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# Purified lignin supplementation on the performance and antioxidant status of broilers subjected to cyclic heat stress

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**ABSTRACT** - The objective of this study was to evaluate the effects of dietary supplementation of purified lignin on the performance, relative organ weights, serum metabolites, and gene expression profiles of broiler chickens subjected to cyclic heat stress (HS). At 22 days old, 280 broilers were distributed in a completely randomized design with four treatments, ten repetitions, and seven birds per experimental unit. The birds were subjected to daily cyclic HS. A high temperature of 32±1 °C was maintained for 10 h/day (08:00–18:00 h), while a temperature of 22±1 °C was maintained for the remaining time. Treatments were a basal diet or basal diet with the addition of 5, 10, or 15 g of purified lignin/kg of diet. Data were analyzed using one-way ANOVA and means were compared by Tukey's test at 0.05 significance. There was no effect of lignin supplementation on performance, carcass yield, relative weights of the bursa, spleen, and liver, or serum levels of glucose, triglycerides, uric acid, malondialdehyde, triiodothyronine, or tetraiodothyronine. The abundance of mRNA of heat shock protein 70, nuclear factor-κB, glutathione peroxidase, and Cu,Zn-superoxide dismutase in the liver was similarly unaffected by treatments. Purified lignin supplementation does not improve performance or the antioxidant response of broiler chickens subjected to HS.

**Keywords:** performance, phenolic compounds, poultry

## 1. Introduction

Heat stress (HS) is a critical problem in broiler production in hot-climate areas, triggering significant economic losses. This condition results from a negative balance between the net energy flowing from the animal's body to its surrounding environment and the amount of heat energy produced by the animal (Lara and Rostagno, 2013). In general, various combinations of factors related to the thermal environment and animal characteristics can trigger this imbalance.

Heat stress can influence performance (Hamidi et al., 2022), immune responses (Hirakawa et al., 2020), and cellular antioxidant system (Habashy et al., 2018; Surai et al., 2019) of broiler chickens. In addition to the utilization of ventilation and cooling systems, nutritional manipulations have been suggested as an alternative to decrease the detrimental impacts of HS on poultry performance and the antioxidant system. Dietary supplementation with polyphenol curcumin improved final body weight, decreased mitochondrial malondialdehyde (MDA) concentration, and enhanced mitochondrial gene expression of superoxide dismutase in broiler chickens subjected to HS (Zhang et al., 2018).

Lignin is a polyphenolic polymer naturally occurring in the cell walls of plants (Vance et al., 1980). In animal nutrition, lignin is mostly regarded as a barrier to nutrient digestibility. Incidentally, in the paper-making industry, purified lignin is recovered as a byproduct of cellulose production after various processes (sulfite, kraft, or alcell). In its purified form, lignin contains several low-molecular-weight phenolic monomers, such as carvacrol and cinnamaldehyde, that possess biological effects not characteristic of native lignin (Bozin et al., 2006; Baurhoo et al., 2007a). Studies have investigated the benefits of these phenolic monomers on the production and health of broilers (Bosetti et al., 2020; Galli et al., 2020), but less research has utilized purified lignin.

In this study, we hypothesized that dietary supplementation of purified lignin can improve performance and the antioxidant responses of broiler chickens subjected to HS. Therefore, we evaluated the effects of dietary supplementation of purified lignin on the performance, relative organ weights, serum metabolites, and gene expression profiles of broiler chickens subjected to cyclic HS.

## 2. Material and Methods

### 2.1. Ethical matters

The Institutional Animal Care and Use Committee approved all animal handling procedures (case number 038/2020), and the experiment was conducted according to the experimental protocol for the use of live birds from the Brazilian College of Animal Experimentation.

### 2.2. Birds, experimental design, and diets

The experiment was conducted in Viçosa, MG, Brazil (20°45'57.19" S, 42°51'35.42" W, and 682 m altitude). The male broiler chickens (Cobb 500) used in the experiment were obtained from a commercial hatchery (Rivelli Alimentos SA, Matheus Leme, MG, Brazil). Chicks were vaccinated against bursal disease and Marek's disease (Serotype 3, Live Marek's Disease Vector, Merial Inc., Athens, GA). From one day old until the beginning of the experiment, the birds were reared in a masonry house divided into protected circular pens containing tube feeders, manual drinkers, and a litter of wood shavings. They had free access to water and were fed *ad libitum* with a corn/soybean meal-based mash diet formulated to meet their nutritional requirements according to Rostagno et al. (2017).

