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Hematology and serum biochemistry of broilers fed diets supplemented with chondroitin and glucosamine sulfates

Hematologia e bioquímica sérica de frangos de corte suplementados com sulfatos de condroitina e de glucosamina na ração

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Highlights

Glucosamine sulfate along with 10% chondroitin sulfate increased lymphocytes.
Glucosamine sulfate reduced chlorides linearly by 21 days.
Glucosamine sulfate increased total calcium linearly by 42 days.
Sulfate interaction on total calcium by 21 days and ionic calcium by 21 and 42 days.
Interaction between the sulfates on phosphorus and chlorides by 42 days.

Abstract

The objective of this study was to evaluate the hematology and serum biochemistry of broilers fed diets supplemented with chondroitin and glucosamine sulfates. An experiment was laid out in a completely randomized design with a 3 × 3 factorial arrangement (three levels of chondroitin sulfate: 0, 0.05 and 0.10%; and three levels of glucosamine sulfate: 0, 0.15, and 0.30%), with each treatment involving six replicates of 30 birds. Hematology (red blood cells, hemoglobin, hematocrit, total plasma protein [TPP], thrombocytes, white blood cells, eosinophils, monocytes, heterophils, and lymphocytes) and serum biochemistry (total

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serum protein [TSP], albumin, globulins, aspartate aminotransferase [AST], gamma-glutamyltransferase [GGT], alkaline phosphatase [AP], total calcium, ionic calcium, phosphorus, sodium, potassium, and chlorides) variables were evaluated at 21 and 42 days. Data were subjected to analysis of variance. When the means differed significantly by the F-test, orthogonal analysis was performed to test the linear and quadratic effects of chondroitin and glucosamine sulfate levels. Glucosamine sulfate reduced the number of monocytes linearly, by 42 days (p = 0.0399). There was an interaction effect between the sulfates on total white blood cells (p = 0.0099) and lymphocytes (p = 0.0004) by 21 days. Chickens supplemented with 0.10% chondroitin sulfate showed a linear increase in white blood cells (p = 0.0287) and lymphocytes (p = 0.0144) with the addition of glucosamine sulfate. Chondroitin sulfate supplementation increased serum albumin linearly (p = 0.0099) and TSP quadratically (p = 0.0140), by 21 days. Glucosamine sulfate induced a quadratic response (p < 0.05) in albumin by 42 days, with the lowest value found with the inclusion of 0.06%. Glucosamine sulfate reduced chlorides linearly (p = 0.0237) by 21 days and increased calcium linearly (p = 0.0012) by 42 days. The interaction between sulfates influenced (p < 0.05) total calcium by 21 days, ionic calcium by 21 and 42 days, and phosphorus, chlorides, and sodium by 42 days. Supplementation with chondroitin and glucosamine sulfates in the broilers' diet favored their immune system and mineral metabolism, increasing serum concentrations of calcium, phosphorus, and sodium.
Key words: Polysulfated glycosaminoglycans. Red blood cells. Serum minerals. Thrombocytes. Total white blood cells.

Resumo

Objetivou-se avaliar a hematologia e a bioquímica sérica de frangos de corte suplementados com sulfatos de condroitina e de glucosamina na ração. Foi conduzido um experimento em delineamento inteiramente casualizado, em esquema fatorial 3 x 3 (três níveis de sulfato de condroitina: 0; 0,05 e 0,10%; e três níveis de sulfato de glucosamina: 0; 0,15 e 0,30%), cada tratamento com seis repetições de 30 aves. Foram avaliadas as variáveis de hematologia (hemácias, hemoglobina, hematócrito, proteínas plasmáticas totais [PPT], trombócitos, leucócitos, eosinófilos, monócitos, heterofilos e linfócitos) e bioquímica sérica (proteínas séricas totais [PST], albumina, globulinas, aspartato aminotransferase [AST], gama glutamiltransferase [GGT], fosfatase alcalina [FA], cálcio total, cálcio iônico, fósforo, sódio, potássio e cloretos) aos 21 e 42 dias. Os dados foram submetidos à análise de variância. Quando as médias diferiram significativamente pelo teste F, a análise ortogonal foi realizada para testar os efeitos lineares e quadráticos dos níveis dos sulfatos de condroitina e de glucosamina. Observou-se efeito linear decrescente (p = 0,0399) do sulfato de glucosamina na quantidade de monócitos aos 42 dias. Houve interação dos sulfatos para leucócitos totais (p = 0,0099) e linfócitos (p = 0,0004) aos 21 dias. Frangos suplementados com 0,10% de sulfato de condroitina mostraram um aumento linear dos leucócitos (p = 0,0287) e dos linfócitos (p = 0,0144) com a inclusão de sulfato de glucosamina. A suplementação com sulfato de condroitina aumentou linearmente (p = 0,0099) a albumina sérica e afetou de forma quadrática (p = 0,0140) as PST aos 21 dias. O sulfato de glucosamina demonstrou um efeito quadrático (p < 0,05) sobre a albumina aos 42 dias, o menor valor foi encontrado para a inclusão de 0,06%, respectivamente. O sulfato de glucosamina reduziu linearmente (p = 0,0237) os cloretos aos 21 dias e aumentou linearmente (p = 0,0012) o cálcio total aos 42 dias. Verificou-se interação (p < 0,05) dos sulfatos para cálcio total aos 21 dias, cálcio iônico aos 21 e 42 dias e para

fósforo, cloretos e sódio aos 42 dias. A suplementação com os sulfatos de condroitina e de glucosamina na ração de frangos de corte favoreceram o sistema imune e o metabolismo de minerais, com aumento nas concentrações séricas de cálcio, fósforo e sódio.

Palavras-chave: Eritrócitos. Glicosaminoglicanos polissulfatados. Leucócitos totais. Minerais séricos. Trombócitos.

Introduction

Intensive farming based on adequate nutrition, environmental and health management, and animal breeding has allowed industrial poultry production to expand exponentially. However, this industry is susceptible to higher rates of locomotor disorders compared with the previous year.

Disorders of movement are a problem of great relevance in poultry production worldwide, since they can cause significant economic losses due mainly to condemnations and the downgrading of carcasses at slaughterhouses. Gocsik et al. (2014) estimated a 1.19% increase in the production cost of broilers in the conventional system due to locomotor deficiencies. Unmeasurable losses are also present, such as the decline in performance due to delayed growth in birds with lameness, which cannot reach the feeders and drinkers and lose weight, displaying worse production indices. Therefore, locomotor diseases are of great importance to the productive efficiency of broilers, as they lead to decreased performance (Shim et al., 2012) and high mortality rates (Schwean-Lardner et al., 2013).

In addition to compromising production and economic efficiency, they negatively affect animal welfare (Shim et al., 2012). Birds with mobility problems are fully or partially deprived of the freedoms described in the animal welfare rules defined by the Farm Animal Welfare Council [FAWC] (2012). As such, locomotor diseases must be prevented, since losses are inevitable once they are established.

Polysulfated glycosaminoglycans (GAGs) have been used as dietary compounds (nutraceuticals), as they constitute a non-invasive method and possess properties capable of preventing and/or treating injuries to bones and cartilage. This ability is provided by their role in maintaining the balance of anabolic and catabolic processes, which can augment chondrocyte proliferation and biosynthesis and decrease the loss of matrix constituents (Castrogiovanni et al., 2016).

Among the GAGs used as nutraceuticals, the chondroitin and glucosamine sulfates are highlighted. Chondroitin sulfate plays an important role in the regulation of anabolic processes of cartilage, e.g. synthesis of proteoglycans and collagen (Kamarul et al., 2011), suppression of inflammatory mediators, and inhibition of cartilage degeneration (Calamia et al., 2012; Taniguchi et al., 2012). Glucosamine is an important precursor of glycoproteins and GAGs (Jerosch et al., 2011), and its exogenous supplementation can stimulate the synthesis of these components in cartilage (Kamarul et al., 2011) and in synovial fluid (Gouze et al., 2001), promoting the restoration of

the locomotor system in case of injury. In addition, similar to chondroitin, it can inhibit degenerative and catabolic processes through its anti-inflammatory (Calamia et al., 2014; Gouze et al., 2001; Kantor et al., 2014; Taniguchi et al., 2012) and antioxidant (Valvason et al., 2008) properties.

