# Leg health and growth performance of broiler chickens supplemented with grape seed extract

***Salud de piernas y comportamiento productivo de pollos suplementados con extracto de semilla de uva***

***Saúde das pernas e comportamento produtivo de frangos suplementados com extrato de semente de uva***

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**Abstract**

**Background:** Active bone remodeling processes can be altered by the presence of reactive oxygen species and, therefore, cause leg problems in broiler chickens. The antioxidant activity present in grape seed extract (GSE) could be a viable alternative to high inclusion levels of vitamin E (VE) as a nutritional strategy to improve the antioxidant capacity of birds and, thus, prevent leg abnormalities. **Objective:** To evaluate the effect of partial substitution of VE with grape GSE on leg health and performance of broiler chickens. **Methods:** Four hundred and twenty newly-hatched Ross 308 male broiler chickens were distributed into three treatments: 1) Control-AL, a diet containing 40 IU/kg of VE and fed *ad libitum* (CAL); 2) Control-FR, the CAL diet but offered through a feed restriction program (CFR); and 3) grape seed extract-AL, a diet containing 10 mg/kg of GSE + 10 IU/kg of VE fed *ad libitum* (GSE-AL). The trial was conducted for 47 days. Feed intake and body weight of the chickens were recorded weekly to evaluate performance. At day 43, gait score (GS), valgus/varus angulation (AngV), foot burn (FB), and hock burn (HB) lesions were evaluated. At day 47, tibia-breaking strength (TBS) and gastrocnemius tendon-breaking strength (GTBS) were evaluated. **Results:** Birds in the GSE-AL treatment showed reduced ($p \leq 0.05$) GS compared to the other treatments. No statistical differences ($p > 0.05$) were found in AngV, HB, FB, TBS, TGBS, and growth performance among treatments. **Conclusions:** These results suggest that GSE may partially replace VE in broiler diets without negative effects on growth performance or leg health. Further research is required to evaluate the potential of grape seed extract to replace VE or other ingredients with antioxidant activity under different rearing conditions and feeding programs.

**Keywords**: *breaking strength; broiler; gait score; gastrocnemius; grape seed extract; leg health, nutrient restriction; performance; tendon; tibia; vitamin E.*

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**Resumen**

**Antecedentes:** Los procesos activos de remodelación ósea pueden verse alterados por la presencia de especies reactivas de oxígeno y, por lo tanto, causar problemas en las patas de los pollos de engorde. La actividad antioxidante presente en el extracto de semilla de uva (GSE) podría ser una alternativa viable a la inclusión de altos niveles de vitamina E (VE) como estrategia nutricional para mejorar la capacidad antioxidante de las aves y así prevenir anomalías en las patas. **Objetivo:** Evaluar el efecto de la sustitución parcial de VE por GSE en la salud podal y el comportamiento productivo de pollos de engorde. **Métodos:** Cuatrocientos veinte pollos de engorde machos Ross 308 se distribuyeron en tres tratamientos: 1) Control-AL, dieta con 40 UI/kg de VE ofrecida *ad libitum* (CAL); 2) Control-FR, la dieta CAL ofrecida bajo restricción alimenticia (CFR); y 3) extracto de semilla de uva-AL; dieta con 10 mg/kg de GSE + 10 UI/kg de VE ofrecida *ad libitum* (GSE-AL). El experimento se llevó a cabo durante 47 días. El consumo de alimento y el peso corporal de los pollos se registraron semanalmente para evaluar su comportamiento productivo. En el día 43 se evaluaron capacidad de caminar (GS), angulación en valgo/varo (AngV), quemadura de pie (FB) y quemadura de corvejón (HB). En el día 47, se evaluaron resistencia a la ruptura de la tibia (TBS) y resistencia a la ruptura del tendón del gastrocnemio (GTBS). **Resultados:** Las aves del tratamiento GSE-AL tuvieron menor GS ($p \leq 0{,}05$) en comparación con los otros tratamientos. No hubo diferencias estadísticas ($p > 0{,}05$) en AngV, HB, FB, TBS, TGBS y comportamiento productivo entre tratamientos. **Conclusiones:** Estos resultados sugieren que GSE puede reemplazar parcialmente a VE en la dieta de pollos de engorde, sin efectos negativos sobre el comportamiento productivo o la salud podal. Se requiere más investigación para evaluar el potencial del extracto de semilla de uva para reemplazar VE u otros ingredientes con actividad antioxidante en diferentes condiciones de crianza y programas de alimentación.