At 22 days old, 280 broiler chickens (983±38 g) were distributed based on body weight in a completely randomized design with four treatments, ten repetitions, and seven birds per experimental unit. They were housed in 40 wire floor cages (1,008 $cm^2$/bird) in a four-level battery equipped with a trough feeder and a nipple drinker.

Birds were subjected to daily cyclic HS in controlled chambers. A high temperature of 32±1 °C was maintained for 10 h/day (08:00–18:00 h), while the temperature was set at 22±1 °C for the remaining time. The relative humidity of the air inside the chambers was maintained at 65±5%.

Treatments were a basal diet or basal diet with the addition of 5, 10, or 15 g of purified lignin/kg of diet. The purified lignin used in this research was extracted from *Eucalyptus urograndis* through the kraft process, used in pulp and paper production. The corn/soybean meal basal diet was formulated to meet the nutritional recommendations given by Rostagno et al. (2017) (Table 1). Purified lignin in the basal diet was used instead of the inert. Diets were prepared in mash form. Free access to water and feed was provided throughout the experimental period (22 to 42 days old). The light program adopted for the entire experimental period was 18 h of light (4:00 to 22:00 h) and 6 h of dark.

**Table 1 -** Ingredients and nutrient composition of basal diet (as-fed basis)

| Ingredient (g/kg) | 22-42 days of age |
|---|---|
| Corn (78.6 g/kg) | 568.2 |
| Soybean meal (450 g/kg) | 322.5 |
| Soybean oil | 60.95 |
| Dicalcium phosphate | 13.28 |
| Limestone | 6.86 |
| Salt | 4.81 |
| DL-Methionine (999 g/kg) | 2.71 |
| L-Lysine HCl (780 g/kg) | 1.92 |
| Vitamin premix[1] | 1.20 |
| Trace mineral premix[2] | 1.00 |
| Choline chloride (600 g/kg) | 0.80 |
| L-Threonine (985 g/kg) | 0.54 |
| L-Valine (990 g/kg) | 0.24 |
| Inert | 15.00 |
| Calculated composition (g/kg, unless shown) | |
| Metabolizable energy (kcal/kg) | 3,200 |
| Crude protein | 195.0 |
| Calcium | 7.05 |
| Available phosphorus | 3.41 |
| Sodium | 2.03 |
| Digestible glycine + serine | 15.52 |
| Digestible lysine | 10.77 |
| Digestible methionine + cysteine | 7.97 |
| Digestible valine | 8.29 |
| Digestible threonine | 7.11 |
| Digestible thyptophan | 2.18 |

[1] Vitamin premix provided per kg of diet: vitamin A, 11,566 IU; vitamin D3, 2,892 IU; vitamin E, 43.3 IU; vitamin K3, 2.32 mg; vitamin B1, 3.12 mg; vitamin B12, 0.019 mg; vitamin B6, 4.33 mg; vitamin B5, 15.54 mg; vitamin B3, 47.0 mg; vitamin B9, 1.08 mg; biotin, 0.11 mg.
[2] Trace mineral premix provided per kg of diet: Mn, 58.36 mg; Zn, 54.21 mg; Fe, 41.68 mg; Cu, 8.31 mg; I, 0.843 mg; Se, 0.250 mg.

## 2.3. Performance and sample collection

Birds and feed leftovers were weighed at 42 days of age to calculate feed intake (FI), weight gain (WG), and feed conversion ratio (FCR). Mortalities were recorded throughout the experimental period, and the necessary corrections of performance data were carried out.

At 42 days old, three birds with weights closest to the average weight for their respective experimental unit were selected. One bird was used for blood collection. After blood collection, the bird was euthanized by cervical displacement and slaughtered. Liver samples were collected, stored individually in cryogenic tubes, and placed in liquid nitrogen. These samples were transferred to freezer storage at −80 °C until the RNA extraction process.