The association of chondroitin and glucosamine sulfates has a proven effect on the bone and cartilaginous development of broilers (Santos et al., 2018, 2019; Sgavioli et al., 2017), consequently preventing locomotor problems (Martins et al., 2020b) and improving performance (Martins et al., 2020b; Martins et al., 2020a; Sgavioli et al., 2017). However, no studies have been done on the hematology and serum biochemistry of broilers fed diets containing GAGs. Therefore, considering that biochemical markers of blood metabolism can be used to characterize and evaluate diseases such as those of the locomotor system, the present study looked into the effect of supplementation with chondroitin and glucosamine sulfates on the hematology and serum biochemistry of broilers.

Material and Methods

Experiment site

The experiment was carried out in the Experimental Broiler Poultry House of the School of Veterinary and Animal Science at the Federal University of Goiás, located in Goiânia - GO, Brazil. All procedures performed in this study were approved by the Ethics Committee on Animal Use at the Federal University of Goiás (approval no. CEUA/UFG 051/16).

Birds and facilities

A total of 1,620 male broiler chicks of the commercial Cobb 500® line, with an average initial weight of 43 ± 0.2 g, acquired from an industrial hatchery (São Salvador Alimentos S/A, Itaberaí, Goiás, Brazil), were raised from one to 42 days of age.

The animals were housed in 54 boxes made of PVC tubes and screens, with dimensions of 1.60 × 1.80 m (2.88 m²), which were set up in the central area of a commercial shed measuring 12 × 125 m (1,500 m²). A negative-pressure ventilation system was present, which was equipped with seven exhaust fans, a misting system, and air inlet with evaporative plate. The shed had short brick walls 0.40 m high, wire mesh 2.80 m high, and a ceiling height of 3.20 m, and the facility was oriented east-west. Housing density was 10 birds/m² (30 birds/box). In the shed was a line with 10 nipple drinkers, one chick-type trough feeder (used up to the seventh day of age), and an adult chicken trough feeder (from eight to 42 days of age).

All birds were vaccinated at the hatchery against Marek's disease, avian infectious bronchitis, and Gumboro disease via drinking water at 14 days of age. Throughout the experimental period, the birds received feed and water *ad libitum.* To ensure adequate ambience throughout the experimental period, the animals were reared following the recommendations for lighting, temperature, humidity, and handling set forth in the Cobb Broiler Management Guide (Cobb, 2008).

Experimental design and treatments

The experiment was laid out in a completely randomized design with a 3 × 3 factorial arrangement (three levels of chondroitin sulfate supplementation in the diet: 0, 0.05 and 0.10%; and three levels of glucosamine sulfate supplementation in the diet: 0, 0.15, and 0.30%). Each treatment involved six replicates of 30 birds, totaling 54 plots. Chondroitin sulfate [$(C_{14}H_{21}NO_{14}S)n$; Biofac A/S, Englandsvej, Kastrup, Denmark] was 91.27% pure and glucosamine sulfate-potassium chloride [$(C_6H_{14}NO_5)$ $2SO_4$ × 2KCl; Zhejiang Golden-Shell Pharmaceutical Co. Ltd., Yuhuan, Zhejiang, China] was 16% pure.

The diets were formulated based on maize and soybean meal and observing the nutritional and energy recommendations proposed by Rostagno et al. (2011) for each rearing phase, namely, pre-starter (one to seven days of age), starter (eight to 21 days of age), grower (22 to 35 days of age), and finisher (36 to 42 days of age). All diets contained a variable portion of 0.4% (chondroitin sulfate and/or glucosamine sulfate and/or inert) according to the treatments (Table 1).

Table 1
Ingredients and calculated nutritional composition of the pre-starter (one to seven days old), starter (eight to 21 days old), grower (22 to 35 days old), and finisher (36 to 42 days old) diets

Ingredient (%)	Pre-starter	Starter	Grower	Finisher
Corn grain	54.46	59.80	63.77	69.94
Soybean meal (45.5%)	35.16	30.78	23.82	15.18
Poultry fat	1.13	1.40	1.87	2.07
Meat and bone meal (47%)	3.67	4.40	3.13	6.87
Offal meal (62.5%)	3.00	1.13	3.53	1.80
Feather meal (84.81%)	-	-	1.53	2.00
Calcitic limestone	0.53	0.53	0.63	0.23
Salt	0.39	0.35	0.27	0.21
Sodium bicarbonate	0.08	0.05	0.10	0.15
Choline chloride (75%)	0.05	0.08	0.05	0.06
DL-methionine (99%)	0.41	0.35	0.27	0.25
L-lysine HCL (64%)	0.33	0.34	0.28	0.45
L-threonine (98%)	0.07	0.08	0.05	0.09
L-valine (96.5%)	0.02	0.01	-	-
[a]Vitamin supplement	0.05	0.05	0.05	0.05
[b]Mineral supplement	0.05	0.05	0.05	0.05
[c]Additives	0.20	0.20	0.20	0.20
[d]Variable portion	0.40	0.40	0.40	0.40
Total	100.00	100.00	100.00	100.00

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Calculad nutritional composition				
Metabolizable energy (kcal/kg)	3,000	3,050	3,150	3,200
Crude protein (%)	25.00	22.50	21.60	19.53
Calcium (%)	0.98	0.98	0.95	0.86
Available phosphorus (%)	0.49	0.48	0.46	0.44
Sodium (%)	0.22	0.21	0.20	0.19
Chlorine (%)	0.30	0.27	0.23	0.20
Potassium (%)	0.90	0.82	0.70	0.57
Digestible methionine + cystine (%)	1.03	0.92	0.85	0.75
Digestible methionine (%)	0.73	0.64	0.56	0.49
Digestible lysine (%)	1.36	1.21	1.10	1.00
Digestible threonine (%)	0.87	0.79	0.72	0.68

[a]Vitamin supplement (provides per kilogram of product): Pre-starter and Starter (vitamin A 20,000,000.00 IU; vitamin D3 5,000,000.00 IU; vitamin E 50,000.00 IU; vitamin K3 4,000.00 mg; vitamin B1 5,000.00 mg; vitamin B2 13,000.00 mg; vitamin B6 7,000.00 mg; vitamin B12 36.00 mg; niacin 84,000.00 mg; pantothenate 30,000.00 mg; folic acid 2,400.00 mg; biotin 160.00 mg; selenium 600.00 mg); grower and finisher (vitamin A 16,000,000.00 IU; vitamin D3 3,800,000.00 IU; vitamin E 40,000.00 IU; vitamin K3 3,600.00 mg; vitamin B1 3,600.00 mg; vitamin B2 11,000.00 mg; vitamin B6 5,200.00 mg; vitamin B12 30.00 mg; niacin 70,000.00 mg; pantothenate 26,000.00 mg; folic acid 1,800.00 mg; biotin 100.00 mg; selenium 600.00 mg).
[b]Mineral supplement (provides per kilogram of product): copper 16.25 g; iron 100.00 g; iodine 2,000.00 g; manganese 150.00 g; zinc 125.00 g.
[c]Additives: pre-starter, starter and grower [maxiban (narasin + nicarbazin) 0.05 g; enradin (enramycin) 0.01 g; microtech (phytase) 0.01g; salmex (formaldehyde, propionic acid, terpenes, and surfactants) 0.10 g; endox (ethoxyquin and butylated hydroxyanisole) 0.004 g; copper sulfate 0.03 g]; final [maxiban (narasin + nicarbazin) 0.05 g; enradin (enramycin) 0.006 g; microtech (phytase) 0.01g; salmex (formaldehyde, propionic acid, terpenes, and surfactants) 0.10 g; endox (ethoxyquin and butylated hydroxyanisole) 0.004 g; copper sulfate 0.03 g].
[d]Variable portion: chondroitin sulfate [$(C_{14}H_{21}NO_{14}S)n$, Biofac A/S, Englandsvej, Kastrup, Denmark] and/or potassium glucosamine sulfate [$(C_6H_{14}NO_5)\ 2SO_4 \times 2KCl$, Zhejiang Golden-Shell Pharmaceutical Co. Ltd., Yuhuan, Zhejiang, China] and/or inert (kaolin) depending on the treatment.

