According to Black et al. (1984), excessive Mn supplementation can lead to unbalanced mineralization, especially when not accompanied by proportional increases in P and Ca levels. However, the maintenance of P content observed in this study suggests that excess Mn did not compromise the activity of phosphate transporters such as type III sodium-dependent co-transporters (Pit1 and Pit2), which are essential for phosphate uptake by osteogenic cells and for proper mineralization of the extracellular matrix (Hasegawa et al., 2022).

Within this context, the Mn concentration recommended by the Broiler Performance and Nutrition Supplement (Cobb 500TM, 2015) appears to be sufficient to support growth plate development and cartilage formation in broiler chickens. Nevertheless, the findings of this study indicate that the highest tibiotarsal Ca content was achieved at an Mn level of 39.5 mg/kg (Fig. 10 and Equation 8), suggesting that moderate Mn levels, regardless of CS inclusion, enhance Ca retention in bones.

Sunder et al. (2006) also demonstrated the effect of Mn on Ca retention. The authors observed increased deposition of Ca, P, and zinc in bones of broilers supplemented with up to 100 mg Mn/kg. Similarly, Pacheco (2012) evaluated increasing levels of organic Mn (25 to 105 mg/kg) in the diets of broilers from 1 to 42 days of age and found that bone mineralization increased with increasing Mn concentrations.

The role of Mn in Ca retention may be explained by its involvement in the formation of the organic bone matrix. Mn acts as a cofactor for galactosyltransferase and glycosyltransferase, enzymes involved in the synthesis of mucopolysaccharides—key constituents of proteoglycans. These macromolecules are fundamental for the structure of the bone matrix and connective tissues (Favero et al., 2013; Reece, 2006; Tufarelli & Laudadio, 2017). In addition to their structural function, proteoglycans increase the viscosity of the extracellular matrix and attract cations such as Ca, thereby promoting its retention. Thus, Mn

contributes to the stability of the extracellular matrix, improving its physical and functional integrity, which positively impacts bone quality.

It is worth noting that, in addition to increasing Ca content and BA, supplementation with moderate levels of Mn also promoted a reduction in chondrocyte density, with a critical point identified at 33.79 mg Mn/kg (Fig. 11 and Equation 9). At first glance, this reduction may suggest that increasing Mn concentration negatively affects chondrocyte proliferation and, consequently, the quality of cartilage and bone. Indeed, using cell models, Liu et al. (2020) demonstrated that excess Mn can induce oxidative stress that leads to mitochondrial dysfunction, increased production of reactive oxygen species, and alterations in cellular signaling pathways, resulting in toxicity and impaired cellular activity.

On the other hand, considering that Mn supplementation levels did not negatively affect zootechnical performance, bone morphometric attributes, BMD, or the mineral profile of the tibiotarsus, it is plausible to interpret the reduction in chondrocyte density as a reflection of an accelerated endochondral ossification process. In this scenario, chondrocytes in the growth plate are rapidly replaced by mineralized bone tissue, simultaneously promoting an increase in Ca deposition and a reduction in chondrocyte density (Matsushita et al., 2020).

According to Ornitz and Marie (2015), this bone growth process may be characterized as premature ossification when it occurs in the early stages of development, potentially leading to early closure of the epiphyseal plate and compromising longitudinal growth. However, since in the present study the reduction in chondrocyte density and the increase in Ca content were observed in broilers at 47 days of age (a more advanced stage of the production cycle), this phenomenon likely reflects a physiological skeletal maturation process. Nevertheless, it is important to emphasize that nutritional or metabolic imbalances may lead to disorganized ossification characterized by irregular mineral deposition, disruption of epiphyseal architecture, and, in extreme cases, impaired bone quality, as discussed by Matsushita et al. (2021).

The results described in Table 5 show that the diets with CS inclusion levels of 0.079 %, 0.14 %, 0.078 %, 0.071 %, and 0.085 % provided the greatest SI, tibial length, relative weight, and proximal and distal epiphyseal perimeters (quadratic effect in Figs. 12–16, respectively). Bone density assessed using the SI refers to material mass per volume of bone and is a measure of BMC, including organic (collagen) and inorganic components (hydroxyapatite) (Rath et al., 2000). Within this context, the higher SI indicated that supplementation with up to 0.12 % CS improved the density of the organic matrix in the tibiotarsus, suggesting an increase in mineral content per unit volume in broiler chickens. Consequently, the greater the bone mineralization, the lower the risk of fractures. Similarly, Muraleva et al. (2012) demonstrated that treating rats with a combination of GAGs (1.26 mg glucosamine + 5.06 mg dihydroquercetin) increased BMD and BS.