**Palabras clave:** *comportamiento productivo; extracto de semilla de uva; gastrocnemio; marcha; pollo de engorda; resistencia a la ruptura; restricción de nutrientes; salud podal; tendón; tibia; vitamina E.*

**Resumo**

**Antecedentes:** Os processos ativos de remodelação óssea podem ser interrompidos pela presença de espécies reativas de oxigênio e, portanto, causar problemas nas pernas dos frangos de corte. A atividade antioxidante presente no extrato de semente de uva pode ser uma alternativa viável ao uso de altos níveis de vitamina E (VE) como estratégia nutricional para melhorar a capacidade antioxidante das aves e, assim, prevenir anormalidades nas pernas. **Objetivo:** Avaliar o efeito da substituição parcial de VE por extrato de semente de uva (GSE) na saúde dos pés e no desempenho produtivo de frangos de corte. **Métodos:** Quatrocentos e vinte frangos de corte machos Ross 308 foram divididos em três tratamentos: 1) Controle-AL, dieta contendo 40 UI/kg de VE e alimentação *ad libitum* (CAL); 2) Controle-FR, a dieta CAL mas oferecida através de um sistema alimentar restrito (CFR); e 3) extrato de semente de uva-AL; uma dieta contendo 10 mg/kg GSE + 10 UI/kg VE com comida oferecida *ad libitum* (GSE-AL). O experimento foi realizado por 47 dias. O consumo de ração e o peso corporal das galinhas foram registrados semanalmente para avaliar o desempenho produtivo. No dia 43, foram avaliados a capacidade de marcha (GS), angulação valgo/varo (AngV), queimadura do pé (FB) e queimadura do jarrete (HB). No dia 47, a resistência à ruptura da tíbia (TBS) e a resistência à ruptura do tendão do gastrocnêmio (GTBS) foram avaliadas. **Resultados:** As aves do tratamento GSE-AL apresentaram menor ($p \leq 0{,}05$) capacidade de marcha GS em comparação com os outros tratamentos.Não houve diferenças estatísticas ($p > 0{,}05$) em AngV, HB, FB, TBS, TGBS e desempenho produtivo entre os tratamentos. **Conclusões:** Esses resultados sugerem que o GSE pode substituir parcialmente o VE em dietas de frangos de corte sem efeitos negativos no desempenho produtivo ou na saúde dos pés. Mais pesquisas são necessárias para avaliar o potencial do extrato de semente de uva para substituir VE ou outros ingredientes com atividade antioxidante em diferentes condições de criação e programas de alimentação.

**Palavras-chave:** *comportamento produtivo; extrato de semente de uva; frango de corte; força de quebra; gastrocnêmio; marcha; restrição de nutrientes; saúde dos pés; tendão; tíbia; vitamina E.*

## Introduction

Leg abnormalities lead to decreased growth performance and negatively impact the welfare of commercial poultry (Danbury *et al.,* 2000). Birds with severe lameness have limited access to feeders and drinkers, causing emaciation and dehydration (Talaty *et al.,* 2010). The economic impact due to lameness could account for up to 30% of the total industry losses (Julian, 1995). According to Waldenstedt (2006), the most common leg problems in commercial broiler facilities are tibial dyschondroplasia, rickets, and angular bone deformities such as valgus/varus deviations (inward curved/outward curved). The bone is a dynamic tissue subject to a continuous process of resorption and formation (Raisz, 2005). During this process, osteoclasts remove old bone, whereas osteoblasts form new bone (Mueller and Russell, 2003). A recent study showed that reactive oxygen species (ROS) such as $H_2O_2$ are related to apoptosis of stem cells in the bone marrow (Chen *et al.,* 2016). The ROS are particularly involved in the homeostasis of mineral tissue by bone resorption (Wauquier *et al.,* 2009). Moreover, ROS could inhibit cartilage formation (chondrogenesis; Zakani *et al.,* 2005). Additionally, high altitude and *ad libitum* feeding are predisposing factors that increase ROS and mortality by ascites (Rodríguez-Ortega *et al.,* 2014).