Hematology and serum biochemistry variables analyzed

At 21 and 42 days of age, 4 mL of blood were collected by cardiac puncture from one bird per replicate, totaling six birds per treatment. Of this amount, 0.5 mL were transferred to pediatric tubes with the 10% ethylenediaminetetraacetic acid (EDTA) anticoagulant for hematological analysis and 3.5 mL to a vacuum tube without anticoagulant and with a separating gel for biochemical analysis.

The hemogram was performed by the manual technique, immediately after blood collection. Cell counts were carried out in a Neubauer chamber, following the method by Natt and Herrick (1952), including red blood cells, total white blood cells, and thrombocytes. The dilution adopted was 10 µL of blood in 1 mL of Natt and Herrick (1952)'s solution.

The red blood cell count was performed in the five medium squares of the large central square of the Neubauer chamber

and the result was multiplied by 5,000 to obtain the count per microliter of blood. White blood cells and thrombocytes were counted throughout the large central square and multiplied by 1,000 as a correction factor. The hematocrit reading was performed in a capillary tube by the microhematocrit method and total plasma protein (TPP) was determined by refractometry. The hemoglobin level was determined by the cyanmethemoglobin method, with a commercial Hemoglobin kit (Labtest®, Lagoa Santa, Minas Gerais, Brazil), using only the sample supernatant to avoid interference from nuclear remnants in the reading. To visualize the cell morphology and differentiation of white blood cells in 100 cells, a blood smear stained with Quick Panoptic (Laborclin®, Pinhais, Paraná, Brazil) was used. After counting heterophils and lymphocytes, the heterophil:lymphocyte ratio was calculated.

The biochemical profile included measurement of analytes, total serum protein (TSP), albumin, and globulins; the enzymes aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), and alkaline phosphatase (AP); and the minerals total calcium, ionic calcium, phosphorus, sodium, potassium, and chlorides. These were measured by spectrophotometry, in an automatic analyzer (CM 250, Wiener®, Rosario, Santa Fe, Argentina), following the instructions recommended by the manufacturer and using commercial analytical kits (Labtest®, Lagoa Santa, Minas Gerais, Brazil).

Albumin and TSP were measured by endpoint reaction, using the bromocresol green and biuret methods, respectively. Globulins were obtained by subtracting albumin from total protein levels. The AST enzyme was determined by the kinetic method proposed by Schumann et al. (2002) - International Federation of Clinical Chemistry and Laboratory Medicine. The GGT enzyme was determined by a modified version of the kinetic method by Szasz (1969) and AP by a modified version of the method by Bowers and McComb (1966).

Total calcium and phosphorus were measured by endpoint reaction, following the modified arsenazo III and ultraviolet colorimetric method of Daly and Ertingshausen (1972), respectively. The other electrolytes were determined by the selective ion method, in a specific apparatus (Cobas B121, Roche®, Rotkreuz, Switzerland).

Statistical analysis

The effects of chondroitin sulfate (CO; 0, 0.05, and 0.10%) and glucosamine (GLU; 0, 0.15, and 0.30%) supplementation and their interaction (CO × GLU) were analyzed according to the experimental model below:

$$Yijk = \mu + (CO)\, i + (GLU)\, j + (CO \times GLU)\, ij + eijk,$$

where Y = response variable; μ = mean of the variable; CO = chondroitin sulfate; GLU = glucosamine sulfate; CO × GLU = interaction between chondroitin and glucosamine sulfates; and eijk = residual error.

The data of all variables were checked for the presence of outliers (Box-and-Whisker Plot), assumptions of normality of observation errors (Cramér-von Mises test), and homogeneity of variances (Bartlett test). After the corrections, the data were subjected to analysis of variance using the Generalized Linear Model (GLM) procedure of SAS® (Statistical Analysis System Institute [SAS

Institute], 2002). When the means differed significantly by the F-test at 5% probability, orthogonal analysis was performed to test the linear and quadratic effects of chondroitin and glucosamine sulfate levels.

Results and Discussion

Neither the isolated chondroitin and glucosamine sulfates nor their interaction influenced ($p > 0.05$) red blood cells, hemoglobin, hematocrit, TPP, or thrombocytes in the blood of the chickens by 21 or 42 days of age. The results found were similar to those reported by Carvalho et al. (2020) in healthy chickens fed basal diets and reared under conditions similar to those described in our study.

Noushi and Naji (2013) investigated the effects of glucosamine sulfate and glucosamine/chondroitin sulfates administered orally to mice for 30 days and did not find differences in red blood cell counts, which is similar to the results presented in this study. Nonetheless, the authors found that administration of glucosamine sulfate increased the number of thrombocytes, demonstrating the hypercoagulation activity of sulfates in rats. Thus, it is possible that this effect does not occur in birds, since the number of thrombocytes remained stable between the different treatments, averaging 51,574 × 103 and 43,370 × 103 by 21 and 42 days of age, respectively.

By 21 days, there were no interactions or differences ($p > 0.05$) between treatments for eosinophils, monocytes, heterophils, or heterophil:lymphocyte ratio. The interaction between chondroitin and glucosamine sulfates influenced total white blood cells ($p = 0.0099$) and lymphocytes ($p = 0.0004$) in the blood of the chickens by 21 days of age (Table 2). Chickens supplemented with 0.10% chondroitin sulfate showed a linear increase in white blood cells ($p = 0.0287$) and lymphocytes ($p = 0.0144$) with the addition of glucosamine sulfate, as shown in Equations 4 and 5, respectively (Table 3).

Table 2
Total white blood cells, eosinophils, monocytes, heterophils, lymphocytes, and heterophil:lymphocyte ratio (H:L) in the blood of broilers fed diets supplemented with chondroitin and glucosamine sulfates, at 21 and 42 days

	White blood cells (×10³)	Eosinophils (µL)	Monocytes (µL)	Heterophils (µL)	Lymphocytes (µL)	H:L
21 days of age						
Chondroitin[1] (CO, %)						
0	8,644.4	203.33	264.71	3,309.4	4,617.5	0.72
0.05	7,833.3	195.63	236.47	3,013.3	4,276.1	0.65
0.10	7,555.6	214.44	260.00	2,821.1	4,207.8	0.62
Glucosamine[2] (GLU, %)						
0	9,922.2	226.25	318.67	3,566.1	5,681.9	0.62
0.15	6,500.0	204.44	208.89	2,690.6	3,393.3	0.79
0.30	7,611.1	186.11	244.44	2,862.4	4,026.1	0.71
Probability						
Regression CO	ns	ns	ns	ns	ns	ns
Regression GLU	ns	ns	ns	ns	ns	ns
CO × GLU	0.0099	ns	ns	ns	0.0004	ns
SEM	635.62	23.08	91.00	215.88	444.98	0.09
CV (%)	35.54	61.16	53.98	45.72	37.10	38.62
42 days of age						
Chondroitin[1] (CO, %)						
0	8,411.8	205.88	255.29	2,795.0	5,131.1	0.60
0.05	6,944.4	176.25	268.33	2,222.4	4,242.9	0.57
0.10	6,941.2	192.94	173.89	2,493.9	4,061.1	0.65
Glucosamine[2] (GLU, %)						
0	8,352.9	203.57	295.88	2,653.1	5,098.3	0.52
0.15	6,666.7	166.67	217.22	2,185.0	4,093.9	0.53
0.30	7,294.1	208.33	186.67	2,682.9	4,242.9	0.64
Probability						
Regression CO	ns	ns	ns	ns	ns	ns
Regression GLU	ns	ns	L[3] (0.0399)	ns	ns	ns
CO × GLU	ns	ns	ns	ns	ns	ns
SEM	435.80	22.62	43.00	179.05	268.99	0.04
CV (%)	31.35	45.23	71.31	34.03	39.59	38.08