The two remaining birds, after 8 h of fasting, were euthanized by cervical displacement and slaughtered to measure the yield of carcass, breast, and thigh with drumstick, as well as the relative weight of the lymphoid organs (bursa and spleen), liver, intestine, and abdominal fat. Carcass yield (CY) was calculated in relation to living weight before slaughter [%CY = (carcass weight × 100)/live weight] and breast and thigh yield with drumstick, and as a function of carcass weight [%Part = (part weight × 100)/carcass weight]. The relative weights of the bursa, spleen, liver, intestine, and abdominal fat were calculated in relation to the live weight of the birds before slaughter.

## 2.4. Serum parameter measurement

The collected blood was used to analyze serum levels of glucose, uric acid, triglycerides (Cobas c 311; Roche Diagnostics GmbH, Basel, Switzerland), and the hormones triiodothyronine (T3) and tetraiodothyronine (T4; Atellica IM, Siemens Healthcare Diagnostics Inc, New York, USA), following the manufacturer's instructions. To measure MDA, 2.5 mL of 20% trichloroacetic acid and 1.0 mL of 0.67% thiobarbituric acid were added to 0.5 mL of serum; the mixture was then heated for 30 min in boiling water. The resulting chromogen was extracted with 4.0 mL of n-butyl alcohol. The absorbance of the organic phase was determined at a wavelength of 530 nm.

## 2.5. Total RNA extraction, cDNA synthesis, and RT-qPCR analysis

Total RNA was extracted from 50 mg of powdered liver samples, using TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The resulting precipitate was rehydrated with 25 µL of UltraPure DNase/RNase-Free water. The RNA concentration was estimated using a NanoDrop™ Lite Spectrophotometer (ThermoFisher Scientific, Beverly, MA, USA). RNA integrity was determined in 1.0% agarose gel. The first cDNA strand was synthesized using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Thermo Fisher Scientific, Beverly, MA, USA). The primer sets used are shown in Table 2; β-actin (*β-ACT*) was used as the reference gene for data normalization. The following target genes were assessed: heat shock protein 70 (*HSP70*), nuclear factor-κB (*NF-κB*), glutathione peroxidase (*GPX*), and Cu,Zn-superoxide dismutase (*SOD1*).

The RT-qPCR analyses were performed in duplicate with an Applied Biosystems™ QuantStudio Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Beverly, MA, USA), using the Relative Quantification method and applying the SYBR® Green system (Applied Biosystems, Foster City, CA, USA) and GoTaq® qPCR Master Mix kit (Promega Corporation, Madison, WI, USA). PCR reactions were subjected to the cycles protocol according to the program: 95 °C for 2 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Threshold cycle (Ct) values obtained were later normalized (ΔCt) based on Ct values of the endogenous control gene *β-ACT*. The calculation of gene expression levels was performed according to the $2^{-\Delta Ct}$ method, as described by Livak and Schmittgen (2001).

**Table 2 -** Primer sequences

| Gene | Forward sequence | Reverse sequence |
|---|---|---|
| *HSP70* | CACCATCACTGGCCTTAACGT | TTATCCAAGCCATAGGCAATAGC |
| *NF-κB* | GTGTGAAGAAACGGGAACTG | GGCACGGTTGTCATAGATGG |
| *GPX* | GACCAACCCGCAGTACATCA | GAGGTGCGGGCTTTCCTTTA |
| *SOD1* | AGGGGGTCATCCACTTCC | CCCATTTGTGTTGTCTCCAA |
| *β-actin* | TGCTGTGTTCCCATCTATCG | TTGGTGACAATACCGTGTTCA |

*HSP70* - heat shock protein 70; *NF-κB* - nuclear factor-κB; *GPX* - glutathione peroxidase; *SOD1* - Cu,Zn-superoxide dismutase.

## 2.6. Statistical analysis

Cage averages were considered as an experimental unit for statistical analysis of growth performance parameters. For analyses of yield of carcass, breast, and thigh with drumstick, as well as the relative weights of the lymphoid organs, liver, intestine, and abdominal fat, the average of two birds per replicate was considered as the experimental unit. For serum and gene expression analyses, one bird per replicate was considered as the experimental unit. Data were analyzed via one-way ANOVA, according to the following general model:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij},$$

in which $Y_{ij}$ is the measured dependent variable, $\mu$ is the overall mean, $\alpha_i$ is the effect of treatments, and $\varepsilon_{ij}$ is the random error.