Vitamin E (VE), a fat-soluble vitamin, has multiple functions for optimal growth and health of broiler chickens. A VE deficiency can cause nutritional encephalomalacia, exudative diathesis, and muscular dystrophy (Ames, 1956). The VE is widely used in poultry diets as an antioxidant because it can neutralize ROS during propagation of free radicals due to its peroxyl radical-scavenging activity (Rizvi *et al.,* 2104; Selvam *et al.,* 2017). A strategy to increase the antioxidant effect of VE is to supplement it in high levels. However, the addition of high levels of VE can increase the cost of the diet (Kennedy *et al.,* 1992). More economically feasible alternatives such as grape seed extract (GSE) could be employed as a nutritional strategy to improve the birds' antioxidant capacity without the need to use high levels of VE. The GSE has antioxidant activity due to its high content of polyphenolic compounds (Brenes *et al.,* 2010), such as procyanidins, catechins, epicatechins, gallocatechins, and epigallocatechins (Chamorro *et al.,* 2013). However, limited information is available evaluating the effect of GSE when it is fed to broiler chickens. An experiment conducted in rats reported that grape proanthocyanidins can increase the quality and strength of jaw bones during growth (Masaru *et al.,* 2004). Another experiment showed that rats fed high-fructose diets and supplemented with GSE had increased activity of hepatic superoxide dismutase, catalase, and suppressed lipid peroxidation when compared to rats fed a high-fructose diet alone (Suwannaphet *et al.,* 2010).

To better understand the potential leg health and performance benefits of supplementing GSE to broiler diets, the present study evaluated the effect of partial substitution of VE by GSE on gait score (GS), valgus/varus angulation (AngV), foot burn (FB), and hock burn (HB) lesions, tibia breaking strength (TBS), and gastrocnemius tendon breaking strength (GTBS), as well as growth performance of broiler chickens.

## Materials and Methods

### *Ethical considerations*

The experimental procedures followed the standards for ethics, biosafety, and animal well-being of the Mexican Official Standard (NOM-062-ZOO-1999; 2001) for the use of animals in research. Euthanasia procedures were performed according to the Mexican Official Norm (NOM-033-SAG/ZOO-2014, 2015).

### *Poultry facilities and management*

The experiment was carried out at the poultry research unit of Colegio de Postgraduados, Campus Montecillo, Estado de México, MX (98º 48' 27" W and 19º 48' 23" N), located at an altitude of 2,278 m above sea level (Barometric Pressure: 581.1 mmHg, partial pressure of oxygen ($PO_2$): 122 mm Hg; (Vázquez-Garcia and Pérez-Padilla, 2000) to increase reactive oxygen species (ROS) production. Four hundred and twenty (420) newly hatched male broiler chickens (Ross

308) were used in this 47-day study. Twenty-one (21) groups of chicks with similar starting weight (45.59 g ± 0.42; p>0.05) were distributed into three experimental treatments using a completely randomized design, with seven replicates per treatment and 20 chickens per replicate pen. The birds were housed in floor pens of three $m^2$ with new wood-shavings litter. Each pen was equipped with a bell drinker and a hanging cylindrical feeder (111 and 121 cm circumference, respectively). Water was offered *ad libitum* throughout the trial. The lighting program was 23 h of light and one hour of darkness during the experimental period. The broiler house was set for 32-33 °C initial temperature and it was gradually reduced after two days (~1 to 2 °C per day) to 24 °C, according to the birds' comfort. The facilities used a curtain-ventilation system.