[1][$(C_{14}H_{21}NO_{14}S)n$, Biofac A/S] 91.27% purity. [2][$(C_6H_{14}NO_5)2SO_4$ × 2KCl, Zhejiang Golden-Shell Pharmaceutical Co. Ltd.] 16% sulfate content.
SEM: standard error of the mean. CV: coefficient of variation. ns: not significant. L: linear.
[3]Monocytes 42 days = - 364.03 GLU + 287.86; R2 = 0.94.

Table 3
Decomposition of the interaction effect between chondroitin and glucosamine sulfates on total white blood cells and lymphocytes in the blood of broilers at 21 days of age

	Chondroitin[1] (CO, %)	Glucosamine[2] (GLU, %)			Regression
		0	0.15	0.30	
White blood cells (×10³) 21 days of age	0	11,600.0	6,500.0	7,833.3	ns
	0.05	11,000.0	6,333.3	6,166.7	ns
	0.10	7,166.7	6,666.7	8,833.3	L[4] (0.0287)
	Regression	ns	ns	ns	
Lymphocytes (µL) 21 days of age	0	7,034.0	3,771.7	3,046.7	ns
	0.05	6,181.7	2,976.7	3,670.0	ns
	0.10	3,830.0	3,431.7	5,361.7	L[5] (0.0144)
	Regression	ns	ns	ns	

[1][($C_{14}H_{21}NO_{14}S$)n, Biofac A/S] 91.27% purity. [2][(C6H14NO5)2SO4 × 2KCl, Zhejiang Golden-Shell Pharmaceutical Co. Ltd.] 16% sulfate content.
ns: not significant. L: linear.
[4]White blood cells 21 days 0.10% CO = 5555.3 GLU + 6722.3; R2 = 0.54.
[5]Lymphocytes 21 days 0.10% CO = 5105.7 GLU + 3442; R2 = 0.56.

The use of glucosamine sulfate along with chondroitin sulfate has been shown to induce lymphocytosis. Meanwhile, there was a decrease in the percentage of monocytes due to the consumption of glucosamine sulfate, demonstrating its potential for fighting against possible infections. According to studies by Sadeghi et al. (2011) and Noushi and Naji (2013), glucosamine has an immunomodulatory function and activates cells of the adaptive immune system, but suppresses the activity of innate immune cells. Treatment with glucosamine increased the capacity of lymphocytes for alloreactive proliferation and increased serum levels of soluble interleukin-2 receptor (sIL-2R), an in vivo marker of T-lymphocyte activation, in addition to reducing serum levels of inflammatory cytokines and having antitumor effect (Sadeghi et al., 2011). Glucosamine can be recovered in the hexosamine pathway and converted to UDP-GlcNAc. Using UDP-GlcNAc as a substrate, golgi N-glycan branching enzymes produce N-glycans, which bind to the T-cell receptor (TCR) and cytotoxic T lymphocyte antigen-4 (CTLA-4), indicating that glucosamine can regulate autoimmunity by modulating the multiple functionalities of T cells and suggesting that it exerts immunoregulation through TCR signaling, increasing the number of T cells (Chen et al., 2013).

A recent study by Ustyuzhanina et al. (2021) in a model of cyclophosphamide-induced immunosuppression in mice demonstrated that chondroitin sulfate can act on the immune system, stimulating hematopoiesis. The sulfate stimulated an increase not only in white, but also red blood cells and thrombocytes due to its effect on bone marrow progenitor cells.

These properties of GAGs allow us to consider them as potentially promising drugs/nutraceuticals for the treatment and prevention of immunological and hematopoietic disorders.

By 42 days of age, glucosamine sulfate supplementation induced a linear decrease ($p = 0.0399$; Equation 3) in the number of monocytes, but neither the interaction nor isolated effects of chondroitin and glucosamine sulfates influenced ($p > 0.05$) the other leukogram values at this age (Table 2).

There was no interaction effect ($p > 0.05$) between the sulfates on albumin, globulins, STP, AST, or GGT. Sulfate supplementation did not affect ($p > 0.05$) globulin, AST, or GGT values at either age. Supplementation with chondroitin sulfate in the diet increased serum albumin linearly ($p = 0.0099$; Equation 6) and induced a quadratic response in TSP ($p = 0.0140$; Equation 7), whose lowest value was found with 0.04% chondroitin sulfate, by 21 days of age. Glucosamine sulfate supplementation resulted in a quadratic response ($p = 0.0450$; Equation 8) in albumin concentration by 42 days of age, whose lowest value occurred with the inclusion of 0.06% glucosamine sulfate in the diet (Table 4).

Increased AST and GGT may indicate the occurrence of liver damage by toxic substances; in the case of AST, due to hepatocyte injury, and in GGT, due to cholestasis and biliary hyperplasia (Thrall et al., 2015). The observed uniformity in the levels of these serum enzymes in this study indicates that the tested concentrations of chondroitin and glucosamine sulfates caused no hepatotoxicity; therefore, these are safe levels to be used.

The concentration of TSP can be indicative of animal health, as it is essential for maintaining colloidal osmotic pressure, regulating the acid-base balance in the blood, transporting various substances, inflammatory reaction, immune reaction, and the processes of healing tissue and repair (Melillo, 2013). Considering that the total protein levels are within the range of 2.5 to 4.5 g/dL expected for birds according to Thrall et al. (2015), the chondroitin and glucosamine sulfates were not harmful to the health of the chickens.

The interpretation of alterations in the TSP content depends on the definition of which is the main protein of the serum that is altered. However, an increase or decrease in albumin or globulin concentration does not always result in a detectable alteration in total protein content. Therefore, when interpreting these alterations, in addition to total protein concentration, albumin, and globulin levels should also be evaluated (Thrall et al., 2015). Accordingly, the influence of sulfates on TSP by 21 days of age was a consequence of alterations in albumin values, which did not occur by 42 days of age (Table 4).

Albumin is the most abundant TSP, accounting for about 75% of the colloid osmotic pressure of vascular plasma, which regulates the water content that diffuses from the blood into the tissues. In addition, it is responsible for the transport of free fatty acids, bile acids, bilirubin, ions, hormones, and medications (Thrall et al., 2015). Thus, the differences found in albumin levels between the birds are probably related to colloid osmotic control.