Analyses were carried out using the GLM procedure of SAS (Statistical Analysis System, version 9.4). Comparison between treatment averages was performed using Tukey's test. A significance level of 0.05 was applied.

## 3. Results

### 3.1. Performance and carcass yield

There was no treatment effect (P>0.05) on performance and carcass yield (Tables 3 and 4).

**Table 3 -** Growth performance of broiler chickens from 22 to 42 days of age

| | Purified lignin (g/kg of diet) | | | | SEM | P-value |
|---|---|---|---|---|---|---|
| | 0 | 5 | 10 | 15 | | |
| Body weight gain (kg/bird) | 1.986 | 1.949 | 1.944 | 1.895 | 0.015 | 0.184 |
| Feed intake (kg/bird) | 3.598 | 3.669 | 3.645 | 3.626 | 0.012 | 0.176 |
| Feed conversion ratio | 1.81 | 1.89 | 1.88 | 1.92 | 0.01 | 0.094 |

SEM - standard error of mean (n = 10 for treatment).

**Table 4 -** Carcass yield, intestine and abdominal fat (% of live weight), and breast and thighs with drumstick (% of carcass) of broiler chickens at 42 days of age

| | Purified lignin (g/kg of diet) | | | | SEM | P-value |
|---|---|---|---|---|---|---|
| | 0 | 5 | 10 | 15 | | |
| Carcass yield (%) | 78.03 | 78.29 | 77.62 | 77.59 | 0.25 | 0.733 |
| Breast (%) | 37.39 | 37.92 | 37.86 | 37.57 | 0.19 | 0.747 |
| Thighs with drumstick (%) | 26.72 | 26.58 | 26.82 | 27.02 | 0.17 | 0.854 |
| Intestine (%) | 3.12 | 3.11 | 3.15 | 3.09 | 0.04 | 0.946 |
| Abdominal fat (%) | 0.79 | 0.80 | 0.71 | 0.69 | 0.03 | 0.481 |

SEM - standard error of mean (n = 10 for treatment).

### 3.2. Relative weights of organs

There was no significant treatment effect (P>0.05) on the relative weights of the bursa, spleen, liver, intestine, and abdominal fat (Table 5).

**Table 5 -** Relative weights of bursa, spleen, and liver (% of live weight) of broiler chickens at 42 days of age

| | Purified lignin (g/kg of diet) | | | | SEM | P-value |
|---|---|---|---|---|---|---|
| | 0 | 5 | 10 | 15 | | |
| Bursa (%) | 0.090 | 0.088 | 0.102 | 0.097 | 0.006 | 0.851 |
| Spleen (%) | 0.107 | 0.104 | 0.113 | 0.105 | 0.003 | 0.789 |
| Liver (%) | 1.528 | 1.586 | 1.529 | 1.600 | 0.021 | 0.515 |

SEM - standard error of mean (n = 10 for treatment).

### 3.3. Serum metabolites

Purified lignin supplementation did not influence (P>0.05) serum levels of glucose, triglycerides, uric acid, MDA, or the T3 and T4 hormones (Table 6).