*Dietary treatments*

All diets were formulated to meet or exceed Ross nutritional requirements for broilers. Chickens were randomly allocated to one of three dietary treatments; 1) Control: basal diet containing 40 IU/kg of VE (DL-α-tocopherol acetate) and fed *ad libitum* (CAL); 2) Control fed restricted (CFR): basal diet containing 40 IU/kg of VE offered through a feed restriction program (feed was offered 16 h per day starting from day 14 until the end of the experiment); and 3) Grape seed extract treatment: a diet containing 10 mg/kg of Procyanidins equivalent to 30 IU/kg VE (according to recommended conversion provided by the product manufacturer) + 10 IU/kg of VE and fed *ad libitum* (GSE-AL). The feeding program was divided into two phases: 1) a starter (0 to 21 days) diet containing 3,025 kcal/kg ME and 22% CP and 2) a grower/finisher (21 to 47 days) diet containing 3,100 kcal/kg ME and 19% CP (Table 1). Birds were fed diets in mash form. The antioxidant activity test (AOA) was evaluated in all diets.

*Antioxidant activity of diets*

The antioxidant activity (AOA) of each diet was measured as % inhibition of the *in vitro* 1, 1-diphenyl-2 picrylhydrazyl (DPPH) radical, according to Rodriguez-Ortega *et al.* (2017) with minor modifications. A sample of 500 g of feed was grounded in a commercial blender (Oster, Owosso, MI, USA). A gram of feed was mixed with 10 mL of methanol (Sigma-Aldrich, St Louis, MO, USA) and incubated at 30 °C for 30 min. The mixture was vortexed for 20 seconds and then centrifuged at 1,342 × *g* for 10 min. The methanol extract was filtered using Whatman (Cytiva, Marlborough, MA, USA) 4 filters. Two hundred µL of the extract was taken. Three mL of methanolic DPPH solution was added (0.11 mM) and stirred for 10 seconds. The extract was kept in a dark room for 20 minutes and then analyzed in a spectrophotometer (Thermo-Scientific, 10SVIS model, Waltham, MA, USA) at 515 nm. The antioxidant activity of the diets was calculated using the following equation:

AOA (% DPPH inhibition) = [(absorbance of DPPH - absorbance of samples) / absorbance of DPPH]*100

*Performance evaluation*

Body weight (BW), BW gain (BWG), feed intake (FI), and feed conversion ratio (FCR) were calculated by phase. Mortality data from the present study is not presented in this paper as it has been previously reported in a separate publication (Rodríguez-Ortega *et al.,* 2017).

*Gait score, valgus/varus angulation, hock, and footpads burn lesion*

On day 43, three birds per pen were randomly selected (21 birds/treatment) to evaluate gait score (GS), valgus/varus angulation (AngV), foot burns (FB) and hocks burn (HB) lesions. Gait score was evaluated according to the methodology described by Kestin *et al.* (1992) and later modified by Garner *et al.* (2002). Six score categories (0 to 5) were used. A score of 0 was given to broilers with normal locomotion and a 5 score to completely lame chickens unable to walk or stand. Two experienced observers viewed and scored each bird individually; when the evaluators did not reach a consensus, they evaluated another bird.


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**Table 1.** Composition of the experimental diets.