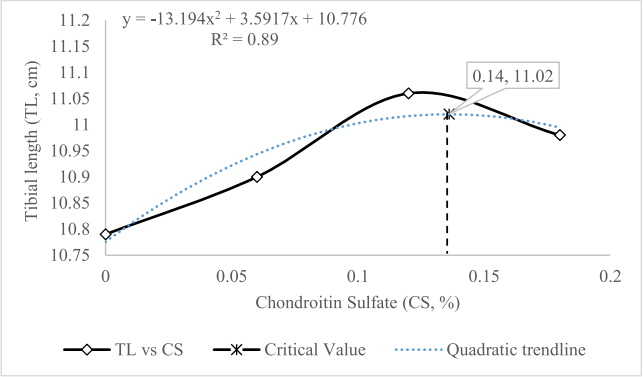

Fig. 16. Regression analysis of tibial length (TL) in broilers fed increasing levels of dietary chondroitin sulfate (CS), regardless of manganese (Mn) inclusion. A quadratic model was fitted to the data ($R^2 = 0.89$), indicating a peak tibial length at 0.14 % CS (11.02 cm).

Regarding morphometric changes, supplementation with CS (diets with 0.06 % and 0.12 % CS) promoted greater tibiotarsal development. According to Souza and Pinhal (2011), Melo et al. (2008), and Vieira et al. (2010), CS has chondrocyte-stimulating properties that increase the synthesis of chondrocytes and of compounds that contribute to maintaining the structural integrity of the extracellular matrix. Furthermore, its negative charge improves the selectivity of the cell's basement membrane in terms of cation binding (sodium, potassium, and Ca) and water retention in tissues. Therefore, CS contributed to maintaining the balance of the organic bone matrix and its interaction with the inorganic matrix, enabling the binding of Ca and P, transporting these ions to the epiphyseal growth plate for precipitation into hydroxyapatite crystals, and thus completing the process of bone calcification (Goff, 2017; Junqueira & Carneiro, 2017).

According to Santos et al. (2019), longitudinal bone growth comprises the proliferation of chondrocytes, production and mineralization of cartilaginous matrix, hypertrophy, and hypertrophic invasion of chondrocytes, as well as the subsequent degeneration of chondrocytes, which results in depressions that must be quickly filled with osteoblasts, called osteocytes. In the present study, supplementation with CS promoted expressive tibiotarsal development, increasing length and epiphyseal perimeters.

In agreement with the present study, Sgavioli et al. (2017) and Martins (2018) found that the inclusion of GAGs (CS and glucosamine sulfate) in the broiler diet at 42 days of age increased morphometric tibiotarsal parameters (length, width of the proximal and distal epiphysis and of the diaphysis). Furthermore, Furthermore, Wolff (2014) reported that the combination of polysulfated GAGs increased cell proliferation (chondrocytes). The author demonstrated that the tibial epiphyseal growth plate promoted longitudinal bone growth after the administration of chondroprotectors. Within this context, the results of the present study suggest that CS added to the broiler diets promoted the characteristic behavior of GAGs, maintaining the balance of anabolic and catabolic processes in articular cartilage, stimulated the synthesis of proteoglycans and collagen, and protected chondrocytes in such a way that they could exert their bone matrix biosynthesis functions, as described by Altman et al. (1989), Clark (1991), and Castrogiovanni et al. (2016). Likewise, GAGs contributed to maintaining the structural integrity of the extracellular matrix by exerting catabolic effects on the suppression of inflammatory mediators and inhibition of degeneration, as reported by Herrero-Beaumont et al. (2008) and Pecchi et al. (2012).

In poultry farming, the main cause of musculoskeletal diseases in broiler chickens is the presence of immature connective tissue that is unable to sustain the weight gain promoted by rapid growth (Almeida-Paz, 2008; Petracci & Cavani, 2012). The selection of birds for rapid growth has been related to an increase in the prevalence of metabolic diseases, body fat deposition, disorders in the locomotor system explained by increased porosity, and reduced mineralization (Araújo et al., 2012; Pratt & Cooper, 2018). Furthermore, the high incidence of leg or locomotion problems in broiler chickens may be associated with low bone strength as a result of skeletons that are not adapted to sustain rapid weight gain (Nääs et al., 2012).

Taken together, the results of this study suggest that GAGs can be used as a nutritional intervention in fast-growing broiler chickens since they contribute to animal development without affecting performance traits, increasing productivity and decreasing animal mortality. Furthermore, GAGs contribute to bone development, preventing structural injuries.

5. Conclusions

- Dietary inclusion of 80 mg Mn/kg does not improve bone quality parameters in broiler chickens and may compromise bone development and BS, depending on the interactions with CS.