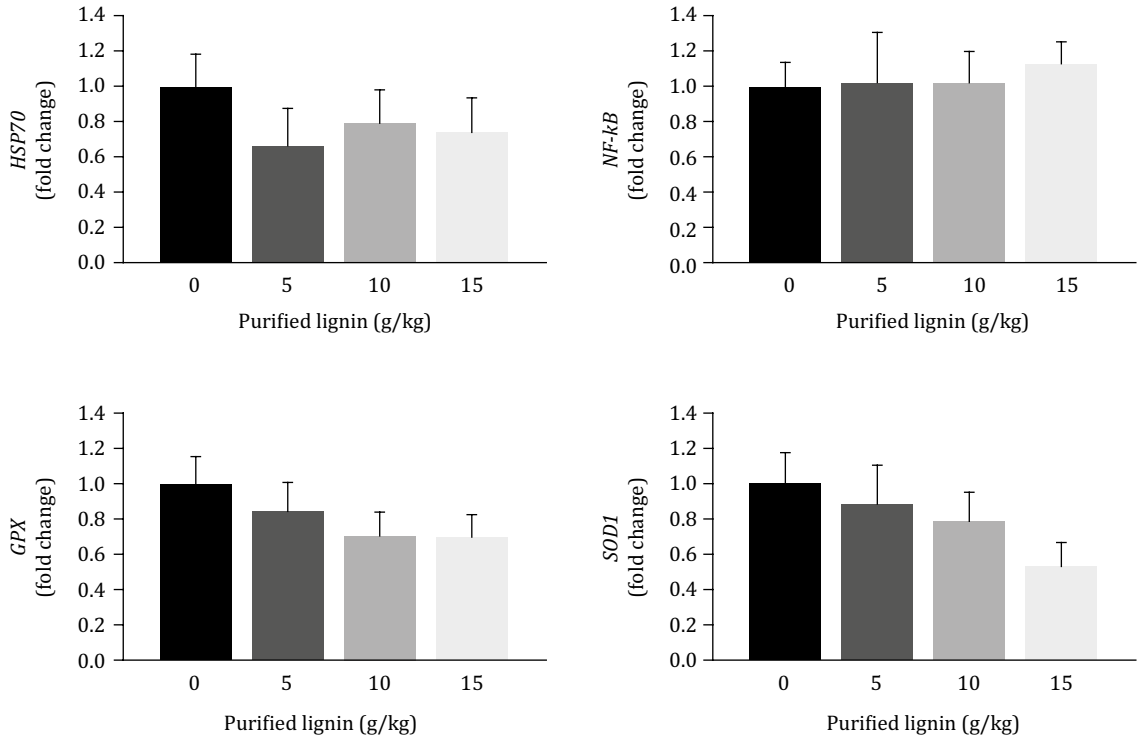
**Table 6 -** Serum metabolites in broiler chickens at 42 days of age

| | Purified lignin (g/kg of diet) | | | | SEM | P-value |
|---|---|---|---|---|---|---|
| | 0 | 5 | 10 | 15 | | |
| Glucose (mg/dL) | 207.6 | 205.3 | 210.7 | 209.3 | 3.7 | 0.965 |
| Triglycerides (mg/dL) | 46.5 | 48.4 | 37.9 | 37.8 | 2.6 | 0.338 |
| Uric acid (mg/dL) | 3.76 | 4.11 | 4.33 | 3.46 | 0.30 | 0.768 |
| Malondialdehyde (nmol/mL) | 2.89 | 2.93 | 2.98 | 2.80 | 0.10 | 0.944 |
| T3 (ng/mL) | 0.39 | 0.30 | 0.35 | 0.42 | 0.04 | 0.726 |
| T4 (mcg/mL) | 0.75 | 0.96 | 0.68 | 0.80 | 0.06 | 0.469 |

T3 - triiodothyronine; T4 - thyroxine; SEM - standard error of mean (n = 10 for treatment).

### 3.4. mRNA content

The abundance of mRNA of *NF-κB*, *HSP70*, *GPX*, and *SOD1* in the liver was not influenced by treatments (P>0.05; Figure 1).




mRNA expression was calculated relative to the control group (0 of lignin/kg of diet).
Means±SEM; n = 8 per treatment.
*HSP70*, P = 0.659; *NF-κB*, P = 0.961; *GPX*, P = 0.413; *SOD1*, P = 0.284.

**Figure 1 -** mRNA expression of heat shock protein 70 (*HSP70*), nuclear factor-κB (*NF-κB*), glutathione peroxidase (*GPX*), and Cu,Zn-superoxide dismutase (*SOD1*) in liver of broilers at 42 days of age in response of addition of purified lignin to the diet.

## 4. Discussion

Heat stress is known to impair the performance of broilers (Hamidi et al., 2022) and cause mitochondrial damage by destabilizing the antioxidant system with an increase in reactive oxygen species (Lu et al., 2017). Furthermore, HS can reduce carcass yield (Baxter et al., 2020). In this study, it was expected that lignin supplementation in its purified form would improve the performance of broiler chickens subjected to HS. However, this hypothesis was not confirmed. No effects of lignin supplementation were observed on broiler performance, carcass yield, or the relative weights of carcass parts. A previous study reported that broilers fed diet supplemented with 12.5 g/kg of purified lignin presented increased villi height and a greater number of goblet cells in the jejunum, along with a lower population of *E. coli* in the litter; however, there was no positive effect on performance (Baurhoo et al., 2007b).