| Ingredient (%) | Starter 0-21 d | | Grower/Finisher 21-47 d | |
|---|---|---|---|---|
| | Control | GSE | Control | GSE |
| Yellow corn | 53.38 | 53.38 | 61.67 | 61.67 |
| Dehulled soybean meal | 39.69 | 39.69 | 31.27 | 31.27 |
| Soybean oil | 2.64 | 2.64 | 2.80 | 2.80 |
| Calcium carbonate | 1.66 | 1.66 | 1.34 | 1.34 |
| Dicalcium phosphate | 1.68 | 1.68 | 1.57 | 1.57 |
| L-Lysine HCl | 0.028 | 0.028 | 0.077 | 0.077 |
| L-Threonine | 0.066 | 0.066 | 0.085 | 0.085 |
| DL-Methionine | 0.122 | 0.122 | 0.088 | 0.088 |
| Mineral premix[1] | 0.100 | 0.100 | 0.100 | 0.100 |
| Vitamin premix[2] | 0.050 | 0.050 | 0.050 | 0.050 |
| Selenium | 0.005 | 0.005 | 0.005 | 0.005 |
| Vitamin E[3] | 0.007 | 0.002 | 0.007 | 0.002 |
| Grape seed extract[3] | 0.000 | 0.001 | 0.000 | 0.001 |
| Choline chloride | 0.213 | 0.213 | 0.213 | 0.213 |
| Coccidiostat4 | 0.050 | 0.050 | 0.050 | 0.050 |
| Pigment (xanthophylls) | 0.000 | 0.000 | 0.370 | 0.370 |
| Salt (NaCl) | 0.300 | 0.300 | 0.300 | 0.300 |
| **Calculated composition (%)** | | | | |
| ME (kcal/kg) | 3,025.00 | 3,025.00 | 3,100.00 | 3,100.00 |
| CP | 22.00 | 22.00 | 19.00 | 19.00 |
| Arginine | 1.50 | 1.50 | 1.50 | 1.50 |
| Lysine | 1.28 | 1.28 | 1.09 | 1.09 |
| Methionine | 0.48 | 0.48 | 0.41 | 0.41 |
| Methionine + Cystine | 0.88 | 0.88 | 0.75 | 0.75 |
| Threonine | 0.90 | 0.90 | 0.80 | 0.80 |
| Calcium | 1.00 | 1.00 | 0.85 | 0.85 |
| Available phosphorus | 0.45 | 0.45 | 0.42 | 0.42 |
| Sodium | 0.16 | 0.16 | 0.16 | 0.16 |
| Chloride | 0.22 | 0.22 | 0.22 | 0.22 |
| Vitamin E (IU) | 40.00 | 10.00 | 40.00 | 10.00 |
| Procyanidins (mg)[3] | 0.00 | 10.00 | 0.00 | 10.00 |
| Choline (mg) | 1,600.00 | 1,600.00 | 1,600.00 | 1,600.00 |

[1]Mineral premix (per kg of premix): Zn 100 g; Fe 50 g; Cu 10 g; Mn 100 g; I 1 g.
[2]Vitamin premix (per kg of premix): vitamin A 24,000,000 IU; vitamin D3 8,000,000 IU; pyridoxine 8 g; thiamine 6 g; riboflavin 16 g; niacin100 g; cyanocobalamin 60 mg; menadione 10 g; calcium pantothenate 28 g; folic acid 3 g.
[3]Vitamin E (DL-α-tocopherol acetate); GSE (Grape seed extract; Procyanidins) are estimated values based on the product manufacturer recommendations.
[4]Coccidiostat, Olistimax®, PiSA Agropecuaria, Guadalajara, Jalisco, Mexico.

Valgus/varus angulation (VAng) measured the angle size of the tibia-metatarsus according to the protocol described by Vargas-Galicia *et al.* (2017). A four-scale lesion score was assigned from 0 to 3, where 0 represented birds with no angulation; Score 1, chickens with slight angulation; Score 2, birds with obvious angulation; and Score 3, birds with severe angulation. Foot and hock burns were measured following the methodology of Su *et al.* (1999). Both legs were evaluated on a scale of 0 to 3, where: 0 represented undamaged feet and hocks; 1, bird with minor damage; 2, presence of obvious damage; and 3, chicken with extensive burns and inflammation.

*Tibia and gastrocnemius tendon breaking strength*

At the end of the experiment, 14 birds per treatment (2 birds/pen) were euthanized by cervical dislocation to evaluate tibia-breaking strength (TBS) and gastrocnemius tendon-breaking strength (GTBS). Both legs were frozen at -20 °C and stored (3 months) until TBS and GTBS assessments were performed. The TBS and GTBS were measured in Newtons (N) using a Vernier force plate (Vernier Software & Technology, Beaverton, OR, USA). The tendon and tibia were dissected the same day they were thawed, as described by Rodríguez-Ortega *et al.* (2022). All measurements were conducted at the animal nutrition laboratory of Universidad Autónoma Chapingo, Mexico.

*Statistical analysis*

Growth performance (BW, BWG, FI, FCR), gastrocnemius tendon-breaking strength (GTBS), and tibia breaking strength (TBS) data were analyzed with one-way ANOVA using the PROC MIXED procedure with orthogonal contrasts using SAS software, version 9.0 (SAS Institute Inc., Cary, NC, USA; 2011). Gait score (GS), valgus/varus angulation (AngV), foot burns (FB), and hocks burns (HB) were analyzed by contrasts using the GLIMMIX procedure (SAS Institute Inc., 2011). Contrasts were analyzed as follows: CAL *vs.* CFR, CAL *vs.* GSE-AL; CFR *vs.* GSE-AL. A statistical significance was considered when p-value was ≤0.05.