Table 4
Albumin, globulins, total serum protein (TSP), aspartate aminotransferase (AST), and gamma-glutamyltransferase (GGT) in the blood of broilers fed diets supplemented with chondroitin and glucosamine sulfates, at 21 and 42 days

	Albumin (g/dL)	Globulins (g/dL)	TSP (g/dL)	AST (IU/L)	GGT (IU/L)
21 days of age					
Chondroitin[1] (CO, %)					
0	1.37	1.41	2.75	258.67	28.28
0.05	1.41	1.32	2.67	266.65	26.17
0.10	1.44	1.46	2.91	260.11	26.53
Glucosamine[2] (GLU, %)					
0	1.49	1.36	2.71	259.59	26.89
0.15	1.38	1.43	2.84	270.06	27.67
0.30	1.36	1.43	2.78	255.39	26.40
Probability					
Regression CO	L[6] (0.0099)	ns	Q[7](0.0140)	ns	ns
Regression GLU	ns	ns	ns	ns	ns
CO × GLU	ns	ns	ns	ns	ns
SEM	0.03	0.06	0.08	10.49	0.95
CV (%)	8.03	18.88	12.69	16.61	15.00
42 days of age					
Chondroitin[1] (CO, %)					
0	1.38	2.04	3.42	912.88	29.83
0.05	1.48	2.12	3.60	714.47	28.89
0.10	1.40	2.01	3.47	756.22	35.93
Glucosamine[2] (GLU, %)					
0	1.37	2.19	3.60	815.65	26.44
0.15	1.38	1.98	3.36	760.61	33.67
0.30	1.52	2.01	3.53	807.06	34.27
Probability					
Regression CO	ns	ns	ns	ns	ns
Regression GLU	Q[8] (0.0389)	ns	ns	ns	ns
CO × GLU	ns	ns	ns	ns	ns
SEM	0.04	0.06	0.07	65.00	1.85
CV (%)	10.42	13.27	8.95	33.57	22.00

[1][$(C_{14}H_{21}NO_{14}S)n$, Biofac A/S] 91.27% purity. [2][$(C_6H_{14}NO_5)2SO_4$ × 2KCl, Zhejiang Golden-Shell Pharmaceutical Co. Ltd.] 16% sulfate content.
SEM: standard error of the mean. CV: coefficient of variation; ns: not significant. L: linear. Q: quadratic.
[6]Albumin 21 days = 0.7 CO + 1.3717; R^2 = 0.99.
[7]TSP 21 days = 64 CO^2 + 4.8 CO + 2.75; R^2 = 0.99.
[8]Albumin 42 days = 2.8888 GLU^2 – 0.3667 GLU + 1.37; R^2 = 0.99.

The inclusion of glucosamine sulfate in the diet reduced chlorides linearly by 21 days of age (p = 0.0237; Equation 9). The chondroitin sulfate levels increased total calcium linearly by 42 days of age (p = 0.0012; Equation 10). There was an interaction effect between the sulfates on total calcium by 21 days (p = 0.0127), ionic calcium by 21 and 42 days (p < 0.0001 and p = 0.0067, respectively), phosphorus by 42 days (p < 0.0001), and chlorides by 42 days of age (p = 0.0003) (Table 5).

The total serum calcium concentration of the chickens by 21 days of age responded quadratically (p = 0.0020; Equation 11) to the inclusion of glucosamine sulfate in diets that contained 0.10% chondroitin sulfate. According to the equation, the lowest total calcium concentration occurred with the inclusion of 0.22% glucosamine sulfate (Table 6).

At 21 days, chickens that were not supplemented with glucosamine sulfate exhibited a higher ionic calcium concentration (p = 0.0354; Equation 12) when 0.05% chondroitin sulfate was included in the diet. In contrast, broilers supplemented with 0.30% glucosamine sulfate showed lower ionic calcium (p = 0.0003; Equation 13) when they received 0.04% chondroitin sulfate. The chondroitin sulfate levels of 0.05% and 0.10% in the diets induced a linear decrease and a quadratic response, respectively, in ionic calcium with the addition of glucosamine sulfate, according to Equations 14 and 15, respectively (Table 6).

At 42 days, the ionic calcium concentration of chickens supplemented with 0.30% glucosamine sulfate increased linearly (p = 0.0165) with the inclusion of chondroitin sulfate (Equation 16). The inclusion of 0.16% glucosamine sulfate in diets without chondroitin sulfate increased ionic calcium (p = 0.0176), according to the quadratic effect described in Equation 17. In the diets with 0.10% chondroitin sulfate, the inclusion of glucosamine sulfate increased the concentration of ionic calcium linearly, as seen in Equation 18 (Table 6).

At 42 days of age, chickens that were supplemented with chondroitin sulfate along with 0.30% glucosamine sulfate showed a linear increase (p = 0.0002; Equation 19) in serum phosphorus concentration. The birds that received 0.05% and 0.10% chondroitin sulfate showed a linear increase in serum phosphorus (p = 0.0295; Equation 20; and p = 0.0002; Equation 21, respectively) with the addition of glucosamine sulfate in the diets (Table 6).

Table 5
Total calcium, ionic calcium, phosphorus, and chlorides in the blood of broilers fed diets supplemented with chondroitin and glucosamine sulfates, at 21 and 42 days of age

	Total calcium (mg/dL)	Ionic calcium (mg/dL)	Phosphorus (mmol/L)	Chlorides (mmol/L)
21 days of age				
Chondroitin[1] (CO, %)				
0	8.82	1.22	6.72	108.58
0.05	8.96	1.13	7.89	109.53
0.10	9.11	1.18	7.80	108.55
Glucosamine[2] (GLU, %)				
0	9.67	1.23	7.04	109.20
0.15	8.52	1.10	7.55	108.83
0.30	8.72	1.19	7.91	108.62
Probability				
Regression CO	ns	ns	ns	ns
Regression GLU	ns	ns	ns	L[9] (0.0237)
CO × GLU	0.0127	< 0.0001	ns	ns
SEM	0.20	0.02	0.21	0.27
CV (%)	8.51	6.85	12.32	1.63
42 days of age				
Chondroitin[1] (CO, %)				
0	7.20	1.13	6.04	113.51
0.05	8.67	1.20	6.13	112.92
0.10	8.70	1.20	7.48	110.35
Glucosamine[2] (GLU, %)				
0	8.77	1.13	6.19	113.36
0.15	8.20	1.20	6.20	112.39
0.30	8.67	1.20	7.26	111.04
Probability				
Regression CO	L[10] (0.0012)	ns	ns	ns
Regression GLU	ns	ns	ns	ns
CO × GLU	ns	0.0067	< 0.0001	0.0003
SEM	0.36	0.02	0.20	0.37
CV (%)	15.62	8.99	14.89	1.76

[1][$(C_{14}H_{21}NO_{14}S)n$, Biofac A/S] 91.27% purity. [2][$(C_6H_{14}NO_5)2SO_4$ × 2KCl, Zhejiang Golden-Shell Pharmaceutical Co. Ltd.] 16% sulfate content.
SEM: standard error of the mean. CV: coefficient of variation. ns: not significant. L: linear.
[9]Chlorides 21 days = - 1.9333 GLU + 109.17; R^2 = 0.98.
[10]Calcium 42 days = 15 CO + 7.44; R^2 = 0.77.

Table 6
Decomposition of the interaction effect between chondroitin and glucosamine sulfates on total calcium and ionic calcium in the blood of broilers at 21 days of age, and ionic calcium, phosphorus, and chlorides in the blood of broilers at 42 days of age

	Chondroitin[1] (CO, %)	Glucosamine[2] (GLU, %)			Regression
		0	0.15	0.30	
Total calcium (mg/dL) 21 days	0	9.47	8.47	8.51	ns
	0.05	9.66	8.39	8.83	ns
	0.10	9.88	8.68	8.76	Q[11]'(0.0020)
	Regression	ns	ns	ns	
Ionic calcium (mg/dL) 21 days	0	1.22	1.22	1.21	ns
	0.05	1.25	1.07	1.04	L[14]'(0.0065)
	0.10	1.22	1.00	1.31	Q[15]'(0.0009)
	Regression	Q[12] (0.0354)	ns	Q[13] (0.0003)	
Ionic calcium (mg/dL) 42 days	0	1.08	1.21	1.11	Q[17]'(0.0176)
	0.05	1.12	1.20	1.14	ns
	0.10	1.18	1.19	1.36	L[18]'(0.0157)
	Regression	ns	ns	L[16] (0.0165)	
Phosphorus (mg/dL) 42 days	0	6.55	5.94	5.62	ns
	0.05	5.96	6.08	6.34	L[20] (0.0295)
	0.10	6.06	6.58	9.81	L[21] (0.0002)
	Regression	ns	ns	L[19] (0.0002)	
Chlorides (mmol/L) 42 days	0	115.27	112.85	112.40	ns
	0.05	113.74	112.88	112.15	ns
	0.10	111.07	111.43	108.56	ns
	Regression	L[22] (0.0229)	ns	L[23] (0.0059)	