Performance is affected when birds are subjected to HS, because their metabolic rates are altered and their consumption is reduced. This is justified by the diversion of nutrients to meet homeostatic activities, in addition to the impairment of lipid and carbohydrate absorption (Montgomery and Turner, 2015) and changes in serum glucose levels due to changes in the gene expression of nutrient transporters, such as the family of glucose transporters (Sun et al., 2015). However, in the present study, according to the performance results, lignin supplementation did not influence the serum levels of glucose, uric acid, or triglycerides.

Atrophy of lymphoid tissues and liver may be associated with a series of HS-induced disorders (e.g., malnutrition, inflammation, and oxidative stress; Quinteiro-Filho et al., 2010; Hirakawa et al., 2020). In the present study, we evaluated the relative weights of the lymphoid organs (bursa and spleen) and liver, and found that lignin supplementation did not influence these variables either.

Metabolic alterations caused by HS are evidenced by changes in the concentrations of hormones responsible for basal metabolism, such as thyroid hormones. Heat stress normally induces reductions in T3 and T4 plasma concentrations. This response is considered an adaptive mechanism to avoid extra heat load by reducing metabolic heat production, thereby reducing maintenance energy requirements (Gonzalez-Rivas et al., 2020). However, no effects were observed on serum levels of the T3 and T4 hormones with lignin supplementation.

Several researchers have reported that thermal stress increases the expression of the *HSP70* gene, which plays an essential protective role against tissue injuries (Yu et al., 2008; Varasteh et al., 2015). In the present study, to assess the ability of lignin to reduce the impact of HS, we measured the mRNA expression of *HSP70* in the livers of broilers and observed no effect.

Heat stress increases the production of reactive oxygen species and may decrease natural antioxidant capabilities; both of these factors can induce oxidative stress (Gonzalez-Rivas et al., 2020). *NF-κB* plays an active role in the inflammatory response of chickens (Lan et al., 2017), and studies have shown an association between increased *NF-κB* expression levels and HS (Sahin and Smith, 2016). Previous studies with broilers indicate that exposure to HS downregulates the mRNA expression of *NF-κB* in the bursa of Fabricius (Liu et al., 2021), while it upregulates the mRNA expression of SOD (Roushdy et al., 2018). Another consequence of HS is an increase in lipid peroxidation, which generates greater production of MDA (Pamok et al., 2009). In a study with the Isa Brown laying strain, lignin supplementation in a diet contaminated with zearalenone prevented an increase in glutathione peroxidase activity in the duodenal mucosa (Grešáková et al., 2012). Thus, it was hypothesized that dietary supplementation of purified lignin could improve the antioxidant response of broiler chickens subjected to HS. To assess the ability of lignin to influence antioxidant response, we evaluated the mRNA abundance of *NF-κB*, *GPX*, and *SOD1* in the liver. However, in accordance with other results observed in this study, these variables were not influenced.

## 5. Conclusions

Supplementation of 5, 10, or 15 g of purified lignin/kg of diet does not improve performance or the antioxidant response of broiler chickens subjected to heat stress.

## Conflict of Interest

The authors declare no conflict of interest.

## Author Contributions

Conceptualization: R.A. Nunes, P.H.R.F. Campos and A.A. Calderano. Data curation: R.A. Nunes, K.A. Costa and A.A. Calderano. Formal analysis: R.A. Nunes, K.A. Costa and A.A. Calderano. Funding acquisition: A.A. Calderano. Investigation: R.A. Nunes, H.R. Salgado, S.O. Borges, R.S. Ferreira and A.A. Calderano. Methodology: R.A. Nunes, L.F.T. Albino, P.H.R.F. Campos, H.R. Salgado and A.A. Calderano. Project administration: A.A. Calderano. Resources: A.A. Calderano. Supervision: L.F.T. Albino, P.H.R.F. Campos and A.A. Calderano. Writing-original draft: R.A. Nunes and R.S. Ferreira. Writing-review & editing: L.F.T. Albino, P.H.R.F. Campos and A.A. Calderano.

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