## Results

The effects of partial substitution of VE by GSE on performance variables and leg health was evaluated. The GSE diets had lower AOA than the control diets (Table 2). No significant differences (p>0.05) in cumulative BWG, FI, and FCR were observed among treatments (Table 3).

**Table 2.** Antioxidant activity of the experimental diets.

| | % inhibition DPPH[1] | |
|---|---|---|
| **Diet** | **Starter** | **Grower/Finisher** |
| Control | 14 | 17 |
| Grape seed extract | 11 | 11 |

[1]The antioxidant activity (AOA) of each diet was measured as % inhibition of *in vitro* 1, 1-diphenyl-2 picrylhydrazyl radical (DPPH).

Birds in the GSE-AL treatment showed the lowest (p≤0.05) GS (worsen walking capacity) with 57% of the birds being classified in Category 3 in comparison with the control treatments. The Control-AL treatment had the highest (p≤0.05) % of birds with a score of 0 (Table 4). There were no significant differences (p>0.05) in FB, HB, AngV (Table 5), TBS, and GTBS (Table 6).

## Discussion

In the literature, proper GSE levels to be included in the diets for broilers have not been conclusive. In this study, no differences in performance were observed in chickens fed GSE *ad libitum*. Our results agree with Brenes *et al.* (2010), who found no significant differences in body weight gain (BWG), feed intake (FI), and FCR among broiler-fed diets GSE at 0.6, 1.8, and 3.6 g/kg of diet. In contrast, Hughes *et al.* (2005) observed a decrease in FI and growth of broiler chickens fed diets that included 30 g/kg GSE, where the GSE product contained 90.2% total extractable polyphenols. In the present study, the GSE product had 85% total extractable polyphenols added at 10 mg/kg of diet; in general, high dietary supplementation of GSE is related to reduced performance (Chamorro *et al.*, 2013); therefore, the low inclusion of GSE in this experiment could explain the performance results.

**Table 3.** Body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) of broilers[1].

| Treatment[¥] | CAL | CFR | GSE-AL | SEM | ANOVA | CAL *vs.* CFR | CAL *vs.* GSE-AL | CFR *vs.* GSE-AL |
|---|---|---|---|---|---|---|---|---|
| **0-21 d** | | | | | | | | |
| BWG (g) | 835 | 828 | 813 | 16 | 0.611 | 0.942 | 0.593 | 0.790 |
| FI (g) | 1,266[a] | 1,205[b] | 1,223[ab] | 12 | 0.006 | 0.005 | 0.052 | 0.554 |
| FCR | 1.53 | 1.47 | 1.50 | 0.02 | 0.249 | 0.220 | 0.667 | 0.667 |
| **21-47 d** | | | | | | | | |
| BWG (g) | 2,525 | 2,496 | 2,579 | 48 | 0.346 | 0.860 | 0.613 | 0.323 |
| FI (g) | 4,764 | 4,724 | 4,730 | 80 | 0.927 | 0.931 | 0.950 | 0.998 |
| FCR | 1.89 | 1.89 | 1.84 | 0.03 | 0.457 | 1.00 | 0.521 | 0.521 |
| **0-47 d[2]** | | | | | | | | |
| BWG (g) | 3,360 | 3,324 | 3,392 | 47 | 0.603 | 0.811 | 0.888 | 0.535 |
| FI (g) | 6,030 | 5,929 | 5,953 | 80 | 0.657 | 0.655 | 0.779 | 0.976 |
| FCR | 1.79 | 1.79 | 1.76 | 0.02 | 0.544 | 0.931 | 0.756 | 0.931 |

Different superscripts letters ([a, b]) within the same row are statistically different at p≤0.05.

[¥]Control-AL: basal diet containing 40 IU/kg of vitamin E (VE) (dl-α-tocopheryl acetate) and fed *ad libitum* (CAL); Control-FR: CAL diet containing 40 IU/kg of VE offered through a feed restriction program (CFR: feed was offered 16 h per day starting from day 14 until the end of the experiment); and grape seed extract (GSE)-AL: a diet containing 10 mg/kg of GSE (equivalent to 30 IU/kg VE) + 10 IU/kg of VE and fed *ad libitum* (GSE-AL).