- Supplementation with CS, as well as inclusion of 40 mg Mn/kg in broiler diets, affects the bone matrix, improving tibiotarsal bone quality, particularly morphometric attributes, Ca content, and BS.
- Broiler performance was not compromised by the inclusion of different levels of CS or Mn in diets. However, supplementation with 0.12 % CS provided benefits by reducing mortality and increasing PEI and may represent a promising nutritional strategy since this CS level could contribute to reducing the incidence of skeletal diseases and improving bird development.

Ethical statement

CERTIFICADO Certificamos que a proposta intitulada "Glicosaminoglicanos e manganês influenciam o desempenho zootécnico e características de carcaça, carne e ossos em frangos de corte.", protocolada sob o CEUA n° 8401,180,618, sob a responsabilidade de Angélica Simone Cravo Pereira e equipe; Julian Andres Muñoz - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais da Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo - FZEA/USP (CEUA/FZEA) na reunião de 22/08/2018. We certify that the proposal "Glycosaminoglycans and manganese influence the zootechnical performance and characteristics of carcass, meat and bone on broilers chickens.", utilizing 1152 Birds (1152 males), protocol number CEUA 8401,180,618, under the responsibility of Angélica Simone Cravo Pereira and team; Julian Andres Muñoz - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was approved by the Ethic Committee on Animal Use of the School of Animal Science and Food Engineering - (São Paulo University) (CEUA/FZEA) in the meeting of 08/22/2018. Finalidade da Proposta: Pesquisa (Acadêmica) Vigência da Proposta: de 07/2018 a 04/2020 Área: Zootecnia Origem: Faculdade de Zootecnia e Engenharia de Alimentos (FZEA/USP) Espécie: Aves sexo: Machos idade: 1 a 49 dias N: 1152 Linhagem: Cobb Peso: 40 a 50 g Resumo: A produção avícola apresenta altos índices produtivos, no entanto, tem-se observado dificuldades que estão representando grandes perdas econômicas para a indústria, no que se refere às miopatias e os distúrbios na estrutura óssea das aves, anormalidades que podem estar associadas com deficiências no tecido conjuntivo, comprometendo nas células musculares o fornecimento de nutrientes e a remoção dos metabólitos ocasionando distúrbios iônicos. Pretende-se estudar o efeito da suplementação de GAGs (sulfato de condroitina) e manganês na dieta de frangos de corte devido a sua importância na matriz óssea, e assim, determinar como estes elementos influenciam as características produtivas, na qualidade de carne e óssea, além de identificar as diferenças entre a dieta que recebeu suplementação e a dieta controle por meio da metabolômica. Serão utilizados 1152 pintos de corte machos da linhagem Cobb, alojados por 49 dias e distribuídos em DIC, sob esquema fatorial 4 × 3, sendo os fatores: quatro doses de sulfato de condroitina (0,00; 0,06; 0,12 e 0,18 %) e três doses de manganês (0, 40 e 80 mg/kg), totalizando 12 tratamentos de oito repetições com 12 aves cada. As aves serão pesadas semanalmente e calculado o peso corporal, ganho de peso, consumo de ração, conversão alimentar, viabilidade criatória e o índice de eficiência produtiva. Aos 49 dias de idade, serão abatidas duas aves por unidade experimental e realizada a análise macroscópica dos peitos na desossa para identificar a presença da miopatia white striping. Em seguida, serão colhidas 5 g de amostra do músculo peitoralis maior e armazenadas a - 80 °C até processamento para análise comparativa entre as amostras pela analise metabolômica. Será calculado o rendimento de carcaça e cortes comerciais: peito, coxas, sobrecoxas e asas. Será avaliada a qualidade da carne nos peitos por meio da determinação do pH, cor, medidas físicas (comprimento, altura e largura do peito), perda de peso por cozimento, força de cisalhamento, composição centesimal, colágeno total, e perfil de ácidos graxos. Para a qualidade de osso das tíbias será realizada an análise macroscópica do osso, índice Seedor, densidade óssea, resistência óssea, perfil mineral e avaliação histopatológica da cartilagem articular. Local do experimento: O experimento será conduzido na no laboratorio de avicultura da Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, Campus Fernando Costa Pirassununga, Localizado na Região Centro-Leste do estado de São Paulo.

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CRediT authorship contribution statement

Julian Andres Muñoz: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Taiane da Silva Martins:** Resources, Methodology, Investigation, Formal analysis. **Pollyana Leite Matioli Garbossa:** Writing – original draft, Methodology, Formal analysis. **Laura Barbosa Ferreira Pimentel:** Methodology, Formal analysis. **Caio Bertasi Barbalho:** Methodology, Formal analysis. **Monica Márcia da Silva:** Methodology, Formal analysis. **André Felipe de Arruda:** Methodology, Formal analysis. **Silvana Martinez Baraldi-Artoni:** Methodology, Formal analysis, Conceptualization. **Cristiane Soares da Silva Araújo:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualization. **Angélica Simone Cravo Pereira:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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