[1][$(C_{14}H_{21}NO_{14}S)n$, Biofac A/S] 91.27% purity. [2][$(C_6H_{14}NO_5)2SO_4$ × 2KCl, Zhejiang Golden-Shell Pharmaceutical Co. Ltd.] 16% sulfate content.
ns: not significant. L: linear. Q: quadratic.
[11]Calcium 21 days $_{0.10\%\ CO}$ = 28.444 GLU² - 12.267 GLU + 9.88; R^2 = 0.99.
[12]Ionic calcium 21 days $_{0\%\ GLU}$ = - 12 CO² + 1.2 CO + 1.22; R^2 = 0.99.
[13]Ionic calcium 21 days $_{0.30\%\ GLU}$ = 88 CO² + 7.8 CO + 1.21; R^2 = 0.99.
[14]Ionic calcium 21 days $_{0.05\%\ CO}$ = - 0.7 GLU + 1.225; R^2 = 0.85.
[15]Ionic calcium 21 days $_{0.10\%\ CO}$ = 11.778 GLU² - 3.2333 GLU + 1.22; R^2 = 0.99.
[16]Ionic calcium 42 days $_{0.30\%\ GLU}$ = 2.5 CO + 1.0783; R^2 = 0.84.
[17]Ionic calcium 42 days $_{0\%\ CO}$ = - 5.1111 GLU² + 1.6333 GLU + 1.08; R^2 = 0.99.
[18]Ionic calcium 42 days $_{0.10\%\ CO}$ = 0.6 GLU + 1.1533; R^2 = 0.80.
[19]Phosphorus 42 days $_{0.30\%\ GLU}$ = 41.9 CO + 5.1617; R^2 = 0.87.
[20]Phosphorus 42 days $_{0.05\%\ CO}$ = 1.2667 GLU + 5.9367; R^2 = 0.96.
[21]Phosphorus 42 days $_{0.10\%\ CO}$ = 12.5 GLU + 5.6083; R^2 = 0.85.
[22]Chlorides 42 days $_{0\%\ GLU}$ = - 42 CO + 115.46; R^2 = 0.98.
[23]Chlorides 42 days $_{0.30\%\ GLU}$ = - 38.4 CO + 112.96; R^2 = 0.80.

When chondroitin sulfate was included, the diets containing 0% and 0.30% glucosamine sulfate provided a linear decrease in blood chloride concentration ($p = 0.0229$ and $p = 0.0059$; Equations 22 and 23, respectively) by 42 days of age as chondroitin sulfate was added (Table 6).

There was no interaction effect between chondroitin and glucosamine sulfates, nor isolated effects ($p > 0.05$), at either age evaluated, on the serum potassium or alkaline phosphatase concentrations. However, the interaction between sulfates had influenced ($p = 0.0108$) sodium by 42 days of age (Table 7). In decomposing the interaction, we observed that the chickens that received the diet with 0.05% chondroitin sulfate displayed a linear increase in blood sodium by 42 days of age ($p = 0.0179$; Equation 24) as glucosamine sulfate was added (Table 8).

Although we did not evaluate differences in alkaline phosphatase between ages, it is important to consider that the activity of this enzyme, which participates in the mineralization process, decreases with bone maturation (Thrall et al., 2015).

The inorganic matrix of bones consists predominantly of calcium and phosphorus ions, in the form of hydroxyapatite crystals; and, in smaller amounts, bicarbonate, magnesium, potassium, sodium, citrate, chloride, and fluoride (Goff, 2017). Adequate extracellular fluid concentrations of ions are required for bone mineralization (Proszkowiec-Weglarz & Angel, 2013). Therefore, the increase in serum minerals following the supplementation with chondroitin and glucosamine sulfates demonstrates greater availability of these ions for bone mineralization and other important functions. According to Proszkowiec-Weglarz and Angel (2013), when the plasma calcium level is high, the C cells of the ultimobranchial glands of birds are stimulated to secrete calcitonin, which increases the entry of calcium and phosphorus into the bone cells and reduces the movement of bone calcium and phosphorus into the plasma.

Imik et al. (2012) showed that serum levels of calcium and phosphorus were lower in chickens that had tibial dyschondroplasia compared with healthy animals, and concluded that locomotor disorders occurred due to incomplete bone and cartilage development. Murakami et al. (2001) suggested that high levels of chloride may increase the likelihood of incidence of tibial dyschondroplasia. According to Oviedo-Rondón et al. (2001), an increase in sodium ion levels reduces the incidence of tibial dyschondroplasia. As observed, the sulfates increased calcium, phosphorus, and sodium ions and reduced chlorides in the chickens' blood, which suggests a possible action of these sulfates in reducing the incidence of tibial dyschondroplasia. This was previously reported in a study by Martins et al. (2020b), who observed not only a reduction in the incidence of tibial dyschondroplasia, but also a decrease in femoral degeneration and valgus and varus angular defects in broilers fed diets supplemented with chondroitin and glucosamine sulfates. These effects consequently influenced performance, which may be in part due to the increased levels of these minerals in the blood.

Table 7
Sodium, potassium, and alkaline phosphatase in the blood of broilers fed diets supplemented with chondroitin and glucosamine sulfates, at 21 and 42 days of age

	Sodium (mmol/L)	Potassium (mmol/L)	Alkaline phosphatase (IU/L)
21 days of age			
Chondroitin[1] (CO, %)			
0	145.25	5.06	3,661.60
0.05	145.73	5.28	4,127.80
0.10	146.09	5.11	3,658.80
Glucosamine[2] (GLU, %)			
0	145.77	5.07	3,642.00
0.15	146.08	5.05	3,779.30
0.30	145.22	5.33	4,024.70
Probability			
Regression CO	ns	ns	ns
Regression GLU	ns	ns	ns
CO × GLU	ns	ns	ns
SEM	0.42	0.24	229.76
CV (%)	1.22	9.22	34.16
42 days of age			
Chondroitin[1] (CO, %)			
0	150.08	4.62	1,961.70
0.05	148.96	4.84	2,257.50
0.10	147.70	4.76	2,473.90
Glucosamine[2] (GLU, %)			
0	149.84	4.72	2,417.60
0.15	148.59	4.86	1,806.61
0.30	148.30	4.63	2,465.72
Probability			
Regression CO	ns	ns	ns
Regression GLU	ns	ns	ns
CO × GLU	0.0108	ns	ns
SEM	0.46	0.14	139.68
CV (%)	1.72	12.36	44.12

[1][$(C_{14}H_{21}NO_{14}S)n$, Biofac A/S] 91.27% purity. [2][$(C_6H_{14}NO_5)2SO_4$ × 2KCl, Zhejiang Golden-Shell Pharmaceutical Co. Ltd.] 16% sulfate content.
SEM: standard error of the mean. CV: coefficient of variation. ns: not significant.

Table 8
Decomposition of the interaction effect between chondroitin and glucosamine sulfates on sodium in the blood of broilers at 42 days of age

	Chondroitin[1] (CO, %)	Glucosamine[2] (GLU, %)			Regression
		0	0.15	0.30	
Sodium (mmol/L) 42 days of age	0	152.98	149.67	147.60	ns
	0.05	148.22	148.62	150.05	L[24](0.0352)
	0.10	148.32	147.47	147.24	ns
	Regression	ns	ns	ns	

[1][($C_{14}H_{21}NO_{14}S$)n, Biofac A/S] 91.27% purity. [2][($C_6H_{14}NO_5$)2SO_4 × 2KCl, Zhejiang Golden-Shell Pharmaceutical Co. Ltd.] 16% sulfate content.
ns: not significant. L: linear.
[24]Sodium 42 days $_{0.05\%\ CO}$ = 6.1 GLU + 148.05; R^2 = 0.90.