[1]Contrasts with p≤0.05 are statistically different; SEM: pooled standard error of the mean.

[2]Cumulative 0-47 d performance data was previously published (Rodríguez-Ortega *et al.,* 2017).

**Table 4.** Gait score (GS) of broilers at 43 days[1].

| Treatment[¥] | Gait score (%) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 |
| CAL | 5 | 23 | 40 | 32 | 0 | 0 |
| CFR | 10 | 38 | 47 | 5 | 0 | 0 |
| GSE-AL | 0 | 0 | 43 | 57 | 0 | 0 |
| | **p-value** | | | | | |
| ANOVA | <0.001 | | | | | |
| CAL *vs.* CFR | 0.042 | | | | | |
| CAL *vs.* GSE-AL | 0.069 | | | | | |
| CFR *vs.* GSE-AL | 0.002 | | | | | |

[¥]Control-AL: basal diet containing 40 IU/kg vitamin E (VE) (dl-α-tocopheryl acetate) and fed *ad libitum* (CAL); Control-FR: CAL diet containing 40 IU/kg VE offered through a feed restriction program (CFR: feed was offered 16 h per day starting from day 14 until the end of the experiment); and grape seed extract (GSE)-AL: a diet containing 10 mg/kg GSE (equivalent to 30 IU/kg VE) + 10 IU/kg VE and fed *ad libitum* (GSE-AL).

[1]Contrasts with p≤0.05 are statistically different.

**Table 5.** Hocks burn (HB), foot burn (FB), and valgus/varus angulation (AngV) frequency of chickens at 43 days[1].

| Treatment[¥] | HB (%) | | | | FB (%) | | | | AngV (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 |
| CAL | 52 | 48 | 0 | 0 | 86 | 14 | 0 | 0 | 5 | 76 | 14 | 5 |
| CFR | 71 | 24 | 5 | 0 | 100 | 0 | 0 | 0 | 5 | 81 | 14 | 0 |
| GSE-AL | 67 | 33 | 0 | 0 | 100 | 0 | 0 | 0 | 5 | 71 | 24 | 0 |
| | **p-value** | | | | | | | | | | | |
| ANOVA | 0.718 | | | | 1.000 | | | | 0.798 | | | |
| CAL *vs.* CFR | 0.466 | | | | 0.983 | | | | 0.686 | | | |
| CAL *vs.* GSE-AL | 0.517 | | | | 0.983 | | | | 0.796 | | | |
| CFR *vs.* GSE-AL | 0.933 | | | | 1.000 | | | | 0.506 | | | |

[¥] Control-AL: basal diet containing 40 IU/kg vitamin E (VE) (dl-α-tocopheryl acetate) and fed *ad libitum* (CAL); Control-FR: CAL diet containing 40 IU/kg VE offered through a feed restriction program (CFR: feed was offered 16 h per day starting from day 14 until the end of the experiment); and grape seed extract (GSE)-AL: a diet containing 10 mg/kg GSE (equivalent to 30 IU/kg VE) + 10 IU/kg VE and fed *ad libitum* (GSE-AL).

[1]Contrasts with p≤0.05 are statistically different.

**Table 6.** Tibia (TBS) and gastrocnemius tendon (GTBS) breaking strength at 47 days[1].

| Treatment[¥] | GTBS (N) | TBS (N) |
|---|---|---|
| CAL | 168 | 404 |
| CFR | 156 | 420 |
| GSE-AL | 184 | 421 |
| SEM | 10 | 20 |
| | **p-value** | |
| ANOVA | 0.139 | 0.799 |
| CAL *vs.* CFR | 0.672 | 0.836 |
| CAL *vs.* GSE-AL | 0.451 | 0.824 |
| CFR *vs.* GSE-AL | 0.120 | 0.999 |

[¥]Control-AL: basal diet containing 40 IU/kg vitamin E (VE) (dl-α-tocopheryl acetate) and fed *ad libitum* (CAL); Control-FR: CAL diet containing 40 IU/kg VE offered through a feed restriction program (CFR: feed was offered 16 h per day starting from day 14 until the end of the experiment); and grape seed extract (GSE)-AL: a diet containing 10 mg/kg GSE (equivalent to 30 IU/kg VE) + 10 IU/kg VE and fed *ad libitum* (GSE-AL).

[1]Contrasts with p≤0.05 are statistically different; SEM: pooled standard error of the mean.

The broilers fed GSE in combination with VE had the lowest GS, which could be due to the number of free radicals released by high metabolic rate (fed *ad libitum* diets) and the high altitude where the experiment was conducted (Kalmar *et al.,* 2013). Rodríguez-Ortega *et al.* (2017) evaluated glutathione peroxidase activity, nitric oxide concentration, and lipid peroxidation [malondialdehyde (MDA) concentration] in plasma, lungs, heart and liver, observing that *ad libitum* diets decreased lung, heart, and liver antioxidant activity in broiler chickens raised at 2,278 m of altitude ($PO_2$ of 122 mm Hg). High altitudes generate tissue hypoxia (Maiti *et al.,* 2006) and decrease electron transport, thus generating free radicals (Poyton *et al.,* 2009). Free radicals affect the formation of bone and cartilage (McAlindon *et al.,* 1996), affecting locomotion. Broilers raised at high altitudes might benefit from high inclusion levels of GSE to effectively prevent oxidative stress when fed *ad libitum* diets.


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High bird density, litter type, moisture, and broiler weight can influence the frequency and severity of HB and FB (Tasistro *et al.,* 2004; Almeida *et al.,* 2010). The present study showed no significant difference between treatments for HB and FB. The lack of effect of GSE on HB and FB could have been partially due to low moisture content of the litter. Future trials should consider the use of higher bird density to introduce a bigger challenge to the litter, thus, increasing the opportunity to observe the GSE effect on the reduction of HB and FB lesions. Angular bone deformity is one of the most common leg defects in broilers (Julian, 1984). In the present study, no significant difference was observed for AngV among treatments. One possible explanation for the results observed in AngV could be birds' age (higher bone maturity at 43 days). These problems are related to lack of maturity of the tissue and BW because the tissue becomes stronger and more resistant with age (Julian, 1998). Applegate and Lilburn (2002) observed that bone deformity problems in broilers are related to constant increase in live weight and immature skeletal tissue.

Feeding ingredients with antioxidant activity can reduce cartilage loss and progression of diseases due to osteoarthritis (Mobasheri *et al.,* 2012). Sanchez *et al.* (2013) observed that chickens fed 80 IU/kg VE and vitamin C at 1 g/kg had greater strength to the gastrocnemius tendon rupture compared to birds fed VE 40 IU/kg only. Moreover, Feresin *et al.* (2013) found that VE improves bone quality, decreases bone resorption, and increases bone formation, but does not restore bone density. One possible reason for the beneficial effects of VE on bone health is because it suppresses inflammatory mediators (prostaglandin E2, $PGE_2$, tumor-α necrosis factor, TNF-α, interleukin 1 (IL-1)) and reduces free radicals that stimulate bone resorption (Garret *et al.,* 1990). We hypothesize that the GSE mode of action could be similar to VE, but further research is needed to find the specific effects of GSE on bone remodeling processes and interaction with other tissues.

In conclusion, GSE may partially replace vitamin E without negative effects on growth performance and overall leg health of broilers. Further research is required to evaluate the potential of the GSE to replace VE or other ingredients with antioxidant activity under different rearing conditions and feeding programs.

## Declarations

### *Conflict of interest*

The authors declare they have no conflicts of interest with regard to the work presented in this report.

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### *Author contributions*

Leodan T. Rodríguez-Ortega: experimental design, data, and sample collection, supervised the health status of the chickens, and manuscript writing. Alejandro Rodríguez-Ortega: experimental design conceptualization. Arturo Pro-Martínez: conceptualization of the study. Filogonio J. Hernández-Guzmán: writing, review, and editing. Eliseo Sosa-Montes: laboratory analysis. Hector Leyva-Jimenez: manuscript writing, and final editing.

### *Use of artificial intelligence (AI)*

No AI or AI-assisted technologies were used during the preparation of this work.

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