Moreover, total extracellular calcium exists in three different forms: bound to proteins, bound to anions, or ionized. Calcium bound to serum proteins, primarily albumin, constitutes a storage pool for ionic calcium, and any alteration in serum proteins will affect total calcium levels. Calcium bound to interstitial ions (lactate, citrate, bicarbonate) is able to diffuse across capillary membranes, like its ionized version. In the ionized form, calcium regulates a number of important biochemical and physiological processes, including neuromuscular excitability, blood clotting, secretory processes, membrane integrity, transport across the plasma membrane, enzymatic reactions, hormone and neurotransmitter release, and intracellular actions of several hormones. Furthermore, adequate concentrations in the extracellular fluid are required for bone mineralization (Pizauro, 2017). Sgavioli et al. (2018) observed an improvement in the mineral profile of calcium and phosphorus in the tibia of broilers fed diets supplemented with chondroitin and glucosamine sulfates.

The increase in calcium, phosphorus, and sodium ions can be explained by the ability of glycosaminoglycans to complex with ions due to the high density of negative charges provided by sulfate and carboxylic groups (Hernandez et al., 2015; Kim et al., 2017).

Conclusion

Supplementation with chondroitin and glucosamine sulfates in the broilers' diet favored their immune system and mineral metabolism, increasing serum concentrations of calcium, phosphorus, and sodium. The uniformity in total serum protein levels and in the serum enzymes AST and GGT indicates that the tested concentrations of chondroitin and glucosamine sulfates caused no hepatotoxicity. Therefore, these are safe levels to be used, with the recommended concentrations being up to 0.10% chondroitin sulfate and 0.30% glucosamine sulfate.

References

Bowers, G. N., & McComb, R. B. (1966). A continuous spectrophotometric method for measuring the activity of serum alkaline phosphatase. *Clinical Chemistry, 12*(2), 70-89. doi: 10.1093/clinchem/12.2.70

Calamia, V., Lourido, L., Fernández-Puente, P., Mateos, J., Rocha, B., Montell, E., Vergés, J., Ruiz-Romero, C., & Blanco, F. J. (2012). Secretome analysis of chondroitin sulfate-treated chondrocytes reveals anti-angiogenic, anti-inflammatory and anti-catabolic properties. *Arthritis Research &Therapy, 14*(5), R202. doi: 10.1186/ar4040

Calamia, V., Mateos, J., Fernández-Puente, P., Lourido, L., Rocha, B., Fernández-Costa, C., Montell, E., Vergés, J., Ruiz-Romero, C., & Blanco, F. J. (2014). A pharmacoproteomic study confirms the synergistic effect of chondroitin sulfate and glucosamine. *Scientific Reports, 4*, R5069. doi: 10.1038/srep05069

Carvalho, G. B., Martins, P. C., Rezende, P. M., Santos, J. S., Oliveira, E., Trentin, T. C., Martins, D. B., Stringhini, J. H., & Café, M. C. (2020). Hematology and serum biochemistry of broilers at the initial and growth stages submitted to different levels of digestible sulfur amino acids. *Ciência Rural, 50*(5), e20180881. doi: 10.1590/0103-8478cr20180881

Castrogiovanni, P., Trovato, F. M., Loreto, C., Nsir, H., Szychlinska, M. A., & Musumeci, G. (2016). Nutraceutical supplements in the management and prevention of osteoarthritis. *International Journal of Molecular Sciences, 17*(12), 2042. doi: 10.3390/ijms17122042

Chen, N. H., Cheong, K. A., Kim, C. H., Noh, M., & Lee, A. Y. (2013). Glucosamine induces activated T cell apoptosis through reduced T cell receptor. *Scandinavian Journal of Immunology, 78*(1), 17-27. doi: 10.1111/sji.12056

Cobb (2008). *Manual de manejo de frangos de corte Cobb.* Cobb-Vantress Brasil.

Daly, J. A., & Ertingshausen, G. (1972). Direct method for determining inorganic phosphate in serum with the centrifichem. *Clinical Chemistry, 18*(3), 263-265. doi: 10.1093/clinchem/18.3.263

Farm Animal Welfare Council (2012). *Fave freedoms.* https://webarchive.nationalarchives.gov.uk/ukgwa/20121010012427/http:/www.fawc.org.uk/freedoms.htm

Gocsik, É., Kortes, H. E., Oude Lansink, A. J. M., & Saatkamp, H. W. (2014). Impact of different broiler production systems on health care costs in the Netherlands. *Poultry Science, 93*(6), 1301-1317. doi: 10.3382/os.2013-0361

Goff, J. P. (2017). Cartilagem, ossos e articulações. In W. O. Reece (Ed.), *Dukes fisiologia dos animais domésticos.* (12a ed., pp. 575-595). Rio de Janeiro.

Gouze, J. N., Bordji, K., Gulberti, S., Terlain, B., Netter, P., Magdalou, J., Fournel-Gigleux, S., & Ouzzine, M. (2001). Interleukin-1beta down-regulates the expression of glucuronosyltransferase I, a key enzyme priming glycosaminoglycan biosynthesis: influence of glucosamine on interleukin-1beta-mediated effects in rat chondrocytes. *Arthritis & Rheumatogy, 44*(2) 351-360. doi: 10.1002/1529-0131(200102)44:2<351::AID-ANR53>3.0.CO;2-M

Hernandez, S. E. R., Streeter, I., & Leeuw, N. H. (2015). The effect of water on the binding of glycosaminoglycan saccharides to hydroxyapatite surfaces: a molecular dynamics study. *Physical Chemistry Chemical Physics, 17*(34), 22377-22388. doi: 10.1039/c5cp02630j

Imik, H., Terim Kapakin, K. A., Gumu, R., Kapakin, S., & Kurt, A. (2012). The effect of tibial dyschondroplasia on metabolic parameters in broiler chickens. *Ankara Üniversitesi Veteriner Fakültesi Dergisi, 59*(4), 271-277. doi: 10.1501/Vetfak_0000002538

Jerosch, J. (2011). Effects of glucosamine and chondroitin sulfate on cartilage metabolism in AO: outllok on other nutrient partners especially omega-3 fatty acids. *International Journal of Rheumatology, 2011*, 1-17. doi: 10.1155/2011/969012

Kamarul, T., Ab-Rahim, S., Tumin, M., Selvaratnam, L., & Ahmad, T. S. (2011). A preliminary study of the effects of glucosamine sulphate and chondroitin sulphate on surgically treated and untreated focal cartilage damage. *European Cells and Materials, 21*, 259-271. doi: 10.22203/ecm.v021a20

Kantor, E. D., Lampe, J. W., Navarro, S. L., Song, X., Milne, G. L., & White, E. (2014). Associations between glucosamine and chondroitin supplement use and biomarkers of systemic inflammation. *Journal of Alternative and Complementary Medicine, 20*(6), 479-485. doi: 10.1089/acm.2013.0323

Kim, H. D., Lee, E. A., An, Y. H., Kim, S. L., Lee, S. S., Yu, S. J., Jang, H. L., Nam, K. T., Im, S. G., & Hwang, N. S. (2017). Chondroitin sulfate-based biomineralizing surface hydrogels for bone tissue engineering. *ACS Applied Materials & Interfaces, 9*(26), 21639-21650. doi: 10.1021/acsami.7b04114

Martins, J. M. S., Santos, L. D., Neto, Gomides, L. P. S., Fernandes, L. P. S., Sgavioli, S., Stringhini, J. H., Leandro, N. S. M., & Café, M. B. (2020a). Performance, nutrient digestibility, and intestinal histomorphometry of broilers fed diet supplemented with chondroitin and glucosamine sulfates. *Revista Brasileira de Zootecnia, 49*, e20190248. doi: 10.37496/rbz4920190248

Martins, J. M. S., Santos, L. D., Neto, Noleto-Mendonça, R. A., Carvalho, G. B., Sgavioli, S., Carvalho, F. B., Leandro, N. S. M., & Café, M. B. (2020b). Dietary supplementation with glycosaminoglycans reduces locomotor problems in broiler chickens. *Poultry Science, 99*(12), 6974-6982. doi: 10.1016/j.psj.2020.09. 061

Melillo, A. (2013). Applications of serum protein electrophoresis in exotic pet medicine. *Veterinary Clinics of North America: Exotic Animal Practice, 16*(1), 211-225. doi: 10.1016/j.cvex.2012.11.002

Murakami, A. E., Oviedo-Rondon, E. O., Martins, E. N., Pereira, M. S., & Scapinello, C. (2001). Sodium and chloride requirements of growing broiler chickens (twenty-one to forty-two days of age) fed corn-soybean diets. *Poultry Science, 80*(3), 289-294. doi: 10.1093/ps/80.3.289

Natt, M. P., & Herrick, C. A. (1952). A new blood diluent for couting eytrocytes and leucocytes of the chicken. *Poultry Science, 31*(4), 735-738. doi: 10.3382/ps.0310735

Noushi, A. A., & Naji, A. S. N. (2013). Possible adverse effects of once-daily oral therapeutic dose of either glucosamine sulfate or glucosamine/chondroitin sulfate on blood cells count in rats. *International Research Journal of Pharmacy, 4*(10), 24-29. doi: 10.7897/2230-8407.041007

Oviedo-Rondón, E. O., Murakami, A. E., Furlan, A. C., Moreira, I., & Macari, M. (2001) Sodium and chloride requirements of young broiler chickens fed corn-soybean diets (one to twenty-one days of age). *Poultry Science, 80*(5), 592-598. doi: 10.1093/os/80.5.592

Pizauro, J. M., Jr., Santos, L. F. J., & Gonçalves, A. M. (2017). Regulação do metabolismo ósseo por hormônios e íons inorgânicos. In M. Macari, & A. Maiorka (Eds.), *Fisiologia das aves comerciais* (pp. 514-530). Jaboticabal.

Proszkowiec-Weglarz, M., & Angel, R. (2013). Calcium and phosphorus metabolism in broilers: effect of homeostatic mechanism on calcium and phosphorus digestibility. *Journal of Applied Poultry Research, 22*(3), 609-627. doi: 10.3382/japr.2012-00743

Rostagno, H. S., Albino, L. F. T., Donzele, J. L., Gomes, P. C., Oliveira, R. F., Lopes, D. C., Ferreira, A. S., Barreto, S. L. T., & Euclides, R. F. (2011). *Tabelas brasileiras para aves e suínos: composição de alimentos e exigências nutricionais* (3a ed.). Universidade Federal de Viçosa.

Sadeghi, B., Hägglund, H., Remberger, M., Al-Hashmi, S., Hassan, Z., Abedi-Valugerdi, M., & Hassan, M. (2011). Glucosamine activates T lymphocytes in healthy individuals and may induce GVHD/GVL in stem cell transplanted recipients. *The Open Transplantation Journal, 5*, 1-7. doi: 10.2174/1874418401105010001

Santos, E. T., Sgavioli, S., Castiblanco, D. M. C., Borges, L. L., Quadros, T. C. O., Laurentiz, A. C., Shimano, A. C., Lizandra, A., & Baraldi-Artoni, S. M. (2019). Glycosaminoglycans and vitamin C affect broiler bone paramenters. *Poultry Science, 98*(10), 4694-4704. doi: 10.3382/ps/pez177

Santos, E. T., Sgavioli, S., Castiblanco, D. M. C., Domingues, C. H. F., Quadros, T. C. O., Borges, L. L., Petrolli, T. G., & Baraldi-Artoni, S. M. (2018). Glycosaminoglycans and vitamin C in ovo feeding affects bone characteristics of chicks. *Revista Brasileira de Zootecnia, 47*, e20170304. doi: 10.1590/rbz4720170304

Statistical Analysis System Institute (2002). *SAS/STAT User's guide: statistics.* Version 9.2. SAS Inst. Inc.

Schumann, G., Bonora, R., Ceriotti, F., Férard, G., Ferrero, C. A., Franck, P. F., Gella, F. J., Hoelzel, W., Jørgensen, P. J., Kanno, T., Kessner, A., Klauke, R., Kristiansen, N., Lessinger, J. M., Linsinger, T.P., Misaki, H., Panteghini, M., Pauwels, J., Schiele, F.,... Siekmann, L. (2002). IFCC primary reference procedures for the measurement of catalytic activity concentrations of enzymes at 37 degrees C. Part 6. *Clinical Chemistry and Laboratory Medicine, 40*(7), 734-738. doi: 10.1515/CCLM.2002.125

Schwean-Lardner, K., Fancher, B. I., Gomis, S., Van Kessel, A., Dalal, S., & Classen, H. L. (2013). Effect of day length on cause of mortality, leg health, and ocular health in broilers. *Poultry Science, 92*(1), 1-11. doi: 10.3382/os.2011-01967

Sgavioli, S., Santos, E. T., Borges, L. L., Andrade-Garcia, G. M., Castiblanco, D. M. C., Almeida, V. R., Garcia, R. G., Shimano, A. C., Nääs, I. A., & Baraldi-Artoni, S. M. (2017). Effect of the addition of glycosaminoglycans on bone and cartilaginous development of broiler chickens. *Poultry Science, 96*(11), 4017-4025. doi: 10.3382/ps/pex228

Shim, M. Y, Karnuah, A. B., Anthony, N. B., Pesti, G. M., & Aggrey, S. E. (2012). The effects of broiler chicken growth rate on valgus, varus, and tibial dyschondroplasia. *Poultry Science, 91*(1), 62-65. doi: 10.3382/ps.2011-01599

Szasz, G. (1969). A kinetic photometric method for serum gamma-glutamyl transpeptidase. *Clinical Chemistry, 15*(2), 124-136. doi: 10.1093/clinchem/15.2.124

Taniguchi, S., Ryu, J., Seki, M., Sumino, T., Tokuhaschi, Y., & Esumi, M. (2012). Long-tern oral administration of glucosamine or chondroitin sulfate reduces destruction of cartilage andu p-regulation of MMP-3 mRNA in a model of spontaneous osteorthritis in Hartley guinea pigs. *Journal of Orthopaedic Research, 30*(5), 673-678. doi: 10.1002/jor.22003

Thrall, M. A., Weiser, G., Allison, R. W., & Campbell, T. W. (Eds.) (2015). *Hematologia e bioquímica clínica veterinária* (2a ed.). Roca.

Ustyuzhanina, N. E., Anisimova, N. Y., Bilan, M. I., Donenko, F. V., Morozevich, G. E., Yashunskiy, D. V., Usov, A. I., Siminyan, N. G., Kirgisov, K. I., Varfolomeeva, S. R., Kiselevskiy, M. V., & Nifantiev, N. E. (2021). Chondroitin sulfate and fucosylated chondroitin sulfate as stimulators of hematopoiesis in cyclophosphamide-induced mice. *Pharmaceuticals, 14*(11), 1074. doi: 10.3390/ph14111074

Valvason, C., Musacchio, E., Pozzuoli, A., Ramonda, R., Aldegheri, R., & Punzi, L. (2008). Influence of glucosamine sulphate on oxidative stress in human osteoarthritic chondrocytes: effects on HO-1, $p22^{Phox}$ and iNOS expression. *Rheumatology, 47*(1), 31-35. doi: 10.1093/rheumatology/